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Preface

The phylum Platyhelminthes, which comprises up to 50,000 species, free-living and parasitic, would be one among the many obscure bilaterian phyla were it not for the fact that some of its parasitic groups (tapeworms, flukes, and relatives) are important scourges to human populations all over the world. However, the free-living Platyhelminthes, still referred to by the old, non-cladistic term 'Turbellaria', do bear two features that make them somewhat more than taxonomical oddities. Firstly, some have extraordinary powers to regenerate a new, whole individual from tiny fragments of their bodies. This has turned them into well known classroom models as well as one of the best model systems for the study of regeneration and pattern formation. Secondly, the structural and functional simplicity of turbellarians, apparent and/or real, has suggested a privileged phylogenetic position as the likely earliest bilaterians and, therefore, a key group in the study of metazoan evolution.

The four years elapsed since the 8th International Symposium on The Biology of the Turbellaria (8th ISBT) held in Brisbane, Australia, in August 1996 have witnessed an increasing interest in Platyhelminthes in general, and turbellarians in particular. Firstly, studies on regeneration have been boosted by the finding of a wealth of genes related to this process, by the setting-up of the first functional genetic assay using interference RNA, and by the recent successful labelling of turbellarian stem-cells or neoblasts. Secondly, molecular phylogenies have shattered the long-held position of Platyhelminthes as the most basal bilaterians. Trees based on 18SrDNA sequences, as well as on other nuclear genes, have reshaped the bilaterian tree and shown that the bulk of Platyhelminthes, the so-called Rhabditophora, can no longer be regarded as primitive but as members of one of the three big clades into which the Bilateria is currently divided: viz. the Lophotrochozoa. However, one order of Platyhelminthes, the Acoela, has been proposed as a true basal bilaterian, thus rendering the Platyhelminthes polyphyletic. Finally, improvements in DNA sequencing, which have already resulted in the elucidation of the full genome sequence of several other organisms, are also fostering elucidation of the full sequence of the genome from the parasitic platyhelminth *Schistosoma mansoni*.

The 9th ISBT Symposium, held in Barcelona, Spain, in June 2000 was the first of the new millenium and also the first set in a mediterranean country. This Symposium tried to bring in and to convey the excitement in the new conceptual and methodological advances, as well as to bridge the gap between those working with free-living flatworms and parasitologists. To that aim, nine world-reknowned specialists were invited to summarize the state-of-the-art in a broad range of relevant scientific fields and topics, and a full plenary session was scheduled on the Biology and Molecular Biology of Parasitism. A fair balance between the more traditional 'morphology-based' fields and those more 'molecularly-driven' was set. To most accounts, the outcome was highly enjoyable and scientifically productive. One of the most interesting conclusions of the meeting was the realisation that merging morphologically-based and molecularly-based approaches to any problem in turbellarian biology is the most fruitful way forward. Also worth mentioning is the special session held to honour the memories of three leading turbellariologists who had passed away since the 8th Symposium in Brisbane: Professors Tor G. Karling, Mario Benazzi and Ian R. Ball. In recognition of their many acomplishments and contributions to the study of turbellarians, their biographies and selected lists of their publications appear on the following pages.

Approximately 85 people from 18 countries attended the meeting, presenting 40 oral communications and 30 posters. In these proceedings, 42 contributions are presented: 22 of them as full papers, 12 as short notes. Eight abstracts summarise contributions the results of which have been published elsewhere. The contributions have been grouped into six "chapters", although several of them touch upon various disciplines and techniques, and the grouping may seem rather arbitrary. Nevertheless, these proceedings represent an excellent "state of the art" publication in flatworm research, and a stepping stone for future research.

Jaume BAGUÑÀ

Barcelona, October 2000

The participants to the 9th International Symposium on the Biology of the Turbellaria. (from left to right)

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In Memoriam: Mario Benazzi (1902-1997)

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Professor Mario Benazzi a few years before passing away

Professor Mario Benazzi and his wife Giuseppina at the Libbie Hyman Memorial Symposium, Chicago, December 1970

Professor Mario Benazzi passed away in Pisa about three years ago, on the 16th December 1997 at the age of 95 years. He was born on August 29th 1902 in Cento a small town in the province of Ferrara, central Italy. At the time of his passing he was still lucid in mind and in a relatively good physical health although often confined to a wheelchair, and his pupils were hoping to celebrate his 100th birthday by organizing a special anniversary event in his honour in the new century. He had recovered from several more or less serious illnesses and fractures and above all he seemed to have coped well with the loss of his beloved wife Giuseppina who had passed away three years earlier, on April 28th, 1994, but unfortunately, a new fall from the wheelchair caused him multiple fresh leg fractures and proved fatal. During the present commemoration I shall devote a few words also to Professor Giuseppina Benazzi-Lentati, who was his faithful companion both in work and personal contexts for about 70 years. She too was an eminent student of turbellarians, produced a large number of outstanding scientific works and attended some of our meetings.

Benazzi was the older son of a well-off cloth tradesman and was destined to succeed his father in the family business, but from the time of his youth he revealed a great passion for animal, plants and all natural phenomena. After repeated unsuccessful attempts to integrate him into

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the cloth trade, his father was obliged to drop his own project, and allow Mario to follow his natural instinct. After lengthy reflection, Benazzi decided to attend the Natural Science course at the University of Bologna where he graduated in 1925 under the guidance of Professor Ercole Giacomini. In the same year, he was summoned by Professor Alfredo Corti, a pupil of Camillo Golgi, to fill the post of assistant professor of Comparative Anatomy and Physiology at the University of Torino. Giuseppina Lentati, three years his junior, being born on October 10th 1905 and curiously enough also the daughter of a well-off carpet dealer, was accepted as a student for her degree thesis into the same Institution at the same time. The two young naturalists immediately discovered many shared interests and a common way of thinking, and in 1931 they married, so becoming an inseparable pair, cemented by a profound connivance and a common passion for nature and biological research. Pina, as he liked to call her, was a tiny woman, fragile in appearance, but with a strong personality; she was the true guide of the family and mistress of the house.

After a brief stay in Sassari, Sardinia (1934-1936) and a longer stay in Siena, Tuscany (1936-1946), where the investigations into triclads began to gain the upper hand over other research projects, at the end of the second World war the Benazzis moved to Pisa where Mario was called to fill the chair of General Biology, Zoology and Genetics at the Faculty of Medicine. He also became the Director of the Institute of Zoology and Comparative Anatomy, where he uninterruptedly continued to work and guide a remarkable group of capable and affectionate students up to 1972 when both he and his wife retired. In the course of his academic career he was also elected fellow of the "Accademia Nazionale dei Lincei", President of the Italian Society of Zoology, Dean of the Faculty of Science and Emeritus Professor of the University of Pisa; he was also a Founding Fellow of various scientific societies including the Italian Group of Embryology (G.E.I.) and the Italian Association of Genetics (A.G.I.), and received several national and international awards, although he was always humble and did not devote much of his time to university political lobbying.

During their time in Pisa, Mario and Giuseppina Benazzi approached morphological, karyological, taxonomic, zoogeographical and evolutionary problems of planarians, and investigated in particular the cytogenetic aspects of the reproductive biology of these worms. Since the study of meiotic oocytes required the collection of abortive, or freshly laid cocoons very early in the morning (usually between 5 and 6 am), they adapted a small area of the Institute as a tiny apartment with two beds, a table, a gas-ring and a bathroom. The whole apartment corresponded to what is now my study. In fact, their physical requirements were very simple, and eating habits particularly frugal (Giuseppina rarely exceeded 40 kg in weight, and Mario was only slightly heavier), as they believed that such a diet was good for their health and could contribute towards having a longer life. Nevertheless, Mario appreciated good cooking as he showed on numerous occasions of graduation or congress dinners, when he was either alone or seated at table at a far distance from Giuseppina. They practically lived permanently in the Institute except for some week-ends and summer holidays when they went to their house in Marina di Pisa or to their cottage in San Marcello Pistoiese on the Appennino mountains, where Mario devoted much of his time to his favourite readings on history, particularly on the origins and development of the Christian religion, and to listening to opera music, and Giuseppina enjoyed gardening. During the week all their fellows and students were able to meet and talk to them from dawn to late at night.

Shortly after moving to Pisa the Benazzis bought a villa in Marina di Pisa and some years later built an annexe in the garden consisting of a small apartment for their domestic staff on the ground floor, and on the first floor a library, a bathroom and a laboratory, inhabited, housed in crowded glass aquaria as well as in small tubes, by thousands of different planarians that they often collected, nourished and cared for personally. Here they continued to study until their deaths.

I met professor Benazzi for the first time at the beginning of the 60's when I was attending the Biology course. He was my Zoology teacher and I found his lessons fascinating and enthusiastically conducted, and at the same time clear, simple and permeated through and through with his immense naturalistic culture. As soon as I had passed his examination he asked me if I would like to prepare my degree thesis with him, an offer that I obviously accepted with great enthusiasm. Because of his heavy academic duties in those days, he sent me to his wife's laboratory for a short training session. She immediately involved me in her research projects and experiments and later guided me towards the attainment of my honours degree.

A few years later, around the end of 60's, I was Assistant Professor of comparative anatomy and I revealed my intentions to dedicate my energies to ultrastructural investigations on triclad oogenesis. When I told Benazzi of my wishes, although he realised that this new experimental approach would progressively exclude both himself and his wife from my research, he wished me well and introduced me to a well-known electron microscopist working at the Institute of Pathological Anatomy in Pisa, Professor Vittorio Marinozzi and later to Professor Giulio Lanzavecchia in Milan. So thanks to Professor Benazzi I was able to embark on the line of research that led me to the Chair of full Professor within about ten years.

In the following years, even when the Benazzis were retired, I often went to visit them in Marina di Pisa to talk about both my and their research, and my family developments, and always I nurtured a filial feeling of love and gratitude towards them, as indeed did all those who had been "brought up", initiated and encouraged along the scientific pathway by Professor Benazzi before me.

Coming now to the scientific work of Professor Benazzi and trying to briefly summarize his main topics of interest and significant results, first of all it must be underlined that he made about 300 publications in total, among them several text books, divulgative articles, obituaries and so on, in addition to scientific papers. All those of you who knew Mario Benazzi personally or indirectly through his papers, know that he was a devoted scholar of planarians, but perhaps only a few, except those studying or working in Pisa, know that he concentrated his attention on these turbellarians only after he moved to Tuscany, especially to Pisa, in the second half of the 40's. In previous years he alternated his occasional investigations into planarians with those into mammals, newts, insects and marine animals (the last investigated during his repeated stays at the famous Stazione Zoologica in Naples). In particular, when he was in Turin and Sassari, he was definable as a comparative anatomist, specialist in histophysiology of the thyroid, pituitary gland and female reproductive apparatus of vertebrates. Only after he gained the Chair of Zoology (in 1940, when he was in Siena) and then taught General Biology and Genetics for the scholars of medicine (from 1946 in Pisa), did he modify his scientific inclinations and goals, directing them to his better known specialities, the systematics, cytogenetics and evolution of freshwater triclads. It is noteworthy in this regard that among his first 87 papers published up to 1940, only 13 concerned triclads and they were initially produced at a rate of one every two years, while among the other 220 scientific papers, more than 190 concerned planarians. Equally noteworthy is that about 70 papers were published after he retired and that the last two were published in 1997, one a few months before, and the other just a few days after his death (23, 24).

His first paper on planarians was published in 1928 and concerned the reproductive biology of *Polycelis cornuta* (1), the second in 1930 dealt with some zoogeographical aspects of *Planaria alpina* (2), the third in 1932 ecological considerations on *Planaria subtentaculata* (3). In 1938 a comprehensive paper on the freshwater triclads of Sardinia (4) and a monograph on the reproduction of planarians (5) were published. Then, starting from 1941, a series of papers on the maternal heredity of body pigmentation was published (6; 7; 8), which some years later, thanks to the careful cytological experiments and observations carried out by his wife and his pupil Glauco Lepori and the brilliant insight of the latter, culminated with the discovery of pseudogamy in planarians (9; 10; 11; 12). In the same period Benazzi initiated a cytogenetic approach to the systematics and microspeciation events of freshwater triclads, and a karyological study of their reproductive biology that carried on together with his wife. These research lines culminated in a number of outstanding papers among which the following deserve to be cited: a review paper on the genetic influence on planarian reproduction published in 1963 by University California Press (13), a work on the nature and role of neoblasts in 1966 (14), which stimulated me to undertake an ultrastructural and karyological approach to the cellular aspects of regeneration, a comprehensive paper on fissioning in planarians from a genetic standpoint presented at the Libbie Hyman memorial symposium held in Chicago, December 1970 and published by Mc Graw-Hill in 1974 (16), and one on the genetic and physiological control of fissioning and sexuality (15). Moreover, Benazzi and his wife wrote a number of monographs that remain milestones for every student of Turbellaria, among which I would like to remember one by Benazzi-Lentati on gametogenesis and egg fertilization in planarians published in 1970 in the International Review of Cytology (17), a very outstanding one on the cytogenetics of Platyhelminthes published in 1976 (18), one on speciation events in Turbellaria (19) and another on developmental biology of planaria (20), both published in 1982 by Alan R. Liss, and a final two, one on sexual differentiation and behaviour (21), and one on asexual propagation and reproductive strategies of Triclads (22) published a few years ago for the multivolume treatise on the reproductive biology of invertebrates by John Wiley and Sons.

To conclude, I would like to remember that a number of foreign, other than Italian, turbellarian specialists, as well as zoologists, cytogeneticists and students of other natural sciences went to Pisa and later to Marina di Pisa to collaborate or simply to meet Professor Benazzi. Among them I would like to remember the late Ian Ball, the President of the organizing committee of this Congress Jaume Baguna, Nicole Gourbault and Elisabeth De Vries. I am sure that each one of them, as well as all other people who had the fortune to meet him, appreciated his calm character, naive curiosity, sincere friendship and profound honesty and will maintain a pleasant memory of him.

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Tor G. Karling – 65 years of research on turbellarian flatworms

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Tor G. Karling making notes during collection of material near Tvärminne Biological Station on the south coast of Finland, 1973.

Tor G. Karling, professor emeritus at the Swedish Museum of Natural History, Stockholm, Sweden, died on the 23rd of September 1998 at the age of 89 years. His first scientific paper, dealing with a rhabdocoel turbellarian, was published in 1930. His two last ones were published in 1995. During this period of 65 years he published a little less than one hundred scientific papers. The majority of them dealt with the morphology, taxonomy and systematics of turbellarian flatworms.

The third international symposium on the biology of the Turbellaria was held in honour of Tor G. Karling, in Diepenbeek, Belgium, in 1980. At that time I was asked to give a biography of him which was published (HENDELBERG, 1981). Much of what was reported then, after his first 50 years of research, will not be repeated here. I will just focus on some of his most important contributions to our knowledge of the Platyhelminthes.

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Karling's doctoral dissertation (KARLING, 1940) was defended during a peace-period of the Second World War in an icy cellar in Helsinki, Finland, with Alex Luther as the opponent. It dealt with the morphology and systematics of the Prolecithophora, a new order Karling recognized for the Cumulata of the divided earlier order Alloeocoela, and the Neorhabdocoela (nowadays just called Rhabdocoela), another new order for the Lecithophora of the earlier order Rhabdocoela which also included the Macrostomida. The thesis meant a big step forwards for the systematics of the turbellarian flatworms. It is interesting to see that many of the conclusions drawn by Karling at that time about the interrelationships of the taxa he included in the Prolecithophora, are strongly supported sixty years later by a parsimony-based hypothesis, based on analysis of 18S rDNA sequences (JONDELIUS *et al*., 2001).

Karling early adopted Hennig's ideas of cladistic analysis. An example of this was given at the first international symposium on the Turbellaria, the Hyman Memorial

Symposium in Chicago, 1970, where he discussed the affinities of the turbellarian sub-groups (KARLING 1970, 1974). He also constructed a theoretical turbellarian archetype. His detailed discussions have been of great value for later generations of turbellarian scientists.

A lot of Karling's papers contained descriptions of new species. Already in the field he made careful notes about the habitat from which he collected his material (Fig.), mostly turbellarians of meiofauna-size. In the beginning he mainly studied turbellarians collected at the Finnish and Swedish coasts of the Baltic, and he described about a fourth of the turbellarian species known from this brackish water sea. Eventually he published a synopsis of the turbellarian fauna of the Baltic (KARLING, 1974). This contains keys to the identification of all the species, based on morphological characters easy to observe, and also valuable information about the ecology and biogeography of the species.

Later on Karling continued to describe turbellarian species from many other geographical regions, always in the same careful way and with concise interpretation of structural details. As an example of this I showed, at this ninth international symposium on the biology of the Turbellaria, in Barcelona 2000, overheads of some of the figures in 'New taxa of Kalyptorhynchia (Platyhelminthes) from the N. American Pacific coast' (KARLING, 1989). Like most of his taxonomic papers with descriptions of new species, this one contains a lot of detailed drawings of live unsqueezed and squeezed specimens, drawings of reconstruction from series of sections, and also photographs of details in squeezed specimens and of sections, all of very high quality.

A rich source of knowledge about turbellarians in the North Sea and the Baltic was published by Josef Meixner just before the Second World War (MEIXNER, 1938). However, this book contained a lot of nomina nuda, as the second special part of the publication, intended to describe the species, was never published. The reason for this was the death of the author in the war. A great number of the nomina nuda belonged to species of the taxon Kalyptorhynchia. Karling, who probably knew this group better than any other scientist, devoted much of his time to identifying the many nomina nuda species of this group (KARLING, 1992). He also gave additional information about their morphology and distribution. This meant that Meixner's book became a much more valuable source of information.

Most of Karling's papers contained a lot of information about the morphology of the free-living flatworms, based on studies by light microscope methods. But he did not only describe the morphology. He also discussed the function and evolution of structures, e.g. the male copulatory organs (KARLING, 1956) and the proboscis (KARLING, 1961) of the Kalyptorhynchia, the defecation apparatus in a genus of the Proseriata (KARLING, 1966b) and the nematocysts and similar structures in some other turbellarians (KARLING, 1966a). One of his ideas was that the evolution from a primitive to a more complex structure often follows the same course in different turbellarian organs, for instance those formed by the body wall, e.g. the pharynx, the copulatory apparatus, and the proboscis starting with a simple folding of the epidermis and its underlying muscular layers, and continuing with more complicated changes (KARLING, 1963)

Karling will not only be remembered for his contribution to our knowledge of the turbellarian flatworms. He was always very helpful and encouraging. He led international courses and took part in organizing symposia. And wherever he was, he made friends who will remember him as the great man he was.

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In Memoriam: Ian R. Ball (1941-2000)

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Ian Raymond Ball (1941-2000) at the height of his scientific career. Picture taken from the group picture of delegates at the 3rd ISBT held in Diepenbeek, Belgium, August 1980

One of the best and most influential papers on triclad phylogeny and biogeography (BALL, 1974a) ends with a quotation from Schopenhauer "Thus, the task is not so much to see what no one has seen yet, but to think what nobody has thought yet, about that which everybody sees". Professor Ian Raymond Ball, a creative scientist, good polemist and excellent writer was the author of the paper (BALL, 1974a) and the quote seems to encapsulate his perspective on science. Ian died in Kingston, Jamaica, on 26th of January 2000 at the young age of 58.

One of Ian's main concerns was to bring some rationality into turbellarian classification, particularly within his favoured group the freshwater triclads, and to relate the phylogeny of the group with ecology and biogeography. He believed classification must be based on phylogeny and not vice versa; hence, recency of common ancestry and not phenetic distances have to be the core of classification. He was the first to bring cladistic analysis to triclad phylogeny and to extrapolate from it a sound biogeographical explanation of today's world-wide triclad distribution. He had a unified, holistic attitude to systematics and was assertive to any new development, conceptual, methodological or technical, helping to improve extant classifications. Thus, to the usual morphological characters, he added karyological characters from the mid 1970s, and embraced molecular techniques from the mid 1980s. Had it not been for several personal problems

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plaguing him since the late 1980s, which undermined both his health and his scientific career, the modern molecular and combined (morphology+molecules) approaches to triclad and turbellarian phylogeny may have had Ian Ball as one of the leading figures.

A BIOGRAPHICAL AND SCIENTIFIC SKETCH

Born in Southport (Lancashire, UK) on June 13, 1941, Ian Ball graduated in Zoology and Botany at the University of Liverpool in 1962. Up to May 1964 he served as Officer/Naturalist at the Letchworth Museum, Hertfordshire (UK) in charge of the educational services of the museum and carried out research on different freshwater invertebrates, planarians among them. His first publications on triclads stemmed from this period (Ball, 1964, 1965, 1967). From 1964-66 he held a position of Research Officer in the Huntington Research Centre (UK) studying the effects of drugs and pesticides on mammals, fish and aquatic invertebrates, and from 1966-67 as Assistant Experimental Officer at the Water Pollution Research Laboratory at Stevenage, UK, carrying out research on the lethal and sublethal effects of pollutants on fish and on biological surveys of polluted rivers.

In 1967 he moved to the University of Waterloo, Ontario (Canada) to undertake a Ph.D. thesis on the systematics and biogeography of freshwater Turbellaria. In April 1971 he was awarded the Ph.D. degree in Biology with a dissertation "A contribution to the Phylogeny and Biogeography of the Freshwater Triclads (Platyhelminthes, Turbellaria)" (Ball, 1971). A year earlier, he received a National Science Foundation award to read, as invited speaker, a summary of this work at the Libbie H. Hyman Memorial Symposium sponsored by the American Society of Zoologists, the Society of Systematic Zoology and the American Microscopical Society within the meeting of the AAAS in Chicago (USA). This work, published 4 years later (Ball, 1974a) has been extremely influential to people working on triclad taxonomy, phylogeny and biogeography. His research on Turbellaria continued as Postdoctoral fellow at the National Museum of Natural Sciences in Ottawa (1971-72) and as Assistant Curator at the Royal Ontario Museum in Toronto (1973-75). Between 1968 and 1976 Ian Ball published almost 30 papers related to turbellarian (mostly triclad) taxonomy and biogeography. Prominent among them are those in biogeography and plate tectonics (Ball & Fernando, 1969), on triclads from the Oriental Region (Ball, 1970), from Central and South America (Ball, 1971), from Australia (Ball, 1974b), a methodological paper on the nature and formulation of biogeographical hypotheses (Ball, 1975) and revisions and monographs on different genera of the family Dugesiidae: *Cura* and *Neppia* (Ball, 1974c) and *Spathula* (Ball, 1977a). He found time to travel and sample in Central America, Brazil, and Australia and to pay visits to different world museums. I regard the 10 years spanning from 1968 to 1977 as the most productive and influential of Ian's career.

Ian Raymond Ball (right) at the first ISBT Meeting, held in Chicago (1970), in honour of Libbie H. Hyman, in which he delivered a talk on his new taxonomy and biogeography of Tricladida. Left: Prof. Th. Lender: center: Prof. M. Kawakatsu. Photograph kindly provided by Prof. M. Kawakatsu.

In 1976, then 35 years old, he was appointed Reader of Special Zoology at the University of Amsterdam (The Netherlands) to become a Professor in 1980, holding the post up to 1985. At Amsterdam, Ball established a productive research programme and gained some funding for his own research. He attracted several promising students, some of them (e.g. Ronald Sluys) now at the forefront of triclad taxonomy and phylogeny. Always ready to challenge authority he followed his path, both personal and professional, with little regard to consequences. He could be fun and charming but also outrageous. His personality frequently drew attention and could engender both good and bad feelings amongst those with whom he interacted. Throughout this 'Dutch period', he extended his description of new species and genera of freshwater triclads from all over the world, and in particular from Australia, North and South America and Europe (Ball, 1977b, 1980; Ball & Tran, 1979; Ball et al, 1981; De Vries & Ball, 1980); reviewed and reshaped his views on the phylogeny of triclads (Ball, 1977c, 1981a); published a series of conceptual papers on taxonomy, phylogeny and biogeography (Ball, 1981b, 1982, 1983) and, with Professor Tom B. Reynoldson, co-authored the handy and useful book on British Planarians within the series of synopses of the British Fauna (Ball & Reynoldson, 1981). Additionally, he supervised the Ph.D. dissertations of his students Elisabeth De Vries and Ronald Sluys, on the taxonomy

and phylogeny of the genus *Dugesia* (De Vries, 1987), and on a taxonomical revision of maricolan triclads (Sluys, 1989). Ball also encouraged them to publish independently (see summaries of their publications in De Vries, 1987; and Sluys, 1989). Altogether, the numbers of papers of him and his team during the 'Dutch period' went up to 40.

In September 1985 he left Amsterdam and moved to the Memorial University of Newfoundland in St. John (Canada) as Professor of Biology and Head of the Department of Biology. There, he tried to initiate a group on molecular evolutionary studies and raise some funds. The outcome was close to a failure since, as far as I am aware, only a short technical note stemmed from that period. He became dispirited with science, and physical accidents and personal troubles soared. In 1992 he made his last professional move, becoming Professor at the Department of Zoology of the University of West Indies (UWI) in Kingston, Jamaica. Unfortunately he could not fulfil the trust UWI put on him to move the Department ahead. Personal and professional problems escalated beyond Ian's control.

SOME PERSONAL RECOLLECTIONS

I first met Ian in Amsterdam in 1979 though we had corresponded earlier. So impressed was I by his 1974 paper (Ball, 1974a) that in February 75 I wrote him a long letter praising his new taxonomy of triclads and dugesiids and his new biogeographical explanation of its world-wide distribution. However, I also expressed my dissent on several aspects of his phylogenetic tree of the genera of Dugesiidae. At that time, I firmly believed karyology was a highly important phylogenetic character. That made it difficult for me to understand his placement of the genus *Schmidtea* as a rather derived group compared to the basal position held in that tree for *Girardia* and related genera. He was kind enough to send me in May 1975 a long and beautiful letter. This letter was my first introduction to the new world of cladistics. I was impressed but, to be fair, not convinced. In 1977 he published in *Acta Zoologica Fennica* (Ball, 1977c) a somehow different view on the phylogeny of dugesiids. There, *Schmidtea* was placed close to *Cura* and to other basal genera, now in the new family erected by Ronald Sluys, the Dimarcusidae (Sluys, 1990). Later on, and recalling these letters, he said I was probably right on the placement of *Schmidtea* but for the wrong reasons. I think he was right. I had the intuition; he had the right methods, which, whilst we should follow them, do not always produce the correct result.

A bit later, and oddly enough, we joined forces on *Schmidtea*. In 1980, one of his students in Amsterdam, Elisabeth De Vries, found an asexual *Schmidtea* population in the island of Mallorca bearing an anomalous karyotype. Back in 1970, I had already observed a similar anomaly in an asexual population of *Schmidtea mediterranea* found in the city of Barcelona (Baguñà, 1973). In the first papers dealing with *Schmidtea mediterranea* (at that time *Dugesia mediterranea*), which I published together with Professor Benazzi and colleagues (Benazzi et al, 1972, 1975) this anomaly was not even mentioned. Professor Benazzi was, to say the least, not prone to even consider a chromosome anomaly as the cause of asexuality in this or any other species. He believed mendelian factors were the actual cause. Now, it is fair to say that asexuality may have different causes, some mendelian, some due to chromosomal changes. Elisabeth De Vries, Ian Ball, and I made a detailed analysis of the anomalous karyotypes of *Schmidtea mediterranea* from Barcelona and Mallorca, and together with rather wild speculations on this species distribution in the Mediterranean based on microplate dispersal, published it in 1984 (De Vries et al, 1984). The chromosomal anomaly, the unequal size of the third chromosomal pair, was interpreted as a duplication. Later on, in our lab in Barcelona, my student Maria Ribas showed very convincingly that such an anomaly was actually not a duplication but a translocation from one of the first pair chromosomes to one of the third pair (Ribas, 1990). These results were formally published, rather ironically, in a paper in tribute to professor Benazzi (Baguñà et al, 1999).

In February 1981 Ian visited Barcelona for the first time. He lectured on 'The problems of historical biogeography' and on 'Philosophy, phylogeny and classification'. Accidentally, his visit coincided with the last, and luckily failed, 'coup d'etat' in Spain. He told me he couldn't imagine I was so kind to organise such an event for him. Shortly after he moved from Amsterdam to Newfoundland (October 1985) I had the chance to referee a grant application from him to the NSERC of Canada: "Evolutionary Genetics and the Systematics and Biogeography of Freshwater Planarians". Even though the application preceded the seminal paper of Hillis & Davis (1986) on the utility of ribosomal DNA restriction patterns, Ball was advocating the use of this tool to place the Platyhelminthes among the Metazoa and to sort out the phylogenetic relationships among freshwater planarians. Although he was not acquainted with molecular techniques he had the feeling, and had the nose, that this was the right way to go. In that sense he was prescient. The last time I met Ian Ball was in Barcelona in January 1989.

From October 1990 I lost track of him. He did not attend the Hirosaki (1990), Abo (1993) and Brisbane (1996) ISBT Meetings. The only news from him came through Ronald Sluys and later on from Tim Littlewood who met him two or three times when vacationing in Jamaica.

In several ways, Ian Ball was a multifaceted figure. Besides science, he was very interested in classical music as listener, performer, teacher and critic. He wrote a book of piano music for beginners. He was fond of history and philosophy and seems to have been good at fencing (as a sport and during controversies). To me he was a friendly

guy, very brilliant, very good at arguing, and extremely good at writing. We clashed on particular musical tastes (he preferred the music-maker, pompous Handel to the true genius of my beloved J.S. Bach, and overrated some 19th and 20th century British composers). Nevertheless, we had a lot of fun together. But he also had a complex and troubled family life. He married twice and had three children and a step-son. And I know he had also, as everybody has, a dark side which other people know better than I do.

But as regards science is concerned, and to flatworm and triclad biology in particular, he made clear and significant contributions. He will be mainly remembered for his contributions on the taxonomy, phylogeny and vicariance biogeography of the Tricladida and, namely, the Dugesiidae, by his contributions to the knowledge of Australasian faunas, by starting to clear the mess of the *Dugesia gonocephala* species complex and by his contributions to systematic and taxonomic theoretical thinking. He published close to 70 papers and could have published a lot more were it not for his policy, rather uncommon those days and today, to encourage graduate students to publish independently. Although Ian Ball's contribution to turbellarian and evolutionary biology was highly influential and earned him an international reputation, I believe that his personal character and circumstances, from the mid 1980s, prevented the full realization of his talents when may be the best was yet to come.

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PHYLOGENY

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Searching for the stem species of the Bilateria

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ABSTRACT. Some recent molecular phylogenetic studies suggest a regrouping of the bilaterian superphyla into Deuterostomia, Lophotrochozoa (Lophophorata, Spiralia and Gnathifera) and Ecdysozoa (Cycloneuralia as the remaining Aschelminthes, and Arthropoda). In some of these trees Platyhelminthes have a more derived position among the Spiralia. On the other hand, taxa within or close to the Platyhelminthes have been singled out as possible plesiomorphic sister groups to all other Bilateria (Acoela and Xenoturbellida). For both proposals there exists conflicting evidence, both when different molecular features are compared and when molecular and phenotypic characters are used. In this paper we summarise the phenotypic models that have been proposed for the transition between diploblastic and triploblastic organisation (Planula- Phagocytella-Archicoelomate-, Trochaea-, Gallertoid-, Coeloplana-, Colonial- concept). With very few exceptions such models construct a vermiform organism (acoelomate/pseudocoelomate or coelomate) at the base of the Bilateria while the finding of similarities in the genetic regulation of segmentation in vertebrates and arthropods has stimulated the search for larger, more complexly designed ancestors. Because of the possible significance of vermiform organisation for understanding the origin of the Bilateria, we present new data concerning the development and evolution of the complex body wall muscle grid of platyhelminths and new findings on their stem cell system (neoblasts). We show that studying the various features of the development of the body wall in a variety of basal platyhelminths (eg. Acoela, Macrostomida) with cytological and molecular techniques would provide essential data for discussions of the diploblast/triploblast transition, because it is the central element in the biology of vermiform organisms.

KEY WORDS: Evolution of Bilateria, diploblastic/triploblastic transition, Acoela, Xenoturbellida, Platyhelminthes, vermiform organisation, body wall muscle differentiation, neoblasts.

INTRODUCTION

The reconstruction of the ancestral organisation of the Bilateria has regained interest rapidly with the accumulation of molecular data (see lit in PETERSON et al., 2000; PETERSON & DAVIDSON, 2000; ADOUTTE et al., 2000; JENNER, 2000). The recent proposals concerning the evolution of the early Metazoa illustrate the major shift in emphasis that has occured over the last decade in the search for clues for the major transitions in animal body plans. Especially the advances in understanding the evolution of Hox genes, from the bilaterian crown groups such as arthropods and vertebrates down to the level of the sponges, together with 18S rDNA sequences form a new backbone for interpretations of the evolution of the metazoan phenotype.

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This paper addresses three points in relation to this topic. We highlight some aspects of body plan evolution and the most recent proposals that Acoela and/or the Xenoturbellida may be the basal-most branches in the Bilateria. Secondly, we give an overview of models, in particular those using the organisation of certain platyhelminth taxa, for the origin of the Bilateria. Finally we give some examples from our own investigations (studies of the platyhelminth body wall musculature and of the neoblast system) which could contribute to an understanding of the origin of the Bilateria.

BODY PLAN EVOLUTION IN LOWER METAZOA AND RECENTLY PROPOSED SISTER GROUPS OF BILATERIA

One of the most significant changes in the phenotype to the level of the Eumetazoa certainly was the appearance of definite epithelial tissue. Steps involved here were specializations of apical junctional complexes to control the

paracellular flow across these covering cell sheaths, as well as the arrangement of the ECM at the base of these sheaths into complex molecular layers, the basal matrices (see RIEGER & WEYRER, 1998 for lit.). A more defined control of two fluid compartments is the result of this evolution: one is surrounded by the basal matrix (the mesogloea of dipoblasts, the primary body cavity, primitive blood vascular systems, and connective tissue fluids of triploblasts), the other one by the apical side of epithelial tissues (the gastrovascular system of diploblasts and the gut, secondary body cavities and duct systems for excretion and for reproduction in the triploblasts).

The most advanced level of eumetazoan organisation is seen in the triploblastic Eumetazoa, the Bilateria. Central for their evolution is the mesodermal tissue developing from the entoderm. This entomesoderm led to the further elaboration of muscle tissues and connective tissue. It occurs in two tissue grades: 1) in the coelomate organisation (the coelom or secondary body cavity) originally as myoepithelial lining, 2) in the acoelomate/pseudocoelomate organisation where muscle tissue is part of the connective tissue. It either fills the space between gut and body wall (acoelomate) or surrounds - in a non-epithelial organisation - fluid-filled compartments derived from the primary body cavity. In the life cycle of such animals, macroscopic adults with coelomic organisation often alternate with mm-sized, acoelomate/pseudocoelomate larvae.

According primarily to molecular studies, the Bilateria have been grouped into three major clades (ADOUTTE et al., 1999; KNOLL & CARROLL, 1999; VALENTINE et al., 1999; GAREY & SCHMIDT-RHEASA, 1998): 1) the Deuterostomia, originally with pseudocoelic larvae and enterocoelous adults, shown by many studies to be most similar to the coelenterate level of organisation (lit in CAMERON et al., 2000), 2) the Lophotrochozoa, which include in addition to the lophophorate phyla the taxon Spiralia and the former aschelminth taxa Rotifera and the Acanthocephala, with the Spiralia very likely representing a monophyletic subunit (BOYER et al., 1998; HENRY et al., 2000). It seems possible that indirect development with a trochophore-like larva is primitive also in the Lophotrochozoa (PETERSEN et al., 2000; PETERSEN & DAVIDSON, 2000). However, ROUSE (2000) concludes that only a lecithotrophic larva and not a planctotrophic trochophore may be plesiomorphic. 3) the Ecdysozoa, direct developers with secondary larvae due to the molting process. They include the Arthropoda and the taxon Cycloneuralia, the remaining aschelminth taxa.

While the concept of the Ecdysozoa is gaining recognition, very little is yet clarified when in comes to deriving segmentation of arthropods and annelids independently or from an unsegmented bilaterian stem species (see KNOLL & CAROLL, 1999; VALENTINE & COLLINS, 1999; JENNER, 2000). In the interpretation of the evolution of the Lophotrochozoa the question whether their common ancestor had an acoelomate/pseudocoelomate design or whether it was a coelomate animal also remains unsolved.

Based on 18S rDNA RUIZ-TRILLO et al. (1999) have singled out one lophotrochozoan taxon, the acoel flatworms, to represent the first branch among all Bilateria. JONDELIUS et al. (this volume) could resolve one major point of critique raised against that data set by showing that the nemertodermatids branch close to the basal branching point of the Acoela. Another recent molecular study using sequences of elongation factor 1-Alpha does not support such an early branching of the Acoela but suggests them to group within the Platyhelminthes (BERNEY et al., 2000).

Phenotypic evidence has led EHLERS & SOPOTT-EHLERS (1997) to suggest a similarly unique basal position for the enigmatic *Xenoturbella bocki* (see also LUNDIN, this volume). There are claims now that this species belongs to the Mollusca and is derived progenetically from protobranch larvae (ISRAELSSON, 1999; NORÉN & JONDELIUS, 1997). *Xenoturbella´s* basiepithelial nervous system and uniquely constructed extracellular matrix (PEDERSEN & PEDERSEN, 1986; 1988) remain an obstacle for morphologists to accept this proposal. In addition, complex character similarities in the epidermal ciliation link *Xenoturbella* and the Acoela (LUNDIN, 1998; this volume). Together with the digestive tract they represent an impressive morphological character sequence that at present can be read only from *Xenoturbella* to the Acoela with the Nemertodermatida as an intermediate taxon (see lit. in LUNDIN, 1998).

A recent cell lineage study shows that the Acoela lack ectomesoderm and that all of the musculature and peripheral parenchyma in the convolutid *Neochildia fusca* is of entomesodermal origin (HENRY et al., 2000). In this feature they are set apart from all other spiralians, but resemble the Ctenophora (MARTINDALE & HENRY, 1999). Ctenophores offer new possible scenarios for the origin of the Bilateria and the description of a fossil ctenophore with 80 comb rows is an unexpected incentive for reconsidering the ctenophores as the plesiomorphic sister taxon of the Bilateria (see MARTINDALE & HENRY, 1998 for lit).

PUBLISHED MODELS FOR THE TRANSITION FROM DIPLOBLASTIC TO TRIPLOBLASTIC ORGANISATION

In the main models proposed for the origin of the diploblastic organisation (Haeckel´s Gastraea-, Bütschli´s Placula-, Lankester´s Planula-, Metschnikoff´s Phagocytella and Gutman´s Gallertoid- hypothesis) original metazoan cell colonies are derived from flagellate unicellular eukaryotes that had developed the characteristic metazoan ECM (see RIEGER & WEYRER, 1998 for lit.). Monociliated cells with one functional cilium and an accessory centriole in a precisely defined position relative to the basal body of the functional cilium represent almost

certainly the plesiomorphic character state of all somatic cells in the early cell colonies.

For the transition from the diploblastic to the triploblastic organisation three groups of models can be distinguished:

The first bilaterians - vermiform organisms with direct development

Vermiform organisation is most often used in reconstructions of the bilaterian stem species. Such organisms have a cylindrical or flattened body profile and use their flexible body wall for locomotion (Fig. 1). The body wall contains helically wound fibers in the cuticle, in the cell web and/or in the basal matrix of the epidermis for retention of shape, and at least two layers of muscles – circular and longitudinal – intimately associated with the basal matrix (RIEGER, 1998). This body wall acts against the extracellular compartments formed by the gut, the pseudocoel and/or the coelom or against intracellular hydrostats of muscle and connective tissue in acoelomates.

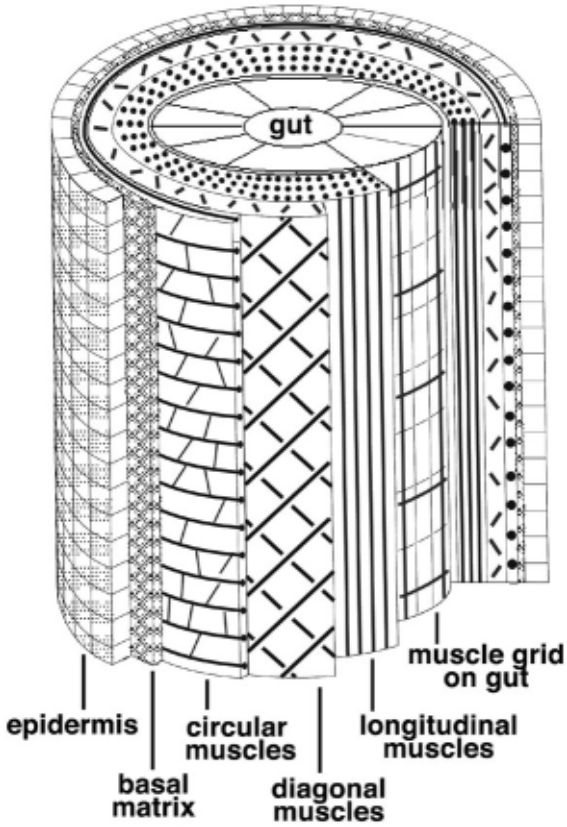

Fig. 1. – Diagram of vermiform body organisation in acoelomates.

Summaries of ideas for the transition at the diplo-/triplobast boundary can be found in REISINGER (1961), SALVINI PLAWEN (1978) and WILLMER (1990). Most widely used are the Planula and Phagocytella (Parenchymula)-concepts where acoels and nemertodermatids are model organisms for the original Bilateria. The main differences between the two models are the assumptions concerning the original organisation of the gastrodermis and the muscle tissue: in the Phagocytella hypothesis the gastrodermis is assumed to be a connective tissue including muscles. In the planula hypothesis gastrodermis and muscles are seen as derived from epithelial tissue.

A basiepithelial nerve plexus with first condensations of longitudinal cords is postulated as ancestral in both the Planula and the Phagocytella concept. The epidermal apical organ of the planula would be the structure giving rise to brain differentiations. In immunocytochemical preparations the organisation of the brain of various Acoela appears rather different from all other platyhelminth taxa (REUTER et al., 2001). These findings underscore the unique and possibly basal position of this taxon among living platyhelminths.

Other proposals are the Gastraea-Bilaterogastrea-concept and the Gastraea-Archicoelomate-concept. Both hypotheses propose an enterocoelic entomesoderm, envisioned to have originated from gastric pouches of the coelenterate organisation. Also these concepts assume a vermiform stem species for all Bilateria, however, this organism would have been a coelomate.

Vermiform organisation is as well assumed in two more hypotheses: LANG´s (1884) Coeloplana-concept and the Gallertoid concept by BONIK et al. (1976) and GUTMAN (1981).

The first bilaterians - vermiform organisms with a biphasic life cycle

The Trochaea hypothesis (Gastraea-Trochaea- concept, see lit. in NIELSEN, 1998), going back to the concept of the benthopelagic life cycle of Jägersten, assumes an acoelomate/pseudocoelomate vermiform ancestor for the protostome line of evolution and a vermiform archicoelomate ancestor for the deuterostome line of evolution. The vermiform benthic adults alternate in a life cycle with pseudocoelic pelagic larvae, the latter ones representing the phylogentically older body plan. This hypothesis is supported by the recent proposal resulting largely from developmental studies (lit. in PETERSON et al., 2000, see ROUSE, 2000 for different view).

The biphasic life cycle with a microscopic larva and a macroscopic adult is also central in the proposal for the ancestral metazoans by one of us (RIEGER, 1994). By progenesis several lines of evolution could have lead from the acoelomate and pseudocoelomate larvae or juveniles of an archicoelomate ancestor to adult acoelomates and pseudocoelomates respectively (see lit. in TYLER, 2000). It should be pointed out that any model using a planula at the diploblast/triploblast transition must be seen as considering progenesis: the planula organisation is only found as larval stage, no planula-like adult organism is known.

The first bilaterians - macroscopic organisms with complex body plan

Similarities in the genetic networks specifying segmentation in such distant groups as vertebrates and arthropods has led to the postulation of more complex macroscopic ancestors for the Bilateria (see discussion in KNOLL & CARROLL, 1999; JENNER, 2000). The scaffolding for this idea is seen in certain conserved genes involved in segmentation (see recent review concerning this issue in DAVIS & PATEL, 1999).

The latest attempt in trying to envision a more complex base for the bilaterian stem species has been presented by DEWEL (2000). Frond-like macroscopic colonial diploblasts, similar to organisms in the Ediacara fauna and to extant Pennatulacea serve as models. Colonial organisation is seen as a condition that might have led to a highly compartmented coelomate ancestor.

Considering all evidence, a vermiform bilaterian ancestor in the size of millimeters remains a realistic model. In the case of the Lophotrochozoa the ancestor could be similar to platyhelminths. The position of the Gastrotricha and the Gnathifera (Gnathostomulida, "Rotifera" and Acanthocephala) may be also close to the bilaterian origin. On the other hand, a cm-sized organism similar to *Xenoturbella* may have led to the evolution of archicoelomates in the sense of Jägersten. Progenesis of larvae or juveniles of such ancestors would have led to today's acoelomate and pseudocoelomate phyla (see TYLER, 2000 for lit.).

AVENUES FOR FUTURE RESEARCH

Given the central position of the vermiform organisation in hypotheses about the bilaterian stem species, as much as possible should be known about the development and organisation of organisms of such a design. In the following we report about work in our laboratory on three research topics related to this aspect.

The fate of the coelenterate aboral-oral axis in the Bilateria

The determination and specification of the bilaterian body axes and their role in development have received special attention again in assessing the evolution of the lower Metazoa (eg. MARTINDALE & HENRY, 1998; GOLDSTEIN & FREEMAN, 1997). Both the anterioposterior (A-P) axis and the dorsoventral (D-V) axis have been studied in detail in a variety of species. As far as the origin of these axes from the aboral-oral (A-B) axis of diploblasts is concerned, it is evident that we particularly need more information concerning the origin of the D-V axis.

In Fig. 2 two different processes that could lead from the A-B axis of a diploblast to the condition of a vermiform, bilaterian ancestor are depicted. In process 1) the diploblastic organism undergoes a 90-degree shift in relation to the substrate, subsequently the original mouth/anus moves forward to the ventral side. In process 2) a compression of the A-B axis takes place. Here the apical brain Anlage is moved forward, the mouth remains in the original position. As a result, the A-B axis is parallel with the bilaterian A-P axis in the first case, a new D-V axis must have been specified. In the second case the A-B axis becomes the D-V axis, a new A-P axis must have evolved in the Bilateria.

Such considerations are in line with models for the origin of the Bilateria mentioned above: The Planula-concept is a clear case of process 1 (REISINGER, 1961, fig. 10), whereas the Coeloplana-hypothesis of LANG (1884) is

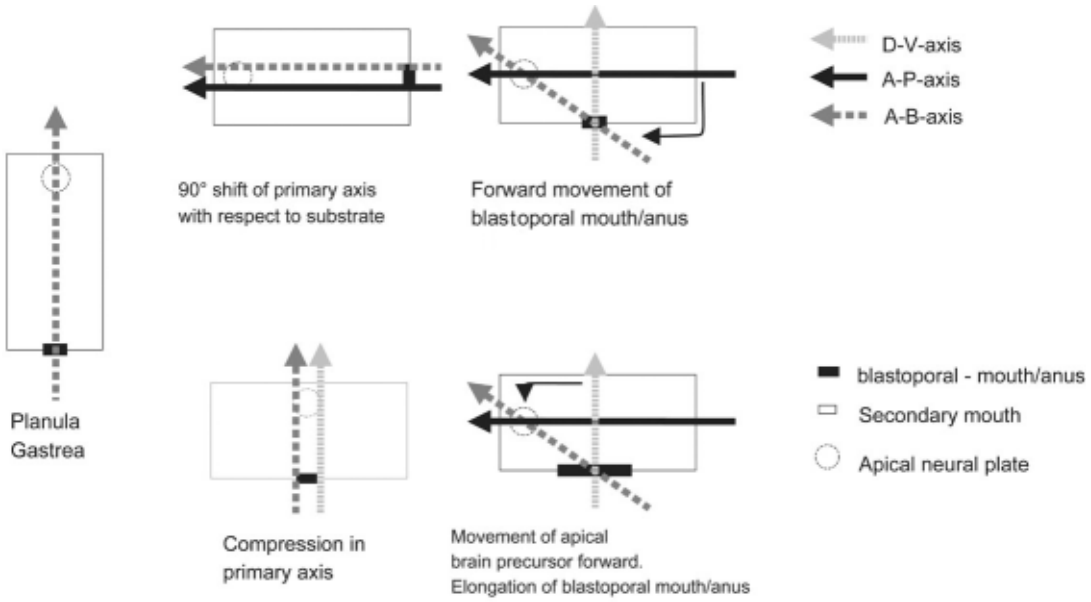

Fig. 2. – Diagram of shifts in body axis at the diploblast/triploblast transition. Upper line, process 1, lower line, process 2, see text for further explanation. Dashed arrow: Aboral-oral axis (A-B), black arrow: anterior-posterior axis (A-P), finely dashed arrow: dorso-ventral axis (D-V).

clearly a case of process 2. During the settling of the Trochaea the mouth remains in the original position and is elongated to form a longitudinal slit (Trochaea-hypothesis: NIELSEN, 1995). The apical plate moves forward, the A-B axis becomes inclined – this behavior and the blastopore in this model are in accordance with model 2.

To study processes that control shifts of the A-B axis we have started to work on the expression of the gene of the Wnt/wingless cascade in *Convolutriloba longifissura* and plan to do this also with *Convoluta pulchra* and *Macrostomum*-species, and the sog/chordin and bmp4/dpp gene family in acoel and macrostomid turbellarians. These genes are part of the genetic network specifying the D-V axis in higher vertebrates and arthropods (see lit. in HOBMAYER et al., in press). Such genes have now also been found in *Hydra*, being expressed in the hypostome in the vicinity of the mouth (HOBMAYER et al., in press). We will trace the expression in the formation of the platyhelminth mouth during embryonic development and during asexual reproduction. We expect that the expression pattern observed in *Hydra* near the mouth will also be found in platyhelminths and will make it possible to follow the separation of the A-B axis from the A-P axis, as depicted in Fig. 2.

In another way axis specificity can also be seen in the muscle development in acoels. Along this route we have studied the formation of the body wall musculature in embryos of *Convoluta pulchra* (LADURNER & RIEGER, 2000) and during asexual reproduction in *Convolutriloba longifissura* (data not shown).

In *Convoluta pulchra,* the cellular orientation relative to the A-P axis is highly specific during the establishment of what we called the original orthogonal muscle grid. Unexpectedly, circular fibers appeared distinctly prior to the longitudinal fibers, and they were oriented along well defined latitudes in embryos at about 50-55% of developmental time (Fig.3A). The A-P axis can be identified from the fiber arrangement because the formation of circular fibers starts closer to the apex of the animal. Since acoels lack any basal matrix of the epidermis, differentiating myocytes may become aligned along differing anterior-posterior densities and/or qualities of receptors in the basolateral epidermal membrane. Ultrastructural data on the cytology of myoblasts actually corroborate this notion (RIEGER, LADURNER, SALVENMOSER, unpublished).

At around 60 % of developmental time from egg laying the longitudinal muscle fibers are visible, forming the primary orthogonal muscle grid (Fig. 3B). As with the original circular fibers, the fibers of the longitudinal layer line up behind each other on defined meridians, in this case parallel to the A-P axis. Differences in this primary muscle grid between the dorsal and ventral side begin to become visible very early, already at about 60 % of developmental time.

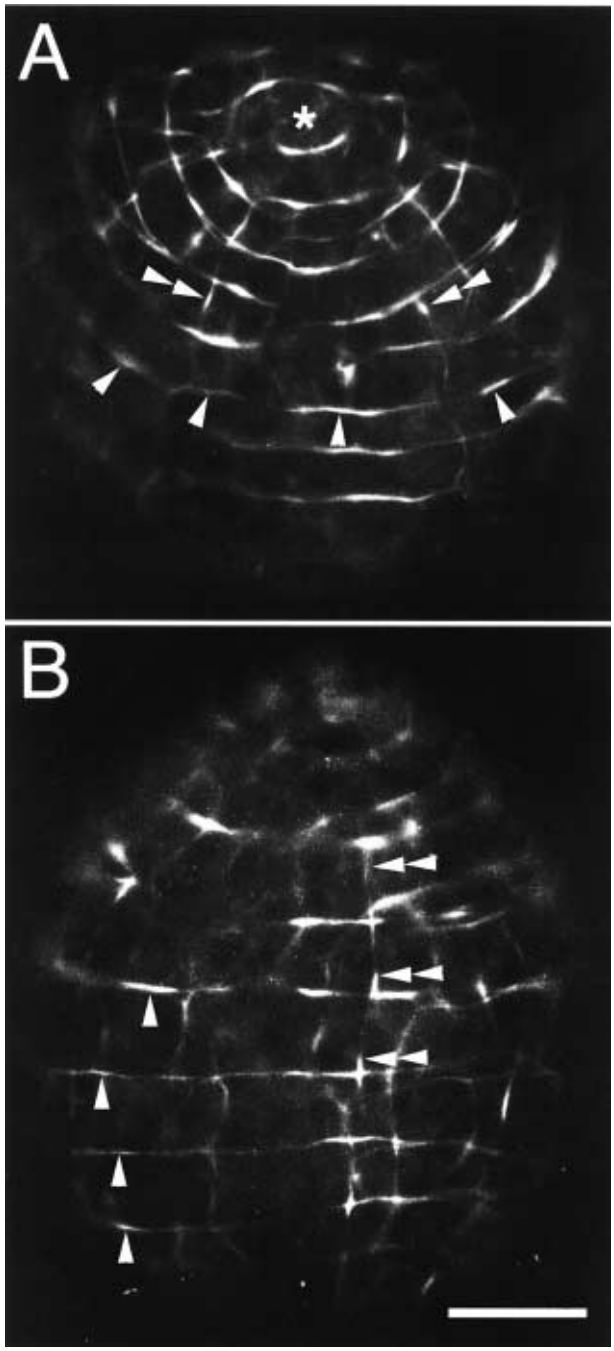

Fig. 3 A, B. – Phalloidin-Rhodamin-stained embryos of *Convoluta pulchra*. A: stage 3-4, about 55% developmental time since egg laying. Note anterior pole of embryo (*), circular muscle fibers aligned on meridians (arrowheads), first longitudinal fibers (double arrowheads). B: stage 4, about 60% developmental time since egg laying. Further development of circular fibers (arrowheads), longitudinal myocytes aligned along meridians (double arrowheads). See also LADURNER & RIEGER (2000). Scale bar: 20 µm.

Transition of the bilayered body wall musculature from two germ layers of diploblasts to one germ layer of tripbloblasts

The second research topic is concerned with the phylogenetic origin of the complicated adult body wall muscu-

latures of Platyhelminthes (see TYLER & RIEGER, 1999 for lit). From our ontogenetic studies (LADURNER & RIEGER, 2000) it seems reasonable to assume that an orthogonal pattern of circular and longitudinal muscles may represent indeed the ancestral condition. In the Acoela unexpected variations of fiber orientation in that orthogonal matrix had been shown (see TYLER & RIEGER, 1999 for lit.), although the responsible mechanisms have yet to be specified.

How did such a primary orthogonal body wall musculature evolve from the conditions seen at the coelenterate level? In the body of *Hydra* as in all cnidarians one finds vermiform parts with orthogonal muscle layers (Fig. 4, see LADURNER & RIEGER, 2000 for lit.). The body column, as well as the tentacles, are vermiform as defined above. Two orthogonal layers are present, the circular fibers being more delicate. All fibers are short when compared with fibers in the microturbellarians, which may run almost the entire length or circumference of the body.

Based on present evidence about body wall muscle fibers in platyhelminths, we can propose two different models for the evolution of the body wall musculature of tripoblasts from diplobasts (Fig. 5): a transition from the diploblastic stage with muscle layers in two germ layers (the cnidarian model), and the origin of all muscle layers from one germ layer (the ctenophore model).

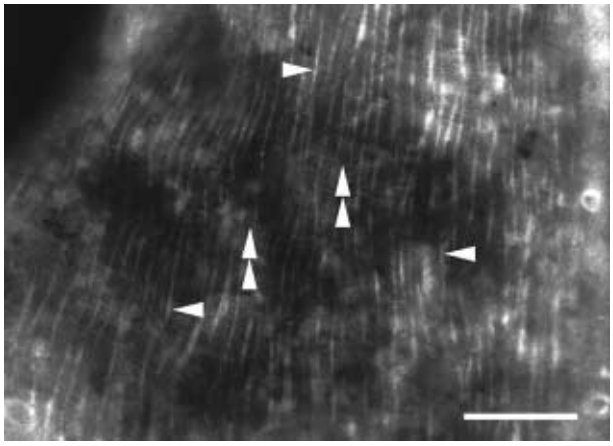

Fig. 4. – Portion of Phalloidin-stained tentacle of *Hydra* sp. showing orthogonal muscle grid. Note longitudinal fibers situated in the epidermis (arrowheads) and circular fibers (double arrowheads) situated in the gastrodermal epithelium. Scale bar: 50 µm.

In both models we assume an important role of the extracellular matrix for the process and start with a random orientation of the fibers, both in the epidermis and the gastrodermis. In the cnidarian model an inversion of the orientation of epidermal longitudinal and gastrodermal circular fibers would lead to a situation known from

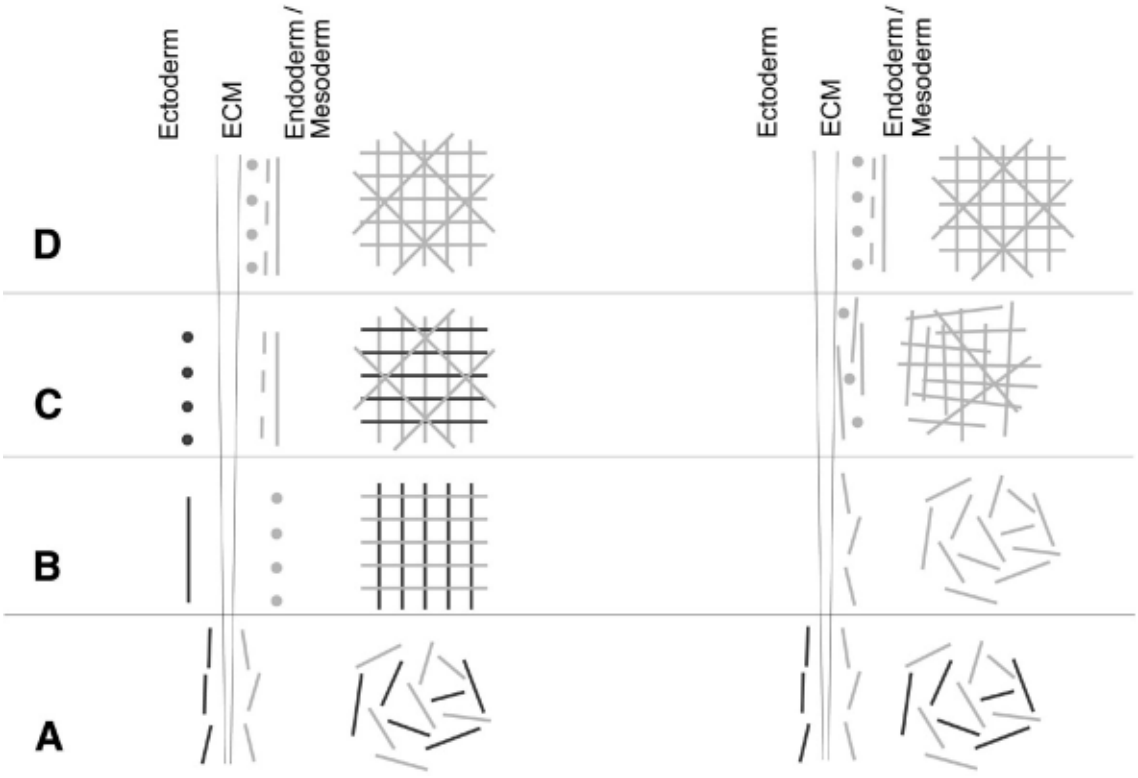

Fig. 5. – Schematic drawing of two possible ways of changes of the body wall muscle grid during the diploblast/triploblast transition (longitudinal sections and head-on views of muscle fibers). Ectodermal muscle fibers in black, entomesodermal muscle fibers in grey. The diagonal fibers are thought to be derived from the longitudinal muscle layer. A-D: evolution from randomly oriented fibers in two germ layers to a muscle grid with outer circular, inner longitudinal and intermediate diagonal fibers in one germ layer. Cnidarian model at left, ctenophore model at right; see text for explanation.

cell lineage experiments in the Müller´s larva of *Hoploplana inquilina* (BOYER et al., 1996; 1998) where the circular muscles, closest to the epidermis, are of ectomesodermal origin while the deeper longitudinal fibers are entomesodermal. A gradual substitution of ectomesodermal muscles by entomesodermal ones is assumed to have occurred in adult spiralians.

Alternatively only the original entodermal muscle grid was retained and became organised later into the orthogonal pattern of the body wall in ancestral bilaterians. This proposal is more in line with the body wall musculature being derived entirely from the entoderm, as has been shown by MARTINDALE & HENRY (1999) for the Ctenophora and by HENRY et al. (2000) for acoels.

The neoblast stem cell system

Investigating the unique neoblast system of the Platyhelminthes with a combination of labelling techniques (eg. BrDU and immunogold-labelling) might reveal another useful set of data for clarifying changes at the diploblast-triploplast transition. As of now (summarized in LADURNER et al., 2000), evidence is increasing that all somatic cells in Platyhelminthes do originate from one cell type. However, more data especially from Acoela, Nemertodermatida and Catenulida are needed before definite conclusions can be drawn (GSCHWENTNER, unpubl. data).

Such a single cell renewal system is not found among the most primitive metazoans, the Parazoa, Placozoa and the coelenterate phyla. What the basal Metazoa seem to suggest is that a combination of the ability of tissue cells to retain mitotic activity and to establish certain stem cell lines is the plesiomorphic condition among the lower Metazoa. Therefore, the neoblast stem cell system may be an autapomorphic trait of the Platyhelminthes.

CONCLUSION

It is of course evident that only the input from many lines of research will lead to further satisfactory progress in reconstructing the body plan of the ancestral bilaterians. As many characters of diploblastic and basal triploblastic animals as possible should be compared, and problematic taxa cannot be disregarded. Contrary to claims in certain molecular studies (ADOUTTE et al., 1999) we think that resolving the original design of the bilaterian body cavity (acoelomate/pseudocoelomate versus coelomate) is still crucial. The free-living platyhelminths are central for answering this question, investigating them with a combination of molecular, cellular, developmental and morphological approaches will be necessary.

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Platyhelminth phylogenetics – a key to understanding parasitism?

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ABSTRACT. The comparative method, the inference of biological processes from phylogenetic patterns, is founded on the reliability of the phylogenetic tree. In attempting to apply the comparative method to the understanding of the evolution of parasitism in the phylum Platyhelminthes, we have highlighted several points we consider to be of value along with many problems. We discuss four of these topics. Firstly, we view the group at a phylum level, in particular discussing the importance of establishing the sister taxon to the obligate parasite group, the Neodermata, for addressing such questions as the monophyly, parasitism or the endo or ectoparasitic nature of the early parasites. The variety of non-congruent phylogenetic trees presented so far, utilising either or both morphological and molecular data, gives rise to the suggestion that any evolutionary scenarios presented at this stage be treated as interesting hypotheses rather than well-supported theories. Our second point of discussion is the conflict between morphological and molecular estimates of monogenean evolution. The Monogenea presents several well-established morphological autapomorphies, such that morphology consistently estimates the group as monophyletic, whereas molecular sequence analyses indicate paraphyly, with different genes giving different topologies. We discuss the problem of reconciling gene and species trees. Thirdly, we use recent phylogenetic results on the tapeworms to interpret the evolution of strobilation, proglottization, segmentation and scolex structure. In relation to the latter, the results presented indicate that the higher cestodes are diphyletic, with one branch difossate and the other tetrafossate. Finally, we use a SSU rDNA phylogenetic tree of the Trematoda as a basis for the discussion of an aspect of the digenean life-cycle, namely the nature of the first intermediate host. Frequent episodes of host-switching, between gastropod and bivalve hosts or even into annelids, are indicated.

KEY WORDS: Platyhelminthes, parasitism, phylogenetics, Monogenea, Cestoda, Digenea, gene trees, life-history evolution

INTRODUCTION

Phylogenies aim to reveal patterns of inter-relatedness and the radiation of constituent taxa. Inferring evolutionary processes from phylogenies, the comparative method (HARVEY & PAGEL, 1991), is a well-established practice and one that has become increasingly more refined as our understanding and development of tree-building methods improves, and more popular as new sources of comparative data, particularly molecular, become available (e.g. PAGEL, 1998). In spite of the early seminal work on platyhelminth phylogenetics coming from turbellarian workers (e.g. EHLERS, 1985a,b) it has been the parasitologists who have embraced the technique wholeheartedly. The nature of parasitism, its commercial and medical consequences and the wealth of comparative information concerning parasites no doubt allow greater scope. Regarding flatworms in this light, perhaps the most significant milestone has been the publication of *Parascript* (BROOKS & MCLENNAN, 1993), a book based on the phylogenetic assessment of numerous parasitic platyhelminths, in which the authors used trees as the basis for addressing and testing many long-standing "myths" associated with the study of evolutionary parasitology. The eloquent story-telling, unravelling of myths and ultimate enlightenment as each tree yields its secrets, herald a most compelling union between systematics and comparative flatworm biology. Alas, the book has not been without its critics as the foundations upon which it draws its strength, namely its trees and the characters that form them, have been denounced, reproached and debated sufficiently (e.g.

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Pearson, 1992; Caira, 1994; Rohde, 1996) to erode them and to induce caution or suspicion. Indeed, whilst phylogenetics clearly holds the key to unravelling evolutionary mysteries, a review of the flatworm literature reveals few single trees that consistently and strictly bifurcate, satisfy all those scrutinising their foundations (i.e. homology assessment, character definition and coding), or provide topologies congruent with those trees derived from additional, independent sources of data.

Whilst congruence and consensus provide us with the necessary confidence to proceed with the comparative method, it is always compelling to take stock of the phylogenetic trees available and interpret the biological consequences of accepting them. Here we take four examples from our own interests and show how the evolution of parasitism can be interpreted from available phylogenies. The first example dwells on the variety of phylum-wide phylogenies and the consequences of accepting any one of them, highlighting the need for congruence and consensus. The second emphasises the need to treat any conflict between gene trees and species trees with care, reviewing the apparent paraphyletic status of the Monogenea based on molecular data. The third and final examples demonstrate the power and frustration of phylogenetics in interpreting the evolution and radiation of the most speciose of platyhelminth and parasitic groups, the digeneans and cestodes. All the examples pose more questions than they answer, hence the title of this contribution.

THE EXAMPLES

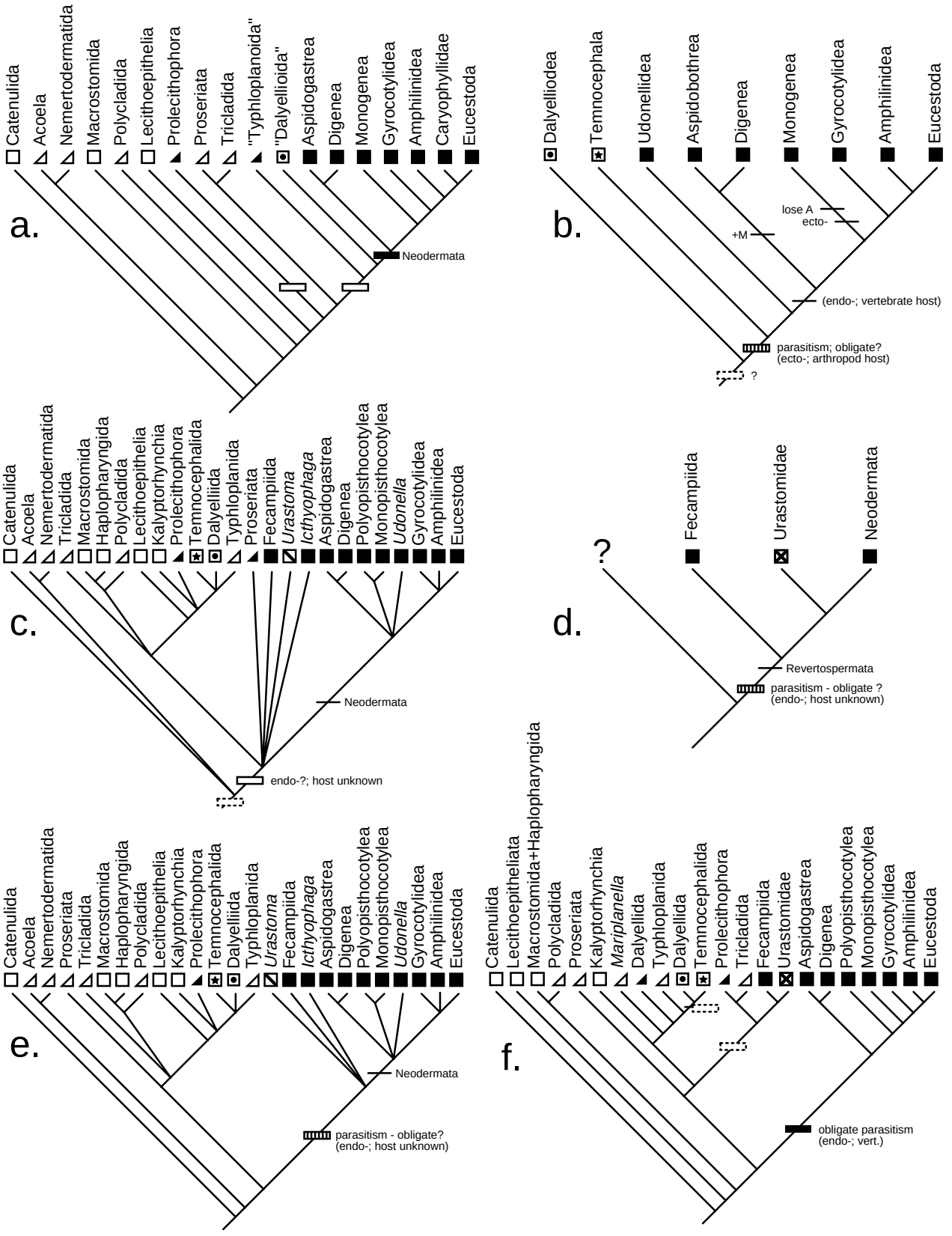
Example 1. Origins and evolution of parasitism in the Phylum Platyhelminthes

The term 'parasitism' means different things to different workers, yet it is generally accepted that an association between host and parasite is to the detriment of the host and the benefit of the parasite. Commensals live in close association with other organisms and, although their existence may appear inextricably linked with another organism, their survival is not thought to depend fully on the association. In contrast, obligate parasites require hosts for the completion of their life-cycle and generally derive some or all of their nutrients from their host during the parasitic phase. The Neodermata include the most familiar of obligate flatworm parasites, and the very presence of a neodermis appears to be inextricably linked at the physical, biochemical and immunological levels (Tyler & Tyler, 1997) with host-parasite interactions that protect and nurture the parasite. Arguably, the neodermis contributes most significantly to the success of the neodermatan taxa. Nevertheless, many 'turbellarians' are found in close association with other taxa; 200 species from 35 families live in permanent association with other animals according to Jennings (1971; 1974). A few groups within this paraphyletic assemblage include flatworms that are also exclusively parasitic: members of the Urastomidae (Rohde, 1994a), and the Fecampiida (Williams, 1988). Members of the Temnocephalida are almost exclusively ectocommensals on a variety of hosts, but predominantly freshwater crustaceans (Cannon & Joffe, in press), and appear to be the most modified of turbellarians for this mode of life. However, among the remaining turbellarian taxa that include species living in close association with other animals, few demonstrate obvious morphological adaptations to a parasitic way of life, such as the development of attachment organs, although many show nutritional and respiratory adaptations to parasitism (Jennings, 1997). The incidence of commensalism is high and few higher order taxa of flatworms appear to include exclusively free-living species. One might argue that generally, the phylum demonstrates a propensity towards parasitism.

Fig. 1 illustrates a selection of six phylogenetic hypotheses that each has a bearing on our understanding of parasitism and commensalism in the phylum (see legend for full details). Figs 1a-e illustrate phylogenies that are based solely on morphological characters, and Fig. 1f represents a tree derived from small subunit (SSU) ribosomal DNA. Ehlers' pectinate scheme (Ehlers, 1985a,b) illustrates the major single evolutionary event that was the emergence of the Neodermata; Fig. 1a. His grouping of the "Dalyellioida" includes many taxa with molluscan, annelid and crustacean hosts, and its sister-group status to the Neodermata would indicate a common ancestry that, based on numbers, has a proclivity for parasitism. Identification of sister-groups is the basis of phylogenetics, and therefore also the comparative method. Brooks' scheme, shown in Fig. 1b (Brooks et al., 1985; Brooks & McLennan, 1993), argues for ectoparasitism and an arthropod host as plesiomorphic conditions for the obligate parasites. Whilst from a strictly parasitological point of view the move from ecto- to endoparasitism seems compelling, the evidence uniting temnocephalans as sister-group to the Neodermata is contentious (Rohde, 1994b; Littlewood et al., 1999a; Cannon & Joffe, 2001). Additionally, both morphological (Boeger & Kritsky, 2001) and molecular data (Littlewood et al.,

Legend to Fig. 1 (see opposite page)

Phylogenetic trees from various sources illustrating the interrelationships of key parasitic platyhelminths, with an indication of the life-history strategy of constituent taxa and the possible origins of obligate parasitism; from a. Ehlers (1985a) based on morphology; b. Brooks & McLennan (1993) based on morphology, indicating intermediate host use and endo/ecto parasitism in/on final host; c. solution based on Rohde's initial matrix argued in Littlewood et al. (1998); d. the interrelationships of the Revertospermata, argued by Kornakova & Joffe (1999); e. solution based on revised matrix of Rohde, argued in Littlewood et al. (1998); f. maximum parsimony solution of 270 complete SSU rDNA genes, excluding acoelomorphs in Littlewood & Olson (2001). Symbols indicate the frequency of commensals and parasites within taxa – see key.


a.
Catenulida
Acoela
Nemertodermatida
Macrostomida
Polycladida
Lecithoepithelia
Prolecithophora
Proseriata
Tricladida
"Typhloplanoida"
"Dalyellioida"
Aspidogastrea
Digenea
Monogenea
Gyrocotylidea
Amphilinidea
Caryophyllidae
Eucestoda
Neodermata
b.
Dalyelliodea
Temnocephala
Udonellidea
Aspidobothrea
Digenea
Monogenea
Gyrocotylidea
Amphilinidea
Eucestoda
lose A
ecto-
+M
(endo-; vertebrate host)
parasitism; obligate?
(ecto-; arthropod host)
?
c.
Catenulida
Acoela
Nemertodermatida
Tricladida
Macrostomida
Haplopharyngida
Polycladida
Lecithoepithelia
Kalyptorhynchia
Prolecithophora
Temnocephalida
Dalyellioida
Typhloplanida
Proseriata
Fecampiida
Urastoma
Icthyophaga
Aspidogastrea
Digenea
Polypisthocotylea
Monopisthocotylea
Udonella
Gyrocotylidea
Amphilinidea
Eucestoda
Neodermata
endo-?; host unknown
d.
?
Fecampiida
Urastomidae
Neodermata
Revertospermata
parasitism - obligate ?
(endo-; host unknown)
e.
Catenulida
Acoela
Nemertodermatida
Proseriata
Tricladida
Macrostomida
Haplopharyngida
Polycladida
Lecithoepithelia
Kalyptorhynchia
Prolecithophora
Temnocephalida
Dalyellioida
Typhloplanida
Urastoma
Fecampiida
Icthyophaga
Aspidogastrea
Digenea
Polypisthocotylea
Monopisthocotylea
Udonella
Gyrocotylidea
Amphilinidea
Eucestoda
Neodermata
parasitism - obligate?
(endo-; host unknown)
f.
Catenulida
Lecithoepitheliata
Macrostomida+Haplopharyngida
Polycladida
Proseriata
Kalyptorhynchia
Mariplanella
Dalyellida
Typhloplanida
Dalyellida
Temnocephalida
Prolecithophora
Tricladida
Fecampiida
Urastomidae
Aspidogastrea
Digenea
Polypisthocotylea
Monopisthocotylea
Gyrocotylidea
Amphilinidea
Eucestoda
obligate parasitism
(endo-; vert.)
free-living
includes some commensals
includes some parasites/commensals
predominantly parasites/commensals
exclusively parasites/commensals
facultative parasites
includes fac./obl. parasites
obligate parasites
+M gain mollusc intermediate host (Trematoda)
lose A arthropod host lost
ecto- ectoparasitic adult
endo- endoparasitic adult
possible origin of parasitism?
origin of parasitism
possible origin of obligate parasitism?
origin of obligate parasitism

1998) argue strongly for udonellids as monopisthocotylean monogeneans. Brooks' eloquent interpretations based on the phylogeny of the 'cercomerians' fails at the point where inferences are drawn on the basis of the positions of Temnocephalida and Udonellidea as critical polarising sister taxa, simply because their position is so poorly supported. Indeed, it is the identity of the sister-group to the Neodermata that is pivotal to the discussion of obligate parasitism.

ROHDE's explicit morphological character matrix, constructed for a phylum-wide estimation (in LITTLEWOOD et al., 1999a), failed to resolve a clear sister-group candidate to the Neodermata (Fig. 1c), in contrast to his earlier prediction based on a non-cladistic assessment, that the Fecampiida occupied this position (ROHDE, 1990). Considering this unresolved, highly polytomous tree, we may infer that the origin of parasitism is as likely to be at the radiation of the Rhabditophora as it is to be at the radiation of the Neodermata. Most recently, the examination of sperm morphology suggested that Urastomidae is the sister-group to the Neodermata with Fecampiida completing a clade of obligate parasites, the Revertospermata (KORNAKOVA & JOFFE, 1999); see Fig. 1d. Fecampiids are parasites of decapod and isopod crustaceans (JENNINGS, 1971), and urastomids are parasites of molluscs and teleosts. If members of the Urastomidae and Fecampiida are truly the closest living relatives of the neodermatans, then it is not possible to predict the plesiomorphic host phylum of the Revertospermata, although, as all revertospermatans are found within their host's tissues, they would presumably have been endoparasitic. Given the very different nature of parasitism and host identity among members of Urastomidae and Fecampiida, these large-scale phylogenetic assessments suggest that finer phylogenetic resolution of these taxa would be well worth pursuing, particularly with a view to tracking the appearance of obligate parasitism. *Ichthyophaga*, unlike other urastomids, is an obligate parasite found embedded in the tissues of teleost fishes and thereby shares features with the predicted ancestor of the Neodermata (LITTLEWOOD et al., 1999b).

The inclusion of the 'revertospermatan' sperm data into ROHDE's morphological matrix yielded a more resolved tree (LITTLEWOOD et al., 1999a) suggesting a Urastomidae+Fecampiida clade as sister-group to the Neodermata; Fig. 1e. The wide distribution of parasites and commensals within the remaining large neoophoran turbellarian clade once again does not rule out the possibility that parasitism originated at the base of the Rhabditophora.

Finally, SSU rDNA sequences of 270 taxa (LITTLEWOOD & OLSON, 2001) maintain the accepted interrelationships of the Neodermata, albeit with the Monogenea paraphyletic (see Example 2 below), with a distinct but poorly supported sister-group of neoophoran turbellarians, and notably including highly derived Fecampiida+Urastomidae. There are few unambiguous morphological characters that support the major groupings of the turbellarians in this molecular tree. Furthermore, obligate parasitic turbellarian groups are dispersed throughout the tree, suggesting no fewer than three distinct origins of obligate parasitism, although again, from a parsimony principle it is equally likely that obligate parasitism was the plesiomorphic condition for the Rhabditophora.

Which scenario is correct? Until the incongruence between the independently derived molecular and morphological trees can be reconciled, and a morphologically based matrix can be refined and improved, including additional information and refinements that reflect the problems associated with character coding (e.g. FOREY & KITCHING, 2000), we are left with few well-resolved clades that reflect the phylogenetic content of independent data sets. Notably, Brooks' interpretation of the radiation of the Neodermata (BROOKS & MCLENNAN, 1993; LITTLEWOOD et al., 1999b) holds true as the interrelationships of its constituent groups appear well resolved (but see Example 2 below). However, the origins of parasitism throughout the phylum cannot, as yet, be recovered. Indeed, if it is the case that a large clade of neoophoran turbellarians is truly the sister-group to the Neodermata, we will neither be able to predict the plesiomorphic host nor have much confidence in whether the first parasites were ecto- or endoparasitic. Nevertheless, the divergence patterns of the neoophoran turbellarians will still hold a key to understanding the prevalence and radiation of parasitic taxa.

It is generally accepted that once a lineage embarks upon parasitism as a way of life (certainly endoparasitism), there is no going back, and there appear to be few phylogenies of any taxonomic group that suggest the adoption of parasitism is a reversible process (see SIDDALL, 1993, and references therein). Even in the Nematoda, a group that includes many parasites and free-living species throughout its constituent taxa, interpretations of phylogenies based on evolutionary parsimony suggest multiple origins of parasitism rather than secondary loss of parasitism (DORRIS et al., 1999). At what point is the irreversibility set? Presumably, only when a species is truly an obligate (endo)parasite, of which there are only a few well-proven examples in the turbellarian flatworms. Thus, although many of the phylogenies in Fig. 1 suggest the appearance and disappearance of commensalism and association with a host on multiple occasions, until finer phylogenetic resolution of the obligate turbellarian parasites is established, we cannot fully resolve the number of times obligate parasitism has arisen in the phylum, nor test whether it may be a reversible process.

Example 2. Monogeneans, morphology, molecules and the question of monophyly

The interrelationships of the Neodermata are well argued from morphology (EHLERS, 1985b), and preliminary combined morphological and molecular evidence is wholly

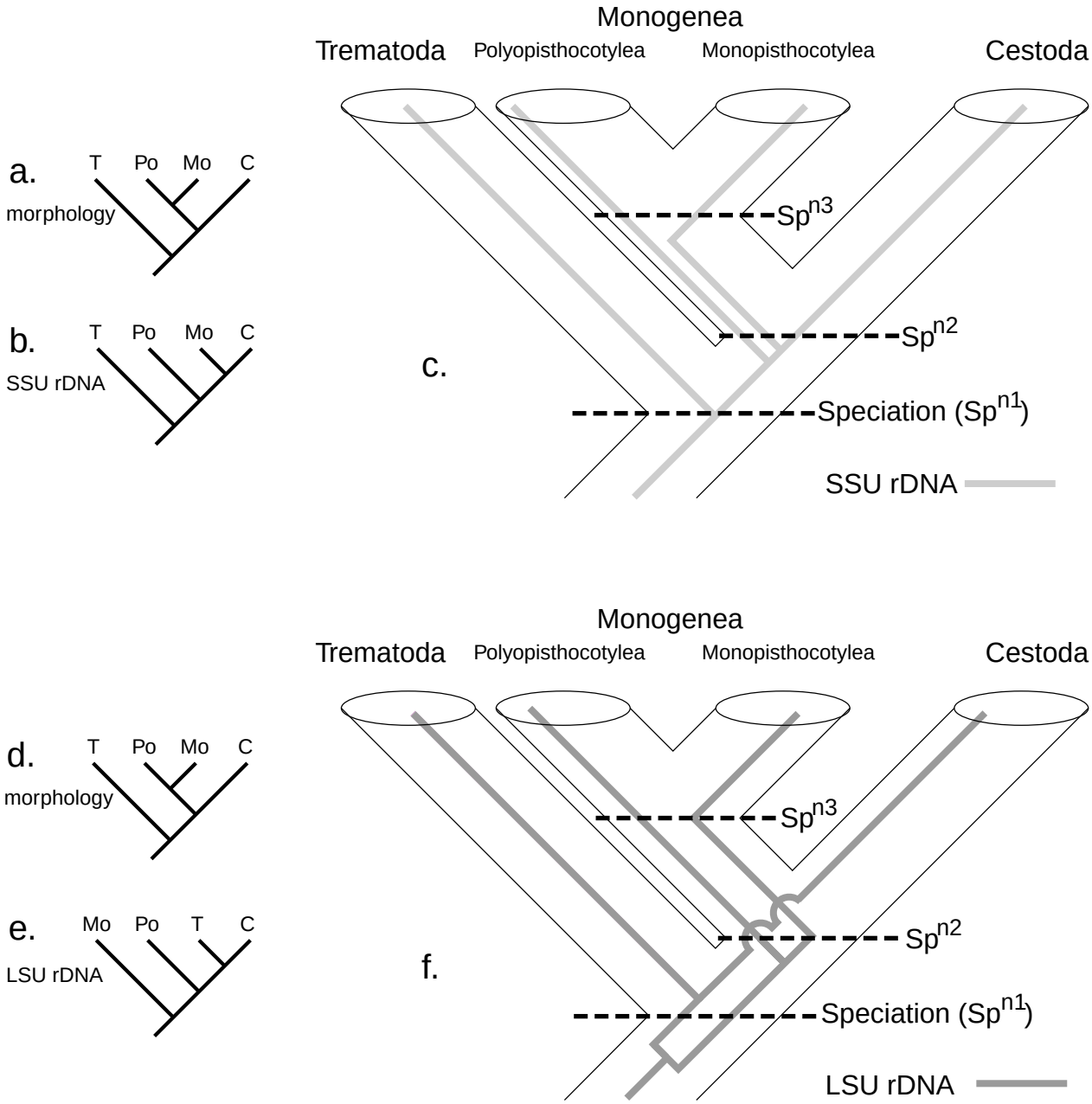


Fig. 2. – Species and gene trees for the Monogenea. Most morphologically based phylogenies argue for monophyly among the Monogenea (a, d). SSU rDNA consistently argues for a paraphyletic solution (LITTLEWOOD et al., 1999a; LITTLEWOOD & OLSON, 2001) with Polyopisthocotylea as basal monogeneans (b). When mapped on to the morphology tree, deep coalescence of the SSU gene prior to the divergence of monogeneans and cestodes, such that the genes had already split during the stem-lineage of the Monogenea, could account for incongruence (c). However, LSU rDNA (e) argues for a very different solution to morphology (e) and problems in lineage sorting are unlikely to account for the incongruence (f). Speciation events (Sp^{n1-3}) are discussed in the text.

congruent (LITTLEWOOD et al., 1999a) with the traditional view (Fig. 2a). Recent morphological data strongly support the contention that the Monogenea is a monophyletic group (BOEGER & KRITSKY, in press), but ribosomal DNA consistently supports paraphyly (MOLLARET et al., 1997; LITTLEWOOD et al., 1998). Disturbingly, the order of paraphyly depends on the gene utilised (SSU and LSU offer different results; Fig. 2b,e) and whilst a call for morphological reassessment is clearly justified (JUSTINE, 1998), in the absence of additional molecular or morphological evidence to support paraphyly, the three possibilities (Fig. 2a, b, e) need to be addressed independently or reconciled. It is worth noting here that LITTLEWOOD et al. (1999b) incorrectly reported that the difference between SSU and LSU was simply the relative placement of the Monopisthocotylea and the Polyopisthocotylea (compare Fig. 2b and 2e); MOLLARET et al.'s (1997) interpretation of neodermatan interrelationships suggested that Trematoda and Cestoda were sister-groups.

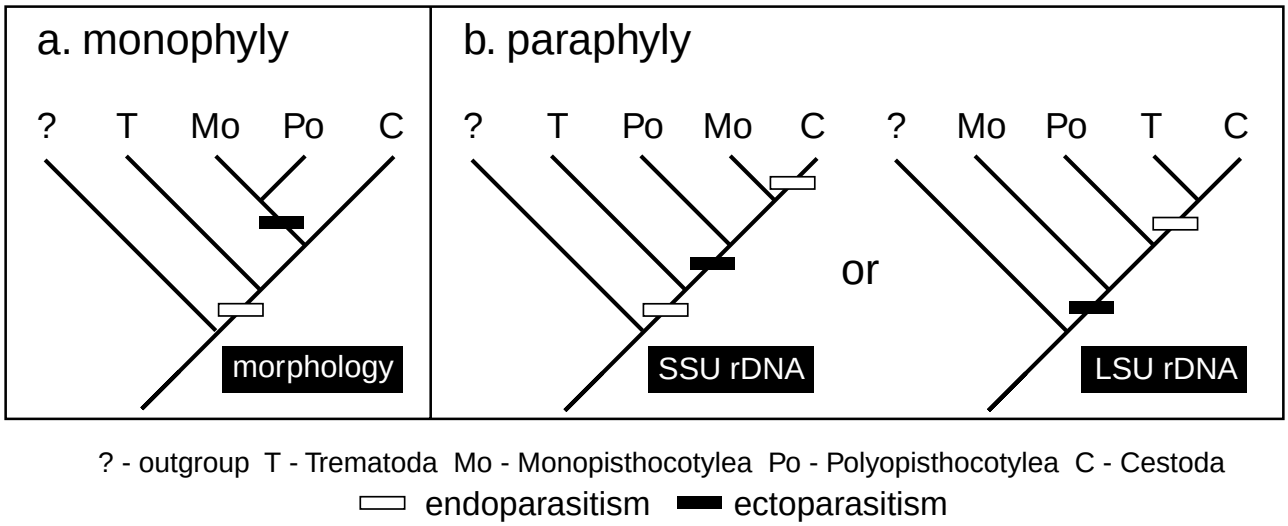


Fig. 3. – Possible origins and evolution of endo- and ectoparasitism in the Neodermata depending on the monophyly/paraphyly of the Monogenea and interrelationships estimated from a. morphology (e.g. EHLERS, 1985b), and b. molecular data; SSU rDNA (e.g. LITTLEWOOD et al., 1999a) and LSU rDNA (MOLLARET et al., 1997).

From an evolutionary parsimony perspective where the number of changes is minimised, a monophyletic Monogenea suggests that the shift toward ectoparasitism was a single event, even when we are uncertain of the sister-group to the Neodermata; Fig. 3a (LITTLEWOOD et al., 1999b). Paraphyly (Fig. 3b) requires that if endoparasitism was the plesiomorphic condition, suggested by morphology and SSU rDNA (LITTLEWOOD et al., 1999b), ectoparasitism arose at the base of the Cercomeromorphae (Cestoda+Monogenea), and along the cestode lineage endoparasitism was reacquired. However, if ectoparasitism was the plesiomorphic condition, suggested by LSU rDNA, then endoparasitism appeared just once, with the divergence of the Trematoda and Cestoda. Intuitively, in the case of paraphyly we might predict that ectoparasitism was the more likely plesiomorphic condition for the Neodermata, unless one considers the neodermis a likely adaptation to endoparasitism, but it seems highly unlikely that Trematoda and Cestoda are sister-groups (e.g. see BYCHOWSKY, 1937; ROHDE & WATSON, 1995; LITTLEWOOD et al., 1999b). Furthermore, the monophyly of the Monogenea remains the favoured solution as neither paraphyletic tree based on molecular data is particularly well supported at its base, there are a number of well-argued morphological synapomorphies for the Monogenea, and paraphyly requires a greater number of life-style switches, at least for the SSU data. If the gene trees are a correct estimation of the divergence of SSU and LSU rDNA, how do we reconcile the morphological solution? Figs 2c and 2f map each of the gene trees onto the morphologically based species tree. A number of possibilities exist for having a gene tree differing from a true species tree and include gene duplication, problems in lineage sorting, and horizontal gene transfer (PAGE & HOLMES, 1998). For the purposes of this discussion we will not discuss the last and, although ribosomal gene paralogy is known with SSU rDNA in triclads (CARRANZA et al., 1996; CARRANZA et al., 1999), there is currently no evidence to suggest this has occurred within the Neodermata. Lineage sorting problems, detected by tracing gene phylogenies back in time, relate to the failure of gene alleles to coalesce before the time when species diverge. In the words of SLOWINSKI & PAGE (1999, p.815) "deep coalescence can produce conflict between a gene tree and the overlying species tree because there is a window of opportunity for a sequence from a less related species to coalesce with one of the descendant sequences of the deep coalescence". However, invoking deep coalescence as the basis for gene and species tree discrepancies for the Monogenea is not wholly satisfactory. The scenario presented for SSU rDNA (Fig. 2c) suggests that between the second and third speciation events (Sp^{n2} and Sp^{n3}), the SSU genes that evolved within the stem lineage of the Monogenea have a more ancient coalescence time, which pre-dates the age of the lineage (see PAGE & HOLMES, 1998 for further examples and rationale), and whilst this single example remains plausible, the situation for LSU rDNA appears highly tenuous (Fig. 2f). The simpler, but incorrect, interpretation of LSU-based paraphyly (Fig. 6b in LITTLEWOOD et al., 1999b) could be explained by deep coalescence time in a way similar to that proposed in Fig. 2c (with the gene lineages of the Monopisthocotylea and Polyopisthocotylea transposed). However, whilst SSU and morphology based trees are compatible under lineage sorting problems, LSU is compatible with neither. LSU and SSU ribosomal genes are members of the same tandemly repeated chromosomal arrays and we would expect concerted evolution to at least provide congruent gene trees (HILLIS & DIXON, 1991).

Another, perhaps more powerful interpretation is that there must have been a relatively short period between the monogeneans diverging from the cestodes and when this stem lineage split into the Monopisthocotylea and

Polyopisthocotylea. Molecular changes accumulated within the stem lineage of the Monogenea have either been obliterated through base saturation, or were very few, and cannot be resolved satisfactorily with either nuclear ribosomal gene fragment. It remains that morphology suggests that a number of important morphological changes took place that unite the Monogenea (for examples see EHLERS, 1985a; LITTLEWOOD et al., 1999a; BOEGER & KRITSKY, 2001) and existing molecular data are incapable of resolving this stem lineage.

Example 3. Cestodes and the evolution of segmentation and attachment

Segmentation is a hallmark of the eucestodes and represents one of the evolutionarily novel means by which the parasitic neodermatans have achieved their enormous reproductive capabilities. It is not universally observed among cestodes, however, and thus the evolution of the trait within the group can be examined by means of a phylogenetic tree derived from other sources of data (e.g. molecules; Fig. 4). The strobilate (segmented) condition may be seen as the result of two separate processes: proglottization, the serial repetition of the gonads; and segmentation, the external division of the proglottids into self-contained compartments that may develop and become fertilized independently of the parental worm. The evolutionary advantages of these processes differ; proglottization increases fecundity, whereas segmentation can allow for development and fertilization to occur in niches other than that occupied by the parental worm (e.g. in the external environment). Evidence for the recognition of strobilation being the result of two processes rather than one is found in the peculiar form of the members of the Spathebothriidea, which exhibit proglottization without external segmentation. Albeit rare, this condition is also found in higher eucestodes such as the pseudophyllidean *Anantrum tortum* Overstreet 1968, and to a lesser extent, in the nippotaeniids which show only weak external segmentation. As the closest relatives of the eucestodes (the cestodarian groups Amphilinidea and Gyrocotylidea) are themselves non-strobilate, it follows that the two non-strobilate eucestode groups, Caryophyllidea and Spathebothriidea, represent the ancestral condition and are placed most parsimoniously at the base of the eucestode tree. The utilisation of oligochaete, rather than arthropod, intermediate hosts has also been argued to provide support for a basal position of the Caryophyllidea (HOBERG et al., 1999); life-histories of spathebothriideans are as yet unknown (BEVERIDGE, 2001). Indeed, BURT & JARECKA (1982) went as far as to propose that the caryophyllidean genus *Archigetes*, species of which may obtain reproductive maturity in oligochaetes, represented the first true tapeworms; however, as the eucestodes are otherwise universally observed to have at least one intermediate host and a vertebrate definitive host, it seems more likely that the life-cycle of *Archigetes* spp. evolved through progenesis of a larval stage (MACKIEWICZ, 1982). Despite such observations, many authors have considered the lack of segmentation in caryophyllideans and spathebothriideans to be secondarily derived from a strobilate ancestor, namely the Pseudophyllidea (e.g. FUHRMANN,

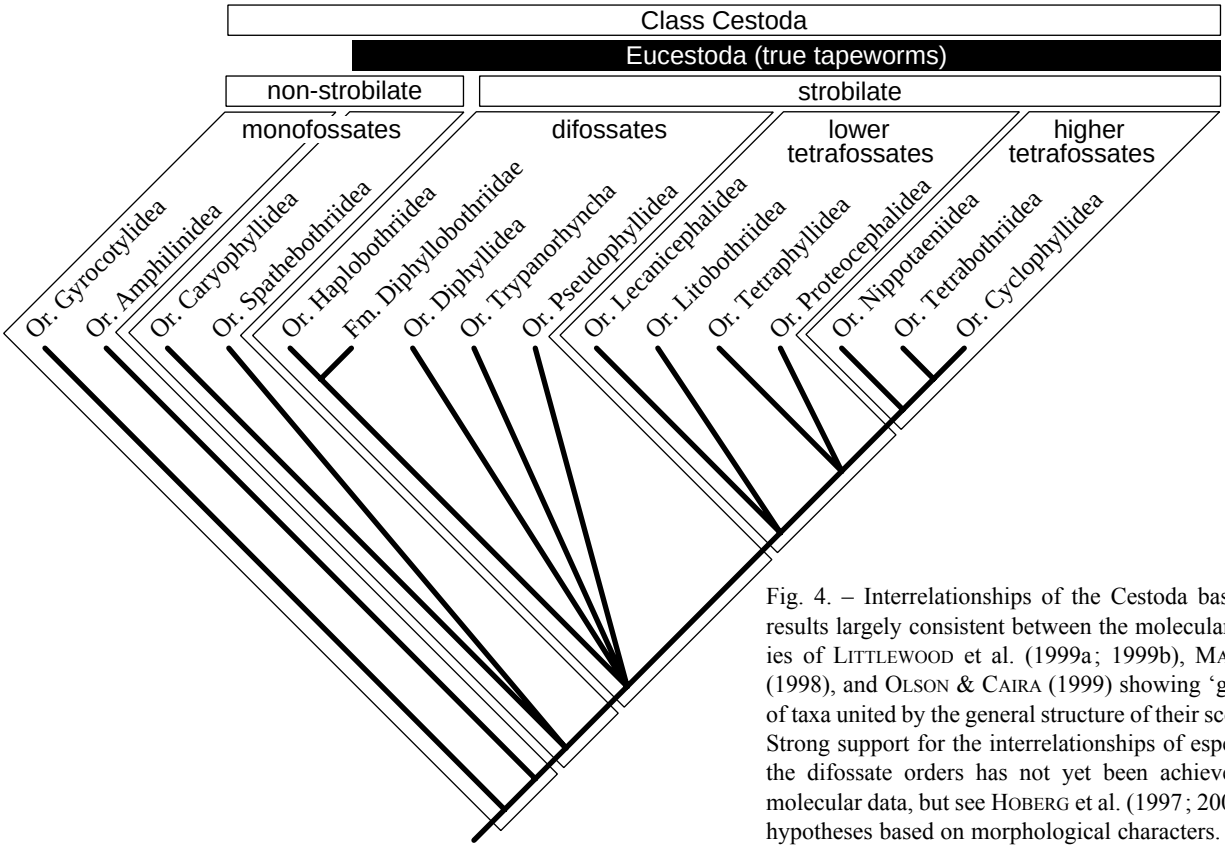


Fig. 4. – Interrelationships of the Cestoda based on results largely consistent between the molecular studies of LITTLEWOOD et al. (1999a; 1999b), MARIAUX (1998), and OLSON & CAIRA (1999) showing 'grades' of taxa united by the general structure of their scolices. Strong support for the interrelationships of especially the difossate orders has not yet been achieved via molecular data, but see HOBERG et al. (1997; 2001) for hypotheses based on morphological characters.

1931; Joyeux & Baer, 1961). Thus, only through an independently derived phylogeny can the evolution of segmentation be addressed objectively.

Molecular data support a basal position of the non-strobilate eucestodes (Mariaux, 1998; Olson & Caira, 1999; Mariaux & Olson, 2001), although the published results differ in regards to which group appeared first. Mariaux's (1998) analysis supported the more commonly hypothesized arrangement inferring strobilation as a step-wise process evolving from non-proglottized, non-segmented worms (Caryophyllidea), to proglottized, non-segmented worms (Spathebothriidea), to the proglottized, segmented condition (higher eucestodes), consistent with previous hypotheses as well as recent analyses based on morphology (Hoberg et al., 1997; Hoberg et al., 2001). Olson & Caira's (1999) work supported a basal position of the Spathebothriidea, whereas the Caryophyllidea was placed in a clade that implied the group to be secondarily non-strobilate. Newer analyses (Olson et al., unpublished data) involving larger numbers of both exemplar taxa and sequence data are showing better support for the former hypothesis (Caryophyllidea (Spathebothriidea(strobilate eucestodes))).

Another classic example from the cestodes is the evolution of their holdfast structures. In their most general form, cestode scolices can be divided among three basic divisions : monofossate, having only a single part to the scolex; difossate, having a bipartite scolex; or tetrafossate, having four parts. Fig. 4 shows 'grades' based on these divisions. Most orders readily fit into one of the grades, whereas the scolex morphology of other groups is more enigmatic. For example, members of Haplobothriidea possess four tentacles and have occasionally been allied with the trypanorhynchs on this basis (Fuhrmann, 1931). Trypanorhynchs also have four tentacles, but their tentacular structures differ significantly and the scolex of a majority of species is otherwise typically difossate. Nippotaeniid scolices possess a single apical sucker thus making them monofossate. Unlike the other monofossate groups (e.g. Caryophyllidea, Spathebothriidea), however, they are strobilate worms. Litobothriids are characterized by a scolex that is cruciform in cross section and differs markedly from the scolex morphologies of other tetrafossate lineages. For groups such as these, little evidence of their phylogenetic affinities can be gained from comparison of scolex features.

Many authors have hypothesized a diphyletic evolution of the cestodes split between difossate and tetrafossate lineages (Fuhrmann, 1931; Euzet, 1959; Euzet, 1974), whereas others show a step-wise evolutionary pattern starting with the monofossate condition and culminating with the tetrafossate condition (Hoberg et al., 1997; Hoberg et al., 2001). Molecular-based results from Olson & Caira (1999) supported a largely diphyletic pattern whereas Mariaux's results (1998) supported a step-wise pattern; results of neither study were strictly diphyletic or strictly bifurcating. Both, however, supported a derived, monophyletic clade of tetrafossate groups that also included the Litobothriidea and Nippotaeniidea (Fig. 4). Within the tetrafossate clade was another derived clade uniting the Cyclophyllidea, Nippotaeniidea, and Tetrabothriidea. Internal branch lengths and levels of support for the internodes subtending the tetrafossate clade and that of the higher tetrafossate clade within it were greater than for any other internode subtending major groupings, with the exception of that separating the Eucestoda from the cestodarian orders. From this we can infer that the monofossate condition seen in the Nippotaeniidea is a reversal to the plesiomorphic condition, and that the affinities of the Litobothriidea are with the Lecanicephalidea, not the Tetraphyllidea as suggested by the classification of Euzet (1994). Likewise, the affinities of the Haplobothriidea are among the difossate group Diphyllobothriidae (Fig. 4) despite their having four tentacles and an undivided scolex.

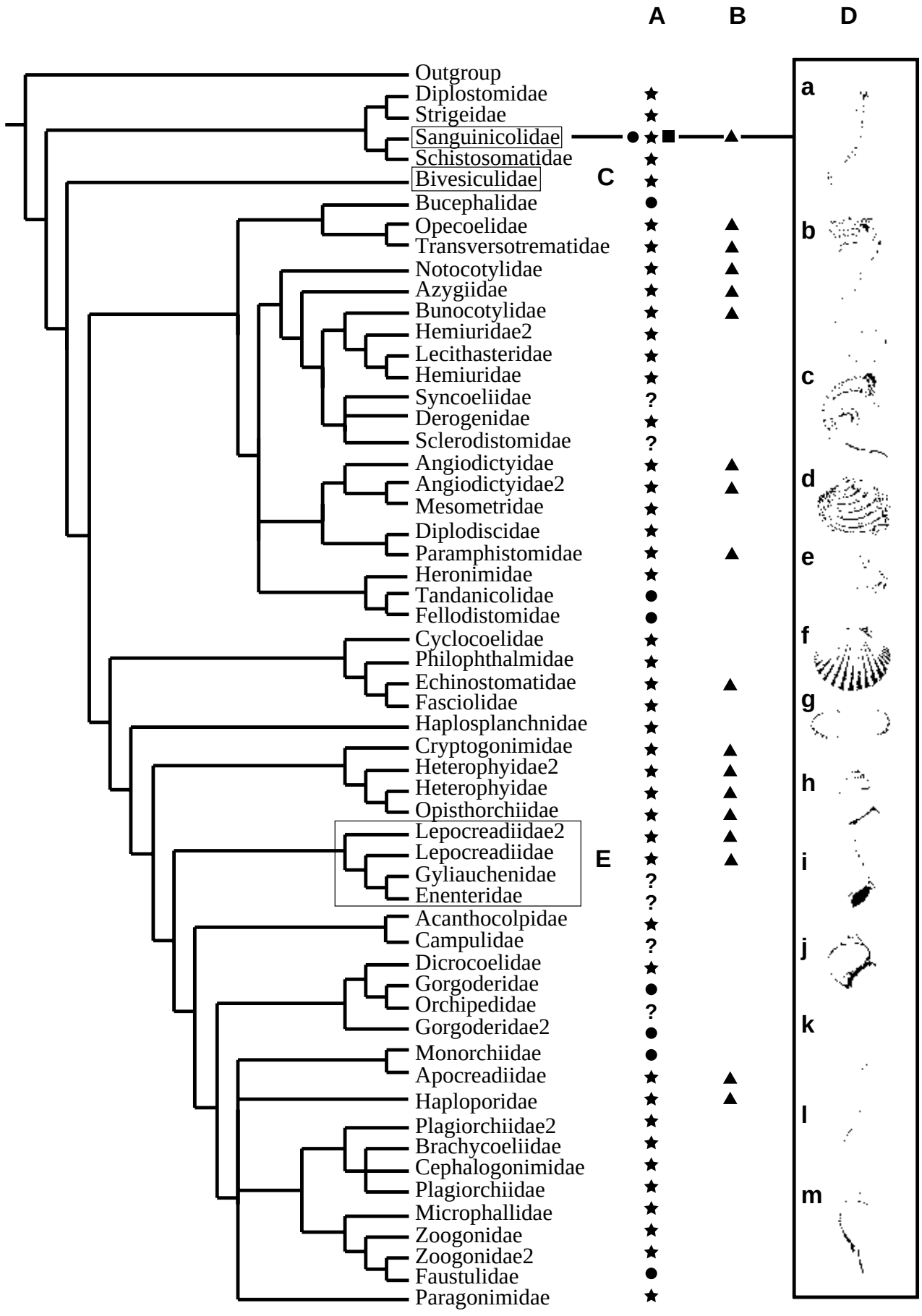
Example 4. Digenean phylogenetics and the evolution of life-history

Digenean trematodes are obligatorily parasitic in invertebrate intermediate and vertebrate definitive hosts. A key aspect of the evolution of parasitism within the group is the exploitation of different taxa as first intermediate hosts, namely gastropods, bivalves, scaphopods and a few polychaete annelids. Clearly, present-day associations are the result of the interaction between the twin processes of coevolution and host-switching but the balance between these two processes is not understood in any detail. Strict host-parasite coevolution could be expected to be reflected by distributions of parasite taxa restricted to closely related molluscs and, as a corollary, mollusc taxa infected with single parasite taxa. A credible phylogeny of the Digenea makes it possible to analyse the significance of present distributions.

To explore these issues we have constructed a database of life-cycle information for the Digenea. The data is derived from the compendium of Yamaguti (1975) and subsequent publications. Here we focus on the inferences that can be drawn by exploring how individual mollusc taxa are exploited by digeneans and how individual digenean taxa exploit the potential range of first intermediate hosts. The distributions are mapped on the maximum parsimony tree inferred from 18S rDNA sequence data (Fig. 5, for details see Cribb et al., 2001);

Legend to Fig. 5 (see opposite page)

Fig. 5. – Phylogeny of the Digenea inferred from 18S rDNA showing distribution of life-cycle attributes for selected taxa. Where parasite taxa appear twice it is as an indication of putative paraphyly. **A.** The class of first intermediate hosts associated with each family: ★ Gastropoda, ● Bivalvia, ■ Polychaeta. **B**. Families that infect hydrobiid gastropods. **C**. The nearly basal taxon Bivesiculidae, which infects only cerithiid gastropods. **D.** Hosts of Sanguinicolidae: Polychaeta – a. Ampharetidae, b. Serpulidae, c. Terebellidae; Bivalvia – d. Veneridae, e. Donacidae, f. Pectenidae, g. Solemyidae; Gastropoda Prosobranchia – h. Bithyniidae, i. Hydrobiidae, j. Viviparidae; Gastropoda Pulmonata – k. Ancylidae, l. Lymnaeidae, m. Planorbidae. **E.** – see text.


A
B
D
Outgroup
Diplostomidae
Strigeidae
Sanguinicolidae
Schistosomatidae
Bivesiculidae
C
Bucephalidae
Opecoelidae
Transversotrematidae
Notocotylidae
Azygiidae
Bunocotylidae
Hemiuridae2
Lecithasteridae
Hemiuridae
Syncoeliidae
Derogenidae
Sclerodistomidae
Angiodictyidae
Angiodictyidae2
Mesometridae
Diplodiscidae
Paramphistomidae
Heronimidae
Tandanicolidae
Fellodistomidae
Cyclocoelidae
Philophthalmidae
Echinostomatidae
Fasciolidae
Haplosplanchnidae
Cryptogonimidae
Heterophyidae2
Heterophyidae
Opisthorchiidae
Lepocreadiidae2
Lepocreadiidae
Gyliauchenidae
Enenteridae
E
Acanthocolpidae
Campulidae
Dicrocoelidae
Gorgoderidae
Orchipedidae
Gorgoderidae2
Monorchiidae
Apocreadiidae
Haploporidae
Plagiorchiidae2
Brachycoeliidae
Cephalogonimidae
Plagiorchiidae
Microphallidae
Zoogonidae
Zoogonidae2
Faustulidae
Paragonimidae
a
b
c
d
e
f
g
h
i
j
k
l
m

A. The distribution of trematodes that use bivalves as first intermediate hosts (Fig. 5A) shows six entirely separate occurrences (members of Allocreadiidae and Gymnophallidae also use bivalves but these taxa are not yet incorporated in the phylogenetic analysis). Because all the other taxa in the analysis use gastropods as first intermediate hosts, this distribution allows the parsimonious inference that parasitism of bivalves has arisen independently within the Digenea at least six times, instead of the other possibilities of being plesiomorphic or suggesting relationship between the taxa concerned. This observation also emphasises how frequent and dramatic host-switching has been in the evolution of the Digenea.

B. The Hydrobiidae is a group of cosmopolitan prosobranch gastropods found in fresh and brackish water. Populations of hydrobiids are frequently extraordinarily dense and heavily infected with trematodes and, as a result, have been studied extensively. Fifteen families of trematodes in the present analysis have been reported from hydrobiids. These are distributed very widely in the phylogeny of the Digenea (Fig. 5B). Such a distribution can be interpreted as the result of either extensive coevolution or of extensive host-switching (or of course as a mixture of both processes). The extent to which these families are also found in other groups of molluscs resolves this question. Of the 15 families reported from hydrobiids, 13 are also known from other gastropod families and 12 from other than the Rissooidea, the superfamily to which the Hydrobiidae belongs. We thus infer that, although some coevolution may be obscured, in general the hydrobiids have become infected through repeated cases of host-switching which relates in turn to their "attractiveness" as intermediate hosts.

C. There is extraordinary variation in the host-specificity shown by families of trematodes. At one extreme the Bivesiculidae is known only from the prosobranch gastropod family Cerithiidae. The implication of such a distribution is, presumably, that the evolutionary history of the parasite family is linked to that of the mollusc family. In the case of the Bivesiculidae this creates a still unresolved problem. The basal position of the family (Fig. 5C) suggests that it may be a relatively ancient taxon (perhaps consistent with elements of its morphology) but there is nothing particularly ancient about the Cerithiidae, known definitively from no earlier than the Upper Cretaceous (HEALY & WELLS, 1998). It seems certain that the Digenea had undergone its major radiations well before the Upper Cretaceous so that this host-parasite distribution is enigmatic.

D. At the other extreme the Sanguinicolidae, the fish blood flukes, are known from 16 families of first intermediate hosts including bivalves, prosobranch and pulmonate gastropods, and polychaete annelids (Fig. 5D). Members of Sanguinicolidae have a broad distribution within fishes (both teleosts and chondrichthyans). Because the Sanguinicolidae falls within the most basal group of Digenea, the host distribution of the Sanguinicolidae is consistent with an ancient coevolutionary radiation between these host groups. However, this hypothesis requires exploration by resolution of phylogenetic relationships within the Sanguinicolidae, information that is not yet available.

E. Finally we can attempt to use the relationships inferred from the phylogeny to predict the intermediate hosts of digeneans for which no first intermediate hosts are known. For example, the Gyliauchenidae and Enenteridae, parasites of marine herbivorous fishes, occur in the clade containing the Lepocreadiidae (Fig. 5E). Many lepocreadiid cercariae are known, but none are known for the Gyliauchenidae or Enenteridae. The first intermediate hosts of lepocreadiids are all gastropods, Subclass Orthogastropoda, Superorder Caenogastropoda, Order Sorbeoconcha and include representatives from four superfamilies (Conoidea, Muricoidea, Naticoidea and Rissooidea) and nine families. Because the parasite phylogeny suggests that the Gyliauchenidae and Enenteridae are families derived from within the Lepocreadiidae we might predict that the first intermediate hosts would be from the Sorbeoconcha. Unfortunately the Sorbeoconcha includes dozens of families so that the predictive power is limited.

For the understanding of the evolution of the digenean life-cycle, the phylogeny of the Digenea solves some questions satisfactorily, leaves others ambiguous, and identifies whole new areas of inquiry. Most importantly, the preliminary nature of the gene tree on which these hypotheses are based must be emphasised (see CRIBB et al., 2001), and our future efforts are set to address this issue as much as to test hypotheses of life-history evolution.

CONCLUSION

As with any evolutionary interpretation dependent upon an estimate of phylogeny, the utility of the approach relies almost exclusively on the validity of the tree and the underlying data that it is founded upon. We have shown that there remain some serious discrepancies between independent estimates of platyhelminth phylogeny, at a multitude of taxonomic levels. Such incongruence needs to be reconciled, as might be possible in some instances of gene/species tree mismatching, or perhaps more likely, additional data needs to be gathered. Even in the absence of conflict we are ever mindful that relatively low nodal support values (estimates of the strength of the tree) limit the confidence in our subsequent interpretations. Nevertheless, with examples from the Cestoda and Digenea, we have shown that phylogenetics remains the most profitable key to understanding the evolution of parasites and parasitism.

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Patterns in the nervous and muscle systems in lower flatworms

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ABSTRACT. In order to test the monophyly of the Plathelminthes and the phylogenetic relationships of Acoela and Nemertodermatida, studies on the neuroanatomy of these groups were performed using anti-serotonin (5-HT) and anti-FMRF related peptides (FaRPs) immunocytochemistry. The Catenulida + Rhabditophora seem to be monophyletic. Four synapomorphies are proposed for these taxa. The presence of: 1. a bilobed brain showing 5-HT- and FaRP immunoreactivity. 2. a distinct neuropile, showing 5-HT and FaRP immunoreactivity. 3. two main nerve cords (MC's) aligned by 5-HT-immunoreactive (IR) marker neurones, 4. FaRP-immunoreactivity in the stomatogastric nervous system (NS). No synapomorphies were detected between Acoela and other Plathelminthes. The IR patterns of Acoela are characterised by: 1. The presence of a 5-HT-IR commissural brain. 2. The presence of clusters of FaRP-IR cells not integrated into a brain of the flatworm type. 3. The absence of a regular orthogon. 4. The absence of serotoninergic marker neurones along the main nerve cords (MC's). 5. The absence of a stomatogastric FaRP positive nervous system (NS). No support was obtained for a taxon Acoelomorpha. Our data are compatible with the hypothesis that both the Acoela and the Nemertodermatida do not belong to the Plathelminthes.

KEY WORDS: neurons, muscles, phylogeny, Platyhelminthes.

INTRODUCTION

In the paper "Is the Turbellaria polyphyletic?" SMITH et al. (1986), were the first to question the monophyly of the Plathelminthes. They pointed out that no synapomorphies were actually known between Catenulida, Acoelomorpha and Rhabditophora. The question of the monophyly of the Plathelminthes still remains open. The Plathelminthes, and particularly the Acoela have been in focus in recent molecular studies (see RUIZ-TRILLO et al., 1999; LITTLEWOOD et al., 1999 BERNEY et al., 2000). Traditionally, the Nemertodermatida and the Acoela have been classified as sister groups within the Acoelomorpha (EHLERS, 1985). In recent molecular investigations this position has been questioned.

In the discussion of the phylogenetic position, the organisation of the nervous system (NS) has been used as one of the discriminating criteria (see REUTER et al., 2001). The application of immunocytochemical (ICC) techniques has proven reliable for studies of flatworm neuroanatomy (see REUTER & GUSTAFSSON, 1995). Particularly informative results have been obtained using antibodies raised against serotonin (5-HT) and FMRF related peptides (FaRPs). By combining phalloidin staining for F-actin and ICC staining of neuroactive substances, it is possible to study the spatial interrelationships between muscles and nerves.

Here data of the 5-HT and FaRP immunoreactivity patterns in the taxa Macrostomida and Catenulida are reviewed (WIKGREN & REUTER, 1985; REUTER & GUSTAFSSON, 1995). Thereafter, recent data on the 5-HT and FaRP immunoreactivity patterns in the taxa Acoela and Nemertodermatida are presented (RAIKOVA et al., 1998, 2000, 2001; REUTER et al., 1998, 2001). Furthermore, new data concerning the spatial relationship between muscles and nerves in all above-mentioned taxa are presented. Finally, the phylogenetic implications of the data are discussed.

MATERIAL AND METHODS

Species

Specimens of 1. *Stenostomum leucops* Dugès, 1828 (Catenulida) were collected from a stock culture main-

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tained in containers with tap water, 2. *Macrostomum lineare* Müller, 1774 (Macrostomida) were collected from brackish water at Stortervo, Pargas (SW Finland), 3. the Acoela: *Anaperus biaculeatus* Boguta, 1970, *Childia groenlandica* Levinsen, 1879, were collected in the vicinity of the White Sea Biological station at Cape Kartesh (Russia), *Faerlea glomerata* Westblad, 1945 and *Paraphanostomum crassum* Westblad, 1942 from the vicinity of Kristineberg Biological station (West coast of Sweden), and specimens of *Avagina incola* Leiper, 1902 were obtained from the gut of the sea urchin *Spatangus purpureus* O.F. Müller, 1776 in the vicinity of Bergen, (Norway). 4. the Nemertodermatida: *Nemertoderma westbladi* Steinbock, 1938 were collected in the vicinity of Kristineberg Marine Research Station (West coast of Sweden) and *Meara stichopi* Westblad, 1949 obtained from the intestine of the holothurian *Stichopus tremulus* Gunnerus, 1767 at Raunefjord near Bergen (Norway).

Immunocytochemistry

For details of the method see KRESHCHENKO et al. 1999 and REUTER et al. 2001.

The specimens were fixed in Stefanini's fixative (2% paraformaldehyde and 15% picric acid in 0.1 M Na-phosphate buffer) at pH 7.6, stored for several weeks in fixative at 4°C, and rinsed for 24-48h in 0.1 M Na-phosphate buffer (pH 7.6) containing 10-20% sucrose. The worms were either handled as whole mounts on poly-L-lysine coated glass slides or embedded in Tissue Tec and sectioned at 10-20µm on a Bright cryostat. Immunostaining was performed according to the indirect immunofluorescence method. The concentrations for the primary antibodies were 1:500. Incubations were performed for 36-48h either with a mixture of goat anti-5-HT (INCSTAR) and rabbit anti-FMRF (INCSTAR) or with a mixture of rabbit anti-5-HT (INCSTAR) and guinea pig antiserum against the native flatworm neuropeptide GYIRF (Bdelloura candida) (Johnstone et al., 1995). Thereafter the incubations were rinsed 3x5 min in PBS-T, followed by consecutive incubations with TRITC- or FITC-labelled secondary antibodies (from DAKO and Cappel).

Phalloidin staining of musculature

Whole mounts or cryosections were stained with TRITC-conjugated phalloidin (Sigma) (1:2000) for 20 min to 2h at 4°C. The phalloidin staining was performed on the same whole mounts or cryosections that had been stained with a-5HT and a-FaRPs, studied in the confocal microscope and photographed.

Microscopy and computer processing of immunocytochemistry micrographs

The preparations were examined in a Leitz Orthoplan microscope combined with filter blocks I2 and N2. A confocal scanning laser microscope (CSLM: Leica TCS 4D) was used to visualise the details of the nervous system.

Files obtained from CSLM were processed with Adobe Photoshop 4.0. Only commands "mode RGB-Grayscale", level of "grey", "brightness" and "contrast" were used, to avoid any distortion of the information contents of the image.

RESULTS

Catenulida

In *Stenostomum leucops,* ICC staining with anti-5-HT and anti-FaRP reveals a bilobed brain with a fibrillar neuropile. Frontally, lateral lobes emerge from the brain *sensu stricto*. The lobes innervate sensory pits, which are connected by transverse muscles (Fig. 1A). 5-HT and FaRP immunoreactivity occur in separate sets of neurones (WIKGREN & REUTER 1985). FaRP immunoreactivity dominates in the ventral cords. 5-HT immunoreactivity occurs in all nerve cords and in the nerve plexus close to the body surface. The two main nerve cords (MC's) are aligned by serotoninergic marker neurons. Only FaRP positive cells and nerves are seen in the stomatogastric NS and they dominate in the ventral cords. By the use of confocal scanning laser microscopy (CSLM) and phalloidin staining of F-actin, we can show that FaRP positive nerves innervate the pharynx (Fig. 1 B) and extend to the muscles connecting the sensory pits (Fig. 1A).

Macrostomida

In *Microstomum lineare*, the 5-HT and FaRP immunoreactivity patterns are similar to those in other Rhabditophora. The peptidergic and aminergic cells surround the neuropile composed of a tangled mass of nerve fibres. Serotoninergic marker neurones are aligned along the MC's. An orthogon and nerve nets close to the body surface are observed (see REUTER & GUSTAFSSON, 1995). FaRP immunoreactivity characterises the stomatogastric NS. Combined staining for F-actin, FaRP and 5-HT immunoreactivity reveals the spatial relationship of neuronal substances and muscle layers. FaRP immunoreactivity occurs in the nerve net around the gut musculature (Figs 1 C-E). FaRP positive cells join the pharyngeal nerve ring and fibres cling to the pharyngeal muscles (Fig. 1 F). In contrast 5-HT immunoreactivity occurs in the subepidermal and submuscular nerve nets of the body wall (REUTER et al., 1995). The difference in distribution patterns is shown in a sagittal section (Fig. 1 G).

Acoela

The immunoreactivity patterns for 5-HT and FaRPs in eight species of Acoela have recently been studied (RAIKOVA et al., 1998; REUTER et al., 1998; 2001).

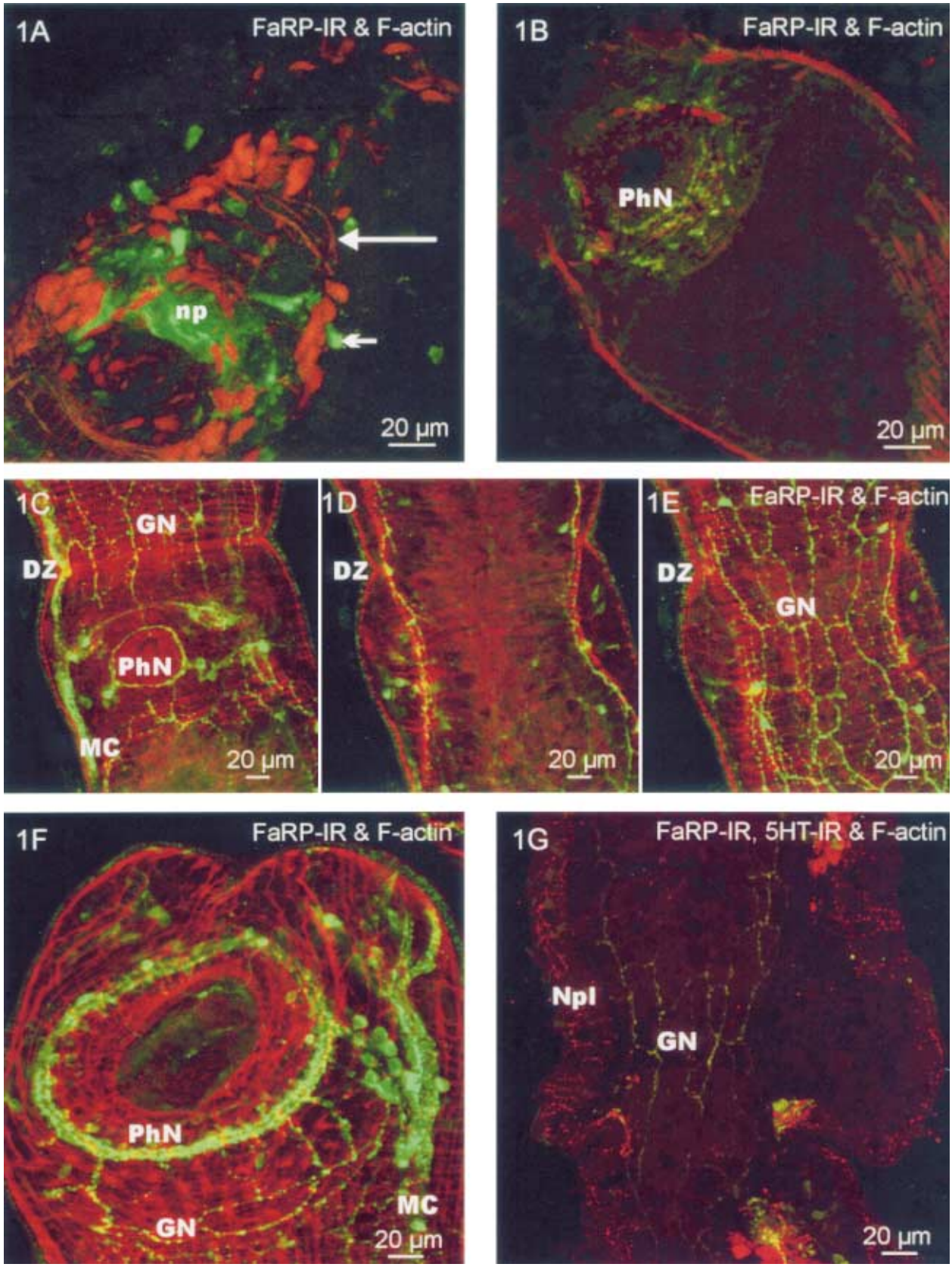
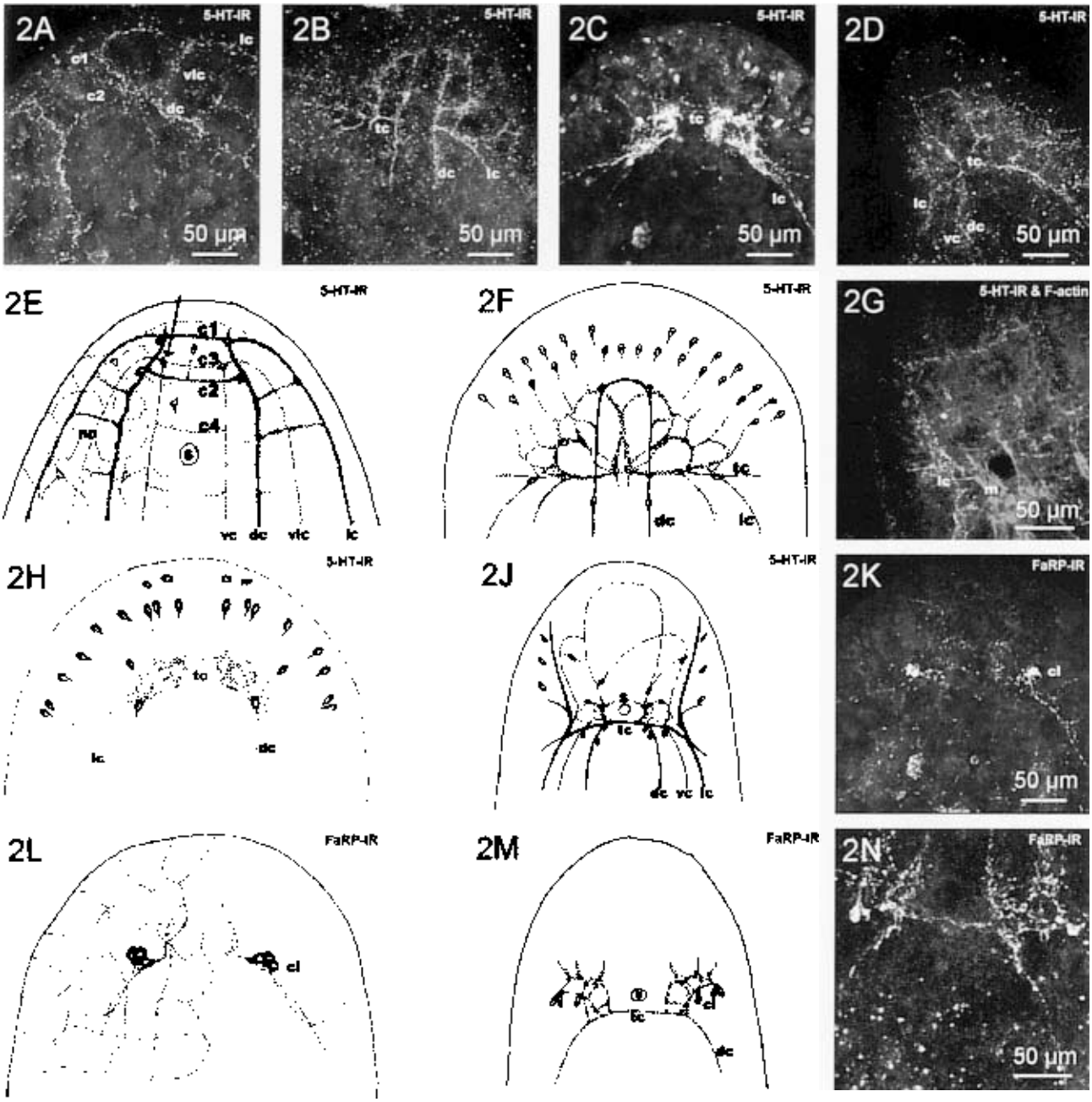


Fig. 1 A-B. – *Stenostomum leucops,* A. Brain and frontal lobes, FMRF-IR (green) in neuropile (np) and in sensory cells in sensory pit (short arrow), phalloidin stained F-actin (red) in transverse muscle fibres (long arrow) between frontal lobes B. FaRP-IR in pharyngeal nervous system (PhN) spatially related to phalloidin stained F-actin in pharyngeal muscles.

Fig. 1. C-G. – *Microstomum lineare.* C-E. Optical sections from three different body levels showing FaRP-IR nerve net (GN) adjoining phalloidin stained F-actin of gut musculature, main longitudinal nerve cord (MC), division zone (DZ). F. FaRP-IR in pharyngeal nervous system (PhN) and gut nerves (GN) in contact with phalloidin stained F-actin in muscles. G. Sagittal section showing FaRP-IR in gut nerve net (GN) and 5-HT-IR in nerve plexus (Npl) of body wall.

***5-HT-IR pattern*.** 4-5 pairs of serotoninergic longitudinal nerve cords form a symmetrical anterior structure composed of nerve fibres, associated with a few cell bodies - a commissural brain. The longitudinal nerves do not form a regular orthogon. 5-HT IR occurs in an irregular network of subepidermal fibres. In addition, submuscular fibres were observed innervating reproductive structures (RAIKOVA et al., 1998). The shape of the brain displays variations in organisation (see WESTBLAD, 1948). According to him a development from a ring-shaped brain to a bridge-shaped brain can be followed. Three terms – barrel-, rosette and bridge-shaped – are proposed for the acoelan brain by REUTER et al. (2000).

The barrel-shaped brain, represented by *Faerlea glomerata*, is characterised by 5-HT-IR longitudinal nerve cords, forming the ribs of the barrel, and transverse fibres, connecting the longitudinal cords and representing the barrel hoops (Figs 2 A, E). The dorsal fibres are stronger than those on the ventral side. All fibres are located close to the body surface. No contact between the 5-HT-IR fibres and the statocyst, lying deep in the parenchyma, was observed. This shape corresponds to the ring-shape of



Figs. 2 A-J. – Patterns of 5-HT-IR in commissural brain of four acoels; A, E. *Faerlea glomerata*, B., F. *Childia groenlandica*, C., H. *Avagina incola*, D., J. *Paraphanostoma crassum*. Transverse brain commissures (c1, c2, c3, c4, tc), dorsal nerve cords (dc), ventrolateral nerve cords (vlc), lateral nerve cords (lc), ventral nerve cords (vc), cell cluster (cl), statocyst (s). In G. phalloidin stained muscles (m) close to the cell cluster and 5-HT-IR in brain loops and lateral nerve cord (lc) in optical section of *P. crassum*.
Figs. 2. K-N. – FaRP-IR patterns in acoels. K., L. *Avagina incola*, M.,N. *Paraphanostoma crassum*. FaRP-IR cell clusters (cl).

WESTBLAD (1948). We prefer the term barrel-shape, because the transverse fibres are clearly weaker than the longitudinal (REUTER et al., 2000).

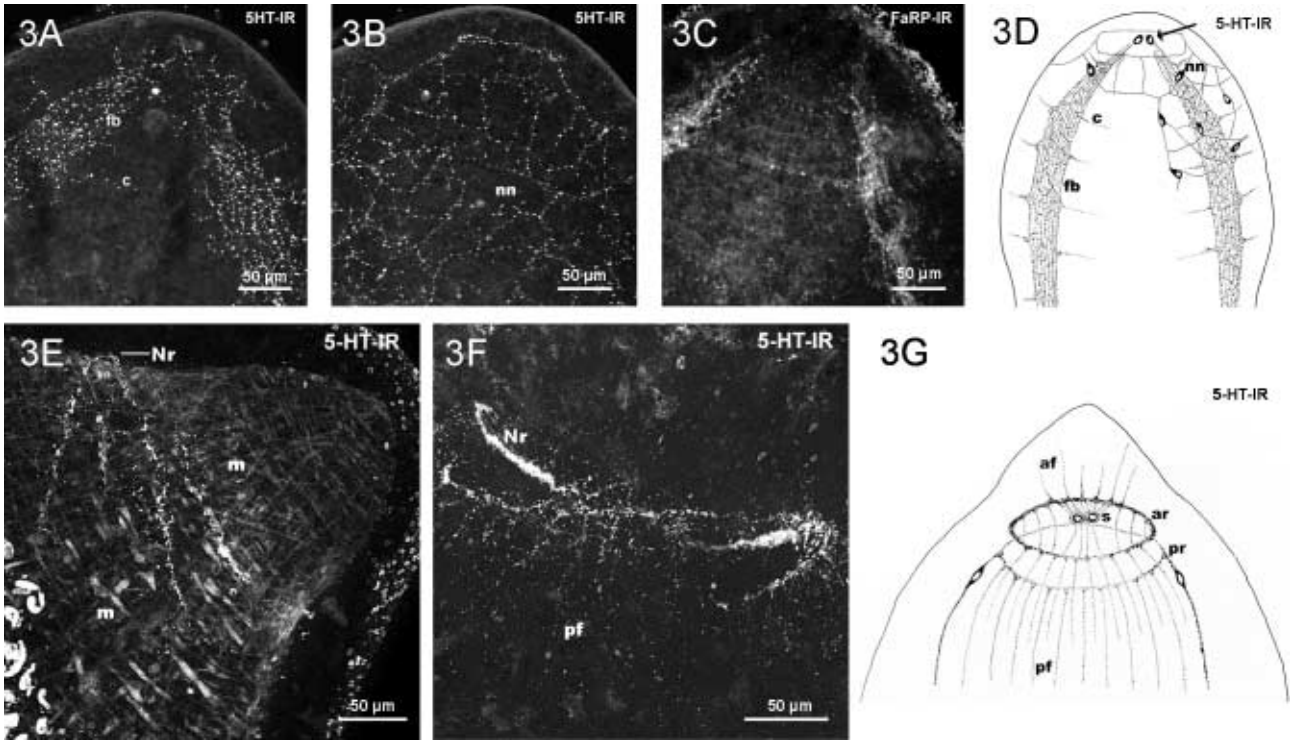
The rosette-shaped brain, represented by *Childia groenlandica,* is characterised by 5-HT-IR anterior loops, joining a common transverse commissure on the dorsal side. In *C. groenlandica*, the loops are large and loosely connected (Figs 2 B, F). When the loops are small and concentrated a bridge-like construction is formed as in *Avagina incola* (Figs 2 C, H).

In the bridge-shaped brain of *Paraphanostoma crassum* (Figs 2 D,G, J) a strongly stained dorsal transverse commissure gives rise to a semi-circle around the statocyst. The construction of the brain and the close association between the nerves and the statocyst indicate a distinct evolutionary development from a superficial centralisation of neurons close to the body surface leading to a true cephalisation.

***FaRP-IR pattern*.** In all the acoels studied the peptidergic patterns differ considerably from the serotoninergic patterns. Symmetrical clusters of cells located peripherally to the 5-HT-IR commissural brain were revealed in all acoels excluding *F. glomerata,* in which no reaction was obtained (see REUTER et al. 2000). By using antibodies against the native flatworm neuropeptide GYIRFamide, nerve fibres around the cell clusters in *A. incola* and *P. crassum* were visualised (Figs 2 K-N). Double-staining with 5-HT and FaRP antibodies show, that the FaRP-IR cell groups are located peripherally to the 5-HT-IR commissural brain.

Nemertodermatida

So far only two species of Nemertodermatida, *Meara stichopi* and *Nemertoderma westbladi*, have been studied with ICC technique in order to compare their neuroanatomy to that of the Acoela (RAIKOVA et al., 2001). The results revealed a surprisingly different pattern between the acoels and the two nemertodermatid species, but also between the last mentioned species themselves. In *M. stichopi* two 5-HT-IR cells occur close to the statocyst. From the cells, symmetrical parenchymal bundles of loosely packed nerve fibres extend posteriorly. A superficial nerve plexus also occurs (Figs 3 A-D). In *N. westbladi,* two basiepthelial 5-HT-IR fibre rings send numerous fine fibres in both posterior and anterior directions. Some of the fibres to the statocyst lie deeper in the parenchyma (Figs 3 E, G). Double-staining with phalloidin for F-actin, clearly shows the basiepithelial position of the nerve ring, outside the body muscles (Fig. 3 F). In *M. stichopi*, the peptidergic pattern corresponds in general to the serotoninergic pattern (Fig. 3 C). In *N. westbladi*, FaRP immunostaining occasionally revealed fibres at the same level as the serotoninergic nerve rings (Fig. 3 G).



Figs. 3. A-D. – *Meara stichopi.* A-B. 5-HT-IR patterns, C. FaRP-IR pattern, D. schematic drawing of 5-HT-IR pattern, bundle of nerve fibres (fb), transverse commissure (c). nerve net (nn), note two frontal nerve cells (arrow) in D.
Figs. 3. E-G. – *Nemertoderma westbladi.* E. 5-HT-IR showing basi-epithelial nerve ring (Nr) located peripherally to the phalloidin stained muscle layer (m). F. 5-HT-IR in two "brain rings" sending nerve fibres posteriorly (pf). G. Schematic drawing, anterior nerve ring (ar), posterior nerve ring (pr), nerve fibres running anteriorly (af) and posteriorly (pf), statocyst (s).

PHYLOGENETIC IMPLICATIONS

Stomatogastric NS

The peptidergic innervation of the stomatogastric NS in Rhabditophora has been described repeatedly (see REUTER & HALTON, 2001, MAIR et al., 1996). The presence of a peptidergic innervation of the stomatogastric NS in Catenulida (WIKGREN & REUTER, 1985, REUTER et al., 1995) and Rhabditophora, points to a synapomorphy for these taxa. The lack of FaRP immunoreactivity in the central parenchyma of Acoela can be explained either as a reduction of the NS in Acoela, lacking a gut, or alternatively as a plesiomorphy. The latter seems more likely, taking into account the basal position of the Acoela in the phylogenetic tree of the Bilateria (see RUIZ-TRILLO et al., 1999). In the nemertodermatid *N. westbladi*, which has an epithelial gut, the absence of FaRP immunoreactivity indicates that the lack of gut innervation represents a plesiomorphy. 5-HT immunoreactivity occurs in the pharynx in all flatworm taxa except Catenulida and Acoelomorpha and thus forms an apomorphy for the Rhabditophora.

Innervation of musculature

Regarding Macrostomida and all other Rhabditophora, as well as the Acoela and the Nemertodermatida, the fine meshed 5-HT-IR nerve plexuses at the body wall – the subepidermal close to the roots of the cilia and the submuscular close to the body wall musculature (REUTER et al., 1995; LADURNER et al., 1997) – may be plesiomorphic. In *Anaperus tvaerminensis* Luther, 1912, EHLERS (1994) described submuscular neurones enveloped by longitudinal muscle cells. A similar close association between 5-HT-IR nerves and longitudinal muscle fibres was observed in *A. biaculeatus*. A double nerve/muscle function of the muscles radiating from the wall of the statocyst in "Paraphanostoma Arten" was suggested by WESTBLAD (1948). In our studies, no 5-HT-IR fibres were observed reaching the strong muscles around the statocyst in *F. glomerata*. Thus some other neuronal signal substances probably innervate the strong muscles radiating from the statocyst wall. The functional aspect of the spatial relationship between muscles and nerves needs further research.

Synapomorphies for Catenulida and Rhabditophora

In the following respects the immunoreactivity patterns of Catenulida correspond to those observed in Rhabditophora.

1. The bilobed brain is composed of 5-HT- and FaRP-IR cells
2. The neuropile is distinct, showing both 5-HT and FaRP immunoreactivity
3. The MC's are aligned by 5-HT-IR marker neurones
4. FaRP-IR occurs in the stomatogastric NS

These features can be regarded as synapomorphies for Catenulida and Rhabditophora.

Acoela vs Plathelminthes; no synapomorphies

Acoela differs from other Plathelminthes by:

1. The presence of a structure named the commissural brain, showing anti-5-HT immunoreactivity, but showing no resemblance to the bilobed brain structure in Plathelminthes, with its nerve cells surrounding a neuropile.
2. The presence of clusters of peptidergic FaRP positive cells, that are not integrated into a brain of the common flatworm type.
3. The absence of a regular orthogon. Only longitudinal nerve cords, connected by irregular nerve fibres forming subepidermal and submuscular nerve plexuses, were observed.
4. The absence of serotoninergic marker neurones along the MC's.
5. The absence of a stomatogastric FaRP positive NS.

Thus no synapomorphies were found in the organisation of the NS of Acoela and Plathelminthes (RAIKOVA et al., 1998; REUTER et al., 2000).

No support for the taxon Acoelomorpha

As to the Nemertodermatida, the presumed sister taxon of the Acoela (see LUNDIN, 1997 for discussion), our studies concerning the organisation of the NS revealed no synapomorphies either with that of the Acoela (RAIKOVA et al., 2000 a) or with that of the Rhabditophora. The basiepithelial position of the NS, the very simple construction of the neuronal centralisation in the frontal end of *N. westbladi* and the absence of any anterior centralisation in *Meara stichopi,* indicate a more primitive nature of the NS in the Nemertodermatida than in the Acoela. However, here further research is needed.

CONCLUSIONS

SMITH et al. (1986) presented five possible cladograms (A-E) for the taxon Plathelminthes. The results of our studies support cladogram B, i.e. that the group Catenulida + Rhabditophora is monophyletic. As to the position of taxon Acoela, recent molecular studies place it separately from flatworms (see RUIZ-TRILLO et al., 1999; LITTLEWOOD et al., 1999). Our results support this view. However, according to BERNEY et al. (2000) the Acoela belong to the Plathelminthes. The position of the Nemertodermatida also seems basal, though no support was found for taxon Acoelomorpha

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SHORT NOTE

Genetic diversity of Japanese Dugesiidae (Platyhelminthes, Tricladida, Paludicola) studied by comparisons of partial 18S rDNA

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The specimens used in this study were collected from seven localities in Japan (Table1). The total DNA prepared from 14 living specimens was extracted according to procedures described in DE Vos & DICK (1). To isolate partial 18S rDNA, Polymerase Chain Reaction (PCR) was used with two primers (5'-TACTGTTGATCCTGCCAGTA-3' AND 5'-ATTACCGCGGCTGCTGGCACC-3', (2)). Amplified DNA fragments were purified with Wizard PCR Preps DNA Purification System (Promega), and were cloned in the pGEM-T Vector (Promega). Positive clones were confirmed with a colony PCR method. The purified PCR fragments were sequenced by the dideoxynucleotide termination method with a HITACHI SQ-5500 sequencer using the Texas Red labelled M13 primers.

TABLE 1

Japanese Dugesiidae sampled for this study

Species	Number of specimens	Number of clones	Locality
D. japonica	2	8	Hirosaki, Aomori Pref.
	1	4	Hashikami, Aomori Pref.
	1	2	Asahikawa, Hokkaido
	2	2	Iruma River, Gifu Pref. (1)
Dugesia sp.	4	9	Narutani River, Mie Pref.
D. ryukyuensis	2	2	Chinen Village, Okinawa Pref. (2)
D. tigrina	2	4	Nagoya, Aichi Pref. (3)

(1) GI strain established by Himeji Institute of Technology.
(2) OH strain established by Hirosaki University.
(3) from laboratory culture in Nagoya University. Details in KAWAKATSU et al. (1985).

The partial 18S rDNA sequence data are shown in Fig. 1. The sequence of *Dugesia japonica* was consistent with all clones from three localities (Hirosaki, Hashikami and Asahikawa). In the specimens collected from Gifu, the sequences of two clones, from two specimens, were different, but another was identical to those found in the other localities. Two types of partial 18S rDNA (*D. tigrina* 1 and *D. tigrina* 2) were also isolated from *D. tigrina* collected in Nagoya (3). The phenomenon has been reported from other populations of *D. tigrina* (4). Among the clones of *Dugesia* sp. from Mie we recognized five distinct sequences (*Dugesia* sp.M1-M5); clones M1 and M2 share 99.4% of sequence identity, the highest among the sequences that were compared among the Dugesiidae characterized here.

To investigate the phylogeny of Japanese Dugesiidae we reconstructed a phylogenetic tree using neighbour joining method as implemented in PHYLIP program version 3.5c (5). Fig. 2 illustrates the phylogenetic tree and the bootstrap values at all nodes. We suggested that M1 and M2 clones belong to type II 18S rDNA, M5 belongs to type I because it corresponds to the type I sequence of *D. japonica* from Gifu, and M3 and M4 perhaps belong to Type II. Of those specimens collected from the Mie population, the frequency of clone types is as follows, (clone type: number): (M1:3), (M2:2), (M1+M5:1), (M3+M4: 1). In *Dugesia ryukyuensis*, only type II of 18S rDNA sequence could be isolated.

KATAYAMA et al. and CARRANZA et al. have already reported the 18S rDNA sequences of *Dugesia japonica* (6, 7). We compared these sequences with our data. The alignments show that our sequences were different from the sequences and we believe this is due possibly to differences in sequencing method and/or genetic variation. The direct sequencing method is not appropriate for Dugesiidae because many species of *Dugesia* have two types of 18S rDNA (4, 8). We detected genetic diversity among *Dugesia* sp. from Mie and *D. japonica* from the

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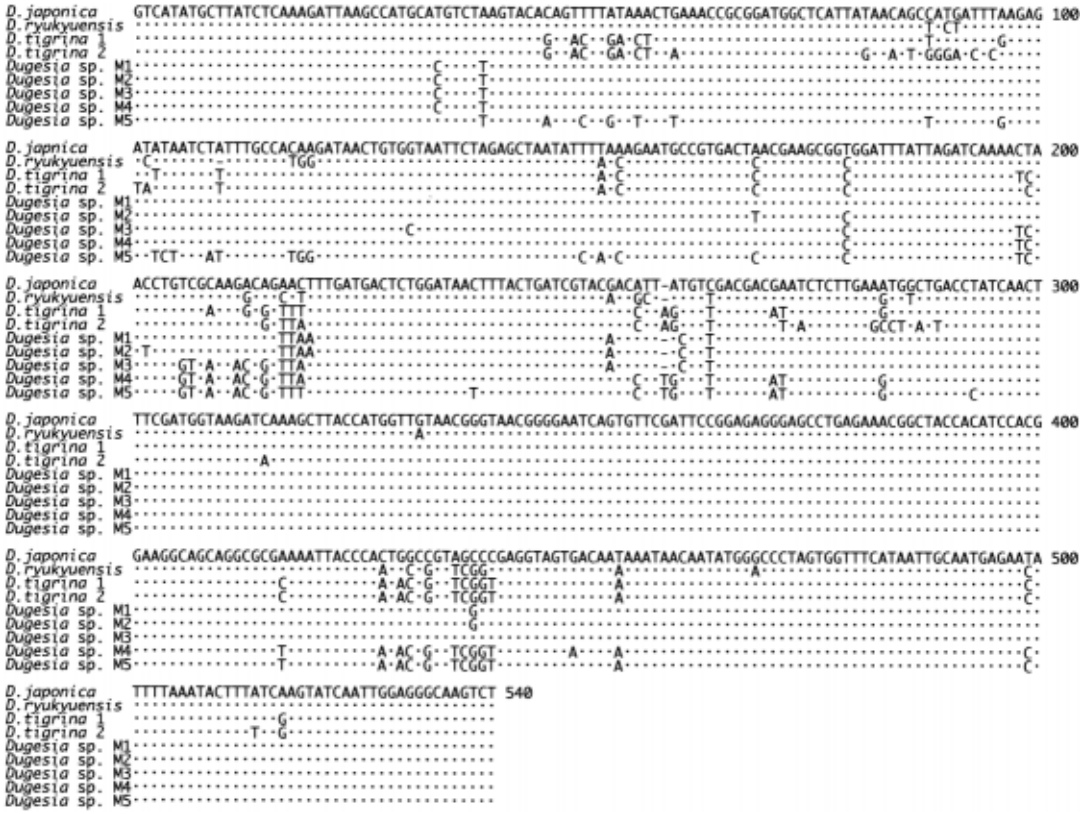

Fig. 1. – Alignment of nine sequences of partial 18S rDNA from Japanese Dugesiidae species. Dots indicate identity with the sequence of *D. japonica*. Dashes indicate deletions.

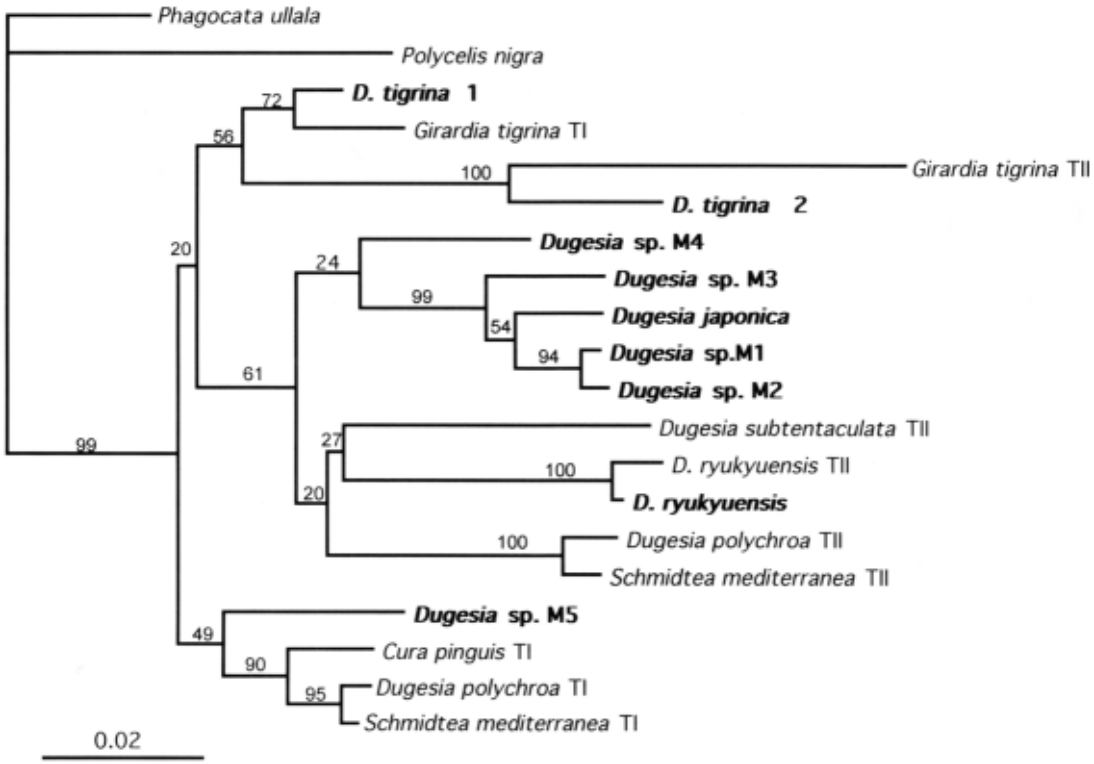

Fig. 2. – The neighbour joining tree for Japanese Dugesiidae and other Dugesiidae from the 18S rDNA sequences. The sequences in bold face were determined by this study and the other sequences have been deposited in GenBank (TI : typeI, TII : typeII). The sequences of *Phagocata ullala* and *Polycelis nigra* were used as outgroups. Bootstrap support (%; n=1000) indicated above the nodes. The scale means that line length equalizes to 0.02 genetic distance calculated by kimura's formula.

northern region of Japan that appears to be geographical intraspecific or interspecific variation.

In the *Dugesia* sp. population of Mie there are probably two types of 18S rDNA and at least two variants, but among *D. japonica* of the northern populations we detected only one type.

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ABSTRACTS

Nemertodermatida, a basal bilaterian group

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The taxon Nemertodermatida was originally classified within the Acoela, but was later separated from the acoels on account of their distinct intestinal lumen. More recently Nemertodermatida was hypothesised as the sister group of the Acoela in the taxon Acoelomorpha owing to similarities in epidermal ciliary systems in the two groups. However, in the first analyses of ribosomal DNA the nemertodermatid *Nemertinoides elongatus* grouped with members of the Rhabditophora and not with acoels.

We sequenced 18S rDNA from four Nemertodermatida and used parsimony analysis and parsimony jack-knifing of nucleotide and nucleotide triplet sequences to generate a hypothesis of the phylogeny of the Nemertodermatida. A secondary structure alignment and five multiple alignments with different gap opening penalties (ClustalW) were evaluated, all yielding similar results. The Nemertodermatida group basally in the bilaterian clade separate from the Acoela in our most parsimonious trees. The same results were obtained when the data set was analysed with maximum likelihood methods.

Branch support is low for the basal clades of the Bilateria in 18S rDNA. When Acoela were excluded from the data set, there was jack-knife support for the Nemertodermatida as the basal bilaterian clade. When the Nemertodermatida were excluded and Acoela left in the dataset, the Acoela was supported as the sister group of other bilaterians. With both Acoela and Nemertodermatida present, support for a basal bilaterian clade was absent.

The sequence labelled *Nemertinoides elongatus* (Genbank Acc no U70083) grouped with Rhabditophora (separately from the Nemertodermatida) also in our analyses. This indicates that the sequence is derived not from a member of the Nemertodermatida, but from a species of the Rhabditophora.

Results can be found in

Ruiz-Trillo, I., M. Riutort, D.T.J. Littlewood, E.A. Herniou & J. Baguñà (1999) Acoel flatworms: earliest extant bilaterian metazoans, not members of Platyhelminthes. *Science*, 283: 1919-1923.

The development of *Neochildia fusca* supports the position of the acoels as basal bilaterians

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The phylogenetic position of the acoel flatworms has long been controversial. They have traditionally been placed within the Platyhelminthes, either at the base of the metazoan tree or in the Lophotrochozoa. However, recent evidence suggests that they belong in a separate phylum of basal, direct-developing triploblastic metazoans (*Science* 283: 1919-1923). Acoel embryos exhibit a unique form of development that previously has been related to that found in polyclad turbellarians and coelomate spiralians, which display typical quartet spiral cleavage. Because developmental characteristics can provide evidence of relationships among metazoan groups, we used modern lineage tracers to generate the cell lineage of the acoel *Neochildia fusca*. Cleavage occurs in a duet pattern in which the second cleavage plane is leiotropically oblique relative to the animal vegetal axis. At the four-cell stage, the plane of first cleavage corresponds to the plane of bilateral symmetry, and subsequent cleavages are symmetrical across the sagittal plane. The first three micromere duets generate only ectodermal derivatives; there is no ectomesoderm. Both third duet macromeres produce the endomesoderm, including the complex musculature, as well as the peripheral and central parenchymas. The cleavage pattern, cell lineage, and mesodermal origins of *N. fusca* share little similarity with those of other metazoans, including the quartet-

cleaving Platyhelminthes such as the polyclads. If acoels flatworms belong to the lophotrochozoan clade, their development appears to represent a degenerate condition related to the abandonment of larval development.

Alternatively, however, we suggest that the acoel developmental program may be related to that of ancestral bilaterians, which were represented by small direct-developing, acoelomate animals exhibiting a form of bilateral (or biradial) cleavage, with mesodermal tissues arising solely from endodermal lineages.

Results can be found in

BOYER, B.C., J.Q. HENRY & M.Q. MARTINDALE (1996). Modified spiral cleavage: The duet pattern and early blastomere fates in the acoel turbellarian *Neochildia fusca*. *Biol. Bull.*, 191: 285-286.

HENRY, J.Q., M.Q. MARTINDALE & B.C. BOYER (2000). The unique developmental program of the acoel flatworm *Neochildia fusca*. *Dev. Biol.*, 220: 285-295.

Molecular markers for taxonomic identification and phylogeny of species of the genus *Dugesia* in the western Mediterranean

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The genus *Dugesia* (Gérard, 1850) comprises up to 70 described species, formerly included within the so-called species group or species complex *Dugesia gonocephala sensu lato* (s.l.), widely distributed in Africa and the Paleartic and Oriental regions. In the western Mediterranean, eight species of *Dugesia* have so far been reported. In this area, however, fissiparous populations clearly outnumber conspecific sexual populations. Because in *Dugesia* most species (the exceptions being *Dugesia hepta* and *Dugesia sicula*) have karyotypes that are a multiple of a basic haploid number of eight with almost identical karyograms, and because polyploidies, aneuploidies and the presence of B-chromosomes have been frequently reported, karyotypic analyses are of little help to assign fissiparous populations to their sexual counterparts. In addition the phylogenetic relationships between these species are still far from clear.

Here, we review the recent application of molecular markers that identify species or groups of species and that lead to a tentative new phylogeny for the species studied. In particular, we discuss results using sequences of the internal transcribed spacer region (ITS-1) of ribosomal DNA, the presence/absence of a family of long interspersed repeated elements (De1) first isolated in *Dugesia etrusca* (BATISTONI et al, 1999) and restriction pattern analysis of rDNA (BATISTONI et al, 1999). Main results were: 1) ITS-1 sequences and De1 contribute useful qualitative markers to identify single species or groups of species; 2) distance and parsimony analyses drawn from ITS-1 sequences show two main phylogenetic assemblages within the species studied, with a good internal resolution; and 3) all asexual populations were unambiguously assigned to particular sexual species.

These results show the usefulness of a molecular approach to taxonomy and phylogeny and the need to make congruent morphologically-based and molecularly-based taxonomies and phylogenies.

Results can be found in

BAGUÑÀ, J., S. CARRANZA, M. PALA, C. RIBERA, G. GIRIBET, M.A. ARNEDO, M. RIBAS & M. RIUTORT (1999). From morphology and karyology to molecules. New methods for taxonomical identification of asexual populations of freshwater planarians. A tribute to Professor Mario Benazzi. *Ital. J. Zool.*, 66: 207-214.

BATISTONI, R., L. ROSSI, A. SALVETTI & P. DERI (1999). A molecular cytogenetic comparison of planarians from the *'Dugesia gonocephala* group' (Platyhelminthes, Tricladida). *Ital. J. Zool.*, 66: 239-244.

EMBRYOLOGY AND DEVELOPMENT

Belg. J. Zool., 131 (Supplement 1): 63-64 April 2001

SHORT NOTES

Analysis of alkaline phosphatase expression during embryogenesis of *Pseudostylochus intermedius* (Platyhelminthes Polycladida)

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The Platyhelminthes have traditionally been considered to be extant representatives of the ancestral triploblastic animals and thus are an important group in studies of phylogeny and germ layer differentiation during development. Both direct and indirect polyclad developments characterize different species of polyclad turbellarian platyhelminths. The cell lineage of the indirect-developing polyclad *Hoploplana inquilina* has been traced using fluorescent markers by BOYER et al. (1, 2). However, direct-developing polyclad embryos have been examined only by observation of living material (3, 4).

The presence of alkaline phosphatase (ALP) as an endoderm-specific marker has been observed during embryogenesis of a number of invertebrates, including ascidians, sea urchins, and starfish (5, 6, 7). The purpose of this study was to characterize ALP expression as a marker of endodermal differentiation in the direct-developing polyclad *Pseudostylochus intermedius* Kato, 1939.

Adult worms were collected in the Natsudomari Peninsula, Aomori Prefecture. Fertilized eggs were obtained by poking the receptaculum seminis of starved mature worms with a needle. The eggs measured approximately 150 μm and developed to juveniles in about three weeks at room temperature. ALP expression was detected using the method of WITTAKER & MEEDEL (8) with some modifications.

ALP expression was observed in the periphery of the nuclei from the fertilized egg to the 4-cell stage. From the 8-cell stage, when the spiral cleavage pattern is first clearly detectable, to the formation of the mesentoblast, ALP is ubiquitously expressed in the micromere. This suggests that maternal alkaline phosphatase may be active in the micromeres at this time. During gastrulation ALP expression in the micromeres disappears but is detected strongly in two groups of cells bilaterally situated between the ectoderm and the yolk mass. This expression may be the initiation of zygotic ALP expression. After gastrulation, only the bilateral, strong expression can be seen in the embryo (Fig. 1A). In the small yolk mass stage, the bilateral staining has extended toward the animal pole and expression also appears in the dividing endodermal cells in the centre of the embryo (Fig.1 B). At the two eye-spot stage, ALP expression is seen in the developing intestines and in the pharynx, as well as in the bilateral regions first observed during gastrulation (Fig. 1C). In the juvenile, expression is obviously observed in the intestine, pharynx and protonephridia (Fig. 1D). Thus, polyclad embryos also exhibit endoderm-specific ALP.

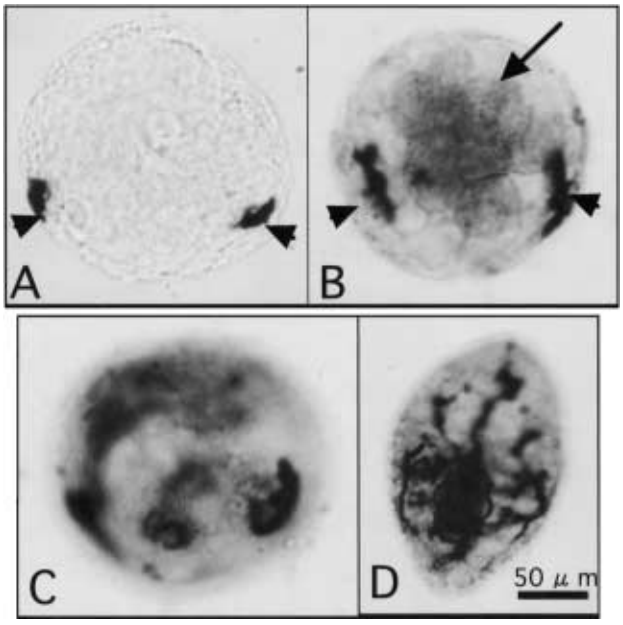

Fig. 1. – ALP expression during embryogenesis of *P. intermedius*.
(A) Bilateral, strong expression after gastrulation. (B) Small yolk mass stage. Expression also appears in the dividing endodermal cells in the centre of the embryo. (C) Two eye-spot stage. Expression is also observed in the developing intestines and in the pharynx. (D) Juvenile. Expression is seen in the intestine, pharynx and protonephridia. Arrowheads in A and B are expression in two groups of cells. Arrow in B indicates the dividing endodermal cell mass. A and B, dorsal view. C and D, ventral view.

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ALP expression in *P. intermedius* is interesting in that it is not confined to the endodermal tissue. The Gomori-Clark staining method indicates that the adults have flame cells that correspond in position to the bilateral regions of ALP expression in the embryo. Furthermore, the typical flame cell was observed at these regions with a transmission electron microscope (Fig. 2). The expression of ALP in nephridia has also been reported in annelids (9, 10). Thus we suggest that the bilaterally-stained regions, which first appear during gastrulation, are the primordia of protonephridia.

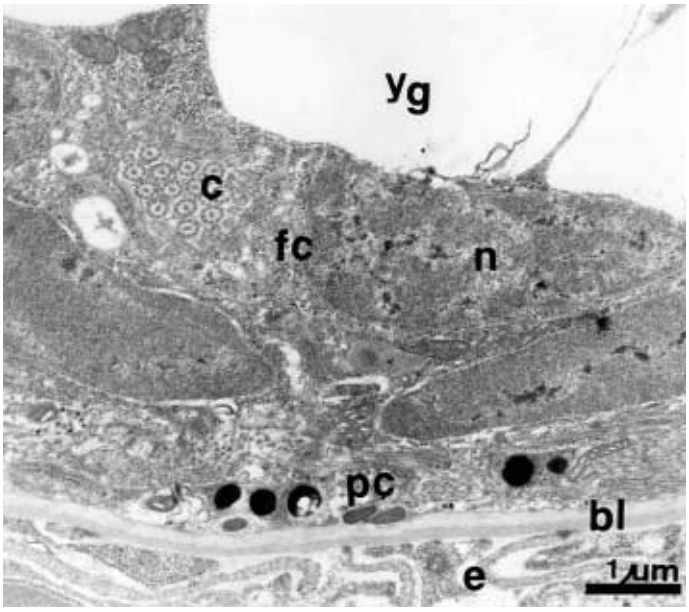

Fig. 2. – Electron micrograph of one side of bilateral ALP expressive region in juvenile (cross section).
A typical flame cell (fc) is situated near yolk granules (yg) in lateral sub-epidermal region. bl: basal layer, c: cilia, e: epidermis, n: nucleus, pc: pigmented cell.

This study provides evidence of ALP expression in both endodermal and mesodermal (protonephridial) tissues, suggesting that it can be used as tissue-specific marker for specific derivatives of both germ layers.

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On mitosis in embryos and larvae of polyclads (Platyhelminthes)

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The epidermis of flatworms is thought to be maintained by cells arising in the parenchyma (1-3). The taxon Catenulida is the only platyhelminth taxon with documented intraepidermal mitoses (4). On the other hand, in the gastrodermis, undifferentiated or proliferating cells are observed in many taxa of the flatworms (5-12), although they are not found in the Tricladida (13) and have not been studied in a number of other taxa of the Platyhelminthes.

There are only fragmentary data on the spatial arrangement of cell proliferation in the epidermis and gastrodermis in the early ontogenetic stages of flatworms. The question of particular interest is at which ontogenetic stage the final localization of stem cells is determined. Comparative data on mitotic activity during ontogeny are required for better understanding of tissue evolution in the flatworms.

We studied cell division in intact embryos and larvae of two polyclad species, *Notoplana humilis* (Stimpson, 1857) (development with an adult-like larva) and *Cycloporus japonicus* Kato, 1927 (development with a Müller's larva). Embryos at the stage of organogenesis (16 and 11 specimens, respectively) and larvae (9 and 15 specimens, respectively) were examined in series of stained paraffin sections.

In the embryos of *N. humilis* during early organogenesis, ectodermal mitoses were rare (~0.5 mitoses/embryo); in late organogenesis and in the larvae no mitotic figures were found in the ectoderm, with the exception of one dubious case. In *C. japonicus* ectodermal mitoses were observed both in the embryos of all stages of organogenesis and in the Müller's larvae (~1 mitosis/specimen). Both in the embryos and the larvae of each species mitotic figures were absent in the intestine, but occurred in mesodermal (mesenchymal) cells.

It has been shown for adult and juvenile polyclads that cell proliferation takes place in the parenchyma and gastrodermis but is absent in the epidermis (14, 15, 11, 12). The above mentioned and present studies support the hypothesis that in polyclads the formation of definitive epidermis is associated with the disappearance of mitotic cells in the integument, which occurs at different stages of morphogenesis. The time of this disappearance seems to depend on the type of development of the polyclad. On the other hand, formation of the definitive gastrodermis is linked with the appearance of mitotic cells in the intestine upon isolation from the parenchyma.

During organogenesis, ectodermal mitoses are known for only two turbellarian species, *Macrostomum appendiculatum* (Macrostomida) and *Minona trigonopora* (Proseriata) (16, 17). The current study has shown that a polyclad with a Müller's larva maintains mitotic cells in the ectoderm not only during embryogenesis, but also in the larva. Thus, *C. japonicus* presents the first example of a turbellarian with ectodermal mitotic activity in the late stages of morphogenesis. Since the existence of intraepidermal mitotic cells, which has been observed only for the Catenulida, may be considered as a plesiomorphic feature for the plathelminths (4), the ectodermal mitosis in Müller's larvae may indicate that the Müller's larva is plesiomorphic for this character.

Our conclusions can be supported by (*a*) analysis of the accumulation of mitotic figures resulting from blocking of mitoses in embryos/larvae and (*b*) more precise identification of cells as being "mitotic" using a suitable label.

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ABSTRACT

Early neurogenesis in flatworms

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We have initiated a series of studies of embryonic neural development in flatworms representing several different taxa, including acoels, macrostomids, polyclads, typhloplanoids, dalyelliids and temnocephalids. In our presentation we surveyed three pertinent studies: 1. We introduce a series of stages defined by easily recognizable morphological criteria that are applicable to all taxa. This staging system will serve us as a framework for our molecular and descriptive analysis of two "laboratory" species, *Macrostomum* and *Neochildia* that produce a sizeable number of eggs for developmental analysis year round. 2. Early neurogenesis: Neural progenitors are formed at an early stage when the flatworm embryo constitutes a multilayered mesenchymal mass of cells. A neurectoderm as in vertebrates or arthropods is absent. Only after neurons in the deep layers of the embryo have started differentiating do superficial cells reorganize into an epithelium that will give rise to the epidermis. Neurons are clustered in two anterior, bilaterally symmetric brain hemispheres. This implies that neurons later found in the trunk migrate out from the brain or are added on by neoblasts. After neural differentiation has set in, an antibody against acetylated beta-tubulin (anti-acTub) that binds neurotubules labels a pattern of pioneer neurons in the brain of midstage embryos of all species investigated. Pioneer neurons are grouped in several small clusters at characteristic positions. They pioneer several commissural tracts of the brain and two pairs of ventral and dorsal connectives, respectively. 3. We have cloned the POU genes *brn-1* and *brn-3*, as well as the homeobox gene *vnd* out of the macrostomid *Macrostomum* and the acoel *Neochildia*. In situ hybridisation carried out with 400bp fragments of these genes labels distinct populations of nerve cells in the brain of juvenile specimens. In situ hybridization on embryos at different stages is ongoing.

Results can be found in

YOUNOSSI-HARTENSTEIN, A., U. EHLERS & V. HARTENSTEIN (2000). Embryonic development of the nervous system of the rhabdocoel flatworm *Mesostoma lingua* (Abildgaard, 1789). *J. Comp. Neur.*, 416: 461-476.

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GENES AND GENE EXPRESSION

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Microsatellite development and inheritance in the planarian flatworm *Schmidtea polychroa*

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ABSTRACT. We developed four polymorphic microsatellite loci for the hermaphroditic planarian *Schmidtea polychroa* and used them to identify differences in recombination rate in the male and female germ line. DNA isolation protocols were optimized for tissue from adults and hatchlings. The final PCR protocols and profiles yielded repeatable and reliable amplification. Null alleles detected at one locus could be avoided by redesigning one primer. Routine genotyping was established using fluorescent-labeled primers and an ABI 310 automated sequencer. They amplify successfully in a number of populations. The four loci are characterized by extreme within-population polymorphism, with 15 to 20 alleles per locus in a standard sample. All four have been submitted to GenBank. Two loci (SpATT16, SpATT18) appeared to be coupled. From parent-offspring comparisons, the recombination fraction could be estimated, which was significantly different for the male (c = 0.07) and female (c = 0.23) line. High overall exclusion rates for first (>0.94) and second parent (>0.98) even with three (unlinked) loci demonstrates the suitability of these microsatellites for other applications such as parentage analysis.

KEY WORDS: Platyhelminthes, hermaphrodite, microsatellites, recombination rate, *Schmidtea polychroa*.

INTRODUCTION

Within the last decade, microsatellites have proven to be extremely powerful codominant genetic markers for parentage as well as population studies (QUELLER et al., 1993; JARNE & LAGODA, 1996; HUGHES, 1998). They consist of 'simple sequences', in which short nucleotide motifs (usually 1-6 bp) are tandemly repeated, and which occur throughout the genomes of all eukaryotic organisms (TAUTZ & SCHLÖTTERER, 1994). Variation in the repeat number of the microsatellite motif can easily be detected electrophoretically as fragment length polymorphism following PCR of the microsatellite locus.

Previously described microsatellite loci (RAMACHANDRAN et al., 1997) in the simultaneously hermaphroditic freshwater planarian *Schmidtea* (formerly *Dugesia*) *polychroa* Ball (1974) could reliably be amplified and scored in some of our study populations but not in others. In particular, samples from some of the most important study populations from Northern Italy showed no or poor amplification for those loci. Therefore, a second genomic library was constructed with DNA samples from individuals collected in Lago di Caldonazzo (Trentino, Italy), where many samples for population genetic analyses as well as paternity studies have also been collected.

METHODS

Microsatellite screening and amplification

Screening was restricted to $(ATT)_n$ trinucleotide repeats, because the first screen had shown their high abundance in the genome of *S. polychroa*. Methods used for constructing the library and screening were the same as described in RAMACHANDRAN et al (1997). Fourteen positive clones were isolated and sequenced, all containing repetitive $(ATT)_n$ sequences (GenBank accession nos.

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AF201314-AF201327). We designed PCR primers for amplification of these regions, and successfully achieved amplification in 5 cases, for which the primer sequences are listed in Table 1. Presence of ATT repeats in the PCR products was verified by blotting and hybridizing with a DIG labeled oligonucleotide $(ATT)_6$ probe. Banding patterns of SpATT19 PCR products on 10% PAGE gels did not suggest within- or between-population polymorphism, while the ones obtained from loci SpATT12, SpATT16, SpATT18 and SpATT20 showed considerable allele size variability. All subsequent analyses of those four loci were done with fluorescent-labeled primers (see table 1) and a PE Applied Biosystems ABI310 genetic analyzer. PCR was performed in 10 μL reactions with the following components (final concentrations): $MgCl_2$ (2.5 mM), BSA (0.1%, bovine serum albumin), dNTPs (0.2 mM each), primers (0.4 μM each), Promega® Taq Polymerase (0.003 units/μL), and Promega® DNA polymerase buffer (as recommended in the product information). 0.4 μL of template DNA solution (50-200 ng/μL) was added to 9.6 μL of premix. PCR conditions included an initial 2´ denaturation step at 94°C prior to cycling, and 30-35 cycles of the following temperature profiles: 40´´ at 94°, 1´ primer annealing, and 1´ at 72°C (loci SpATT12, SpATT16, SpATT20), and 50´´ (94°C), 1´ (55°C) for SpATT18.

TABLE 1

Characteristics of PCR primers for amplification of the four microsatellite loci in *S. polychroa* (FL = fluorescent label used for detection on Abi310; B = length of the primer in nucleotide numbers)

Locus/primer	Name	Nucleotide sequence	FL	B
SpATT12/forward	ATT12U	5'TTAGATTTTGCTGGATGAA 3'	-	19
SpATT12/reverse	ATT12L	5'TTGCCACTGAAATAATAA 3'	TET	18
SpATT16/forward	ATT16U	5'TTGATGAGAAATTATTGAAA 3'	6-FAM	20
SpATT16/reverse	ATT16L	5'CTTGCATTTTGCTCTGATAA 3'	-	20
SpATT18/forward	ATT18U	5'TACATTATTCGCAACAAAA 3'	HEX	19
SpATT18/reverse	ATT18BL	5'TTGGTAAAATCTCTTGAACA 3'	-	20
SpATT20/forward	ATT20BU	5'CCAGGAGATTGACAAAGACT 3'	6-FAM	20
SpATT20/reverse	ATT20L	5'ATGTTTACCACTAAAATTATTG 3'	-	22

Sample origins

Adult individuals collected at various sites from four different localities in Northern Italy provided DNA samples for the characterization of the variability within and between populations at the four loci, which enabled us to evaluate their use for parentage testing and population studies. Mendelian inheritance could be demonstrated using known mother-offspring pairs from our laboratory culture animals (data not shown). In order to study sex-specific linkage, parent-offspring comparisons with suitable variability (heterozygous genotypes in the parental genotype at both loci) were analysed. They allowed calculation of the recombination fraction between the two loci separately for the male and female line. 342 father-offspring pairs from 40 different sperm donors could be used for analysis of the male gametes, and 299 mother-offspring pairs from 41 different mothers for analysis of the female gametes.

DNA isolation

Samples obtained from adults

Tissue for DNA extraction was cut off the posterior part of individuals and stored in pure absolute ethanol at -20°C or -80°C. Tissues were transferred to empty 1.5 ml tubes, air-dried in the open tubes (3 min) before adding 400 μL of DNA extraction buffer (10 mM Tris/HCl, 2 mM EDTA, 10 mM NaCl, 1% SDS, 0.4 mg/ml Proteinase K). Samples were incubated at 50°C for 2 h until the tissue was completely dissolved, followed by heating the mixture to 90°C for 5 min in order to stop Proteinase K activity. After chilling the samples on ice, 200 μL of 4.5 M NaCl solution was added in order to precipitate proteins. After adding 600 μL of Chloroform-Isoamylalcohol (24:1), samples were mixed and shaken for 10 min by inverting the tubes continuously. Aqueous and organic phases were separated by centrifuging at 16000g for 10 min at RT. From the aqueous phase, 500 μL of each sample were transferred to new tubes. DNA was precipitated by adding 500 μL of isopropanol. Centrifugation (16000g, RT) resulted in brownish pellets containing DNA and epidermal pigments of the individuals. The pellets were washed (70% EtOH), air-dried at RT for about 20 min, and redissolved in TE at room temperature.

Samples obtained from hatchlings

Hatchlings provide only very limited material for DNA extraction, and therefore complete individuals were extracted using a commercial DNA extraction kit (Nucleon BACC1, Amersham™). We used the protocol provided for extracting DNA from mammalian blood cells, but with reduced volumes. Hatchlings were transferred to Eppendorff tubes containing 100 μL of *Reagent*

A from the kit, which then were shock-frozen in liquid nitrogen, and stored at -80°C.

RESULTS

Characterization of the four microsatellite loci

All four microsatellite loci were highly polymorphic (Table 2). Locus SpATT16 has only been tried on samples from two populations, while the three other loci could successfully be amplified in all diploid sexual as well as tri- and tetraploid parthenogenetic *S. polychroa* populations that we sampled across Europe (data not shown). Data analyses concerning population differentiation in sexuals, and genetic diversity in parthenogenetic populations will be presented elsewhere. Amplification of the loci failed in two closely related sister species (Benazzi´s biotypes E and F; BENAZZI, 1982).

TABLE 2

Characteristics of the four microsatellite loci in 13 subpopulations collected from four locations in Northern Italy (Lago di Levico, Lago di Caldonazzo, river Sarca near Arco, Lago d'Iseo). Details of the population studies will be published elsewhere.

Locus	SpATT12	SpATT16	SpATT18	SpATT20
A	30	24	36	28
BP	199	299, 353-365	255-261	220, 226
	226-262	413, 422, 425	346, 388	232-304
	268-314	452-482, 497[a]	397-487	310
R	4-42	47-116[a]	see text	19-49
H_{obs}	0.83	0.61	0.86	0.85
H_{exp}	0.89	0.88	0.89	0.89
N	640	67	613	644

A Allele numbers
BP Allele size range (base pairs)
R Allele size range (repeat numbers)
H_{obs} Observed heterozygosity (mean over 13 subpopulations)
H_{exp} Expected heterozygosity (mean over 13 subpopulations)
N Sample size (number of adults analysed)
a plus 3 alleles > 500 bp

The allele size ranges comprised almost continuous allele arrays consisting of 13-42 (SpATT12), about 50 to 80 (SpATT18, see below), and 19-47 (SpATT20) repeat units (Table 2). SpATT16 showed a highly discontinuous allele size range, with extremely large allele size differences (> 200 bp). Large allele size differences were also found at locus SpATT18. Apart from the pure *ATT* repeat motifs, the repeat region of SpATT18 contains other derived motifs (*ACT*, *ATTT*), and therefore assigning repeat numbers as allele labels would have required an arbitrary definition of what is considered the repeat array. Sequencing of the short alleles showed that they lacked almost the entire core sequence (data not shown).

Unusual banding patterns

Detection of a third allele in offspring samples

Individuals at hatching are full of swallowed yolk cells, some of which are still intact (MARINELLI & VAGNETTI, 1975). Since yolk cells provide an excellent source for maternal DNA, it is likely that hatchling DNA samples can contain small amounts of maternal DNA extracted from yolk cells that are occasionally amplified during PCR. This was concluded from the analysis of 198 offspring samples, for which the maternal genotypes were known, and which had an additional band that was much less intense (by at least one order of magnitude) than the other (one or two) bands defining the genotype. The respective banding patterns were clearly different from the ones obtained from triploid individuals, and therefore did not indicate triploidy. Out of 198 offspring samples with known maternal genotypes, 190 supported the interpretation that the additional weak bands represented the second maternal allele, amplified from small amounts of DNA from yolk cells extracted together with the hatchlings. Eight cases only were not consistent with this hypothesis, probably due to mutation or genotyping errors.

Peak intensity patterns at SpATT12

Locus SpATT12 showed particular peak intensity patterns that depended on the allele size combinations in heterozygotes. The allele frequency distribution is bimodal, with a shorter range (SR) of alleles 15 to 25 repeats long, and a longer one (LR) comprising alleles from 27 to 41 repeats in length. Usually, when two peaks indicate a heterozygous genotype, the peak representing the longer allele appears less intense. This was also the case in SpATT12 when the genotype was either composed of two SR or two LR alleles. However, when an SR allele occurred together with an LR allele, the intensity of the former was much weaker than the one of the LR allele. A more detailed treatment of these particular banding patterns, including pictures of the respective electropherograms, is given elsewhere (PONGRATZ, 2000).

Null alleles

A common problem with microsatellites is the occurrence of null alleles (PEMBERTON et al., 1995). Null alleles are alleles that are not amplified through PCR. They are often caused by a mutation within the primer region, which prevents proper annealing under stringent conditions, and inhibits or completely prevents

amplification (CALLEN et al., 1993). Samples with one null and one amplified allele appear as homozygous genotypes. This can result in significant heterozygote deficiency. Null alleles can impair the use of a microsatellite locus for paternity when they remain unnoticed. In practice, the best way to detect null alleles is by following inheritance of the alleles in known parent-offspring combinations (CALLEN et al., 1993). Indications for the presence of null alleles have been found at SpATT16 (N=66) where a significant heterozygote deficiency was observed (H_{obs}=0.61, H_{exp}=0.88 ; χ^2 = 37.18 ; P < 0.001). With genotype comparisons of known parent-offspring pairs it could be confirmed that some genotypes that appear as homozygotes, because only one allele is seen, must have a second allele that is not or only poorly amplified and cannot be detected.

Linkage

Parent-offspring comparisons as well as disequilibrium analyses of field samples did not show indications for linkage between pairs of loci except for SpATT16 / SpATT18. The loci are not strictly linked, but allelic combinations for those loci among the offspring show significant deviations from the ones expected under random association. The two loci therefore appear to be on the same chromosome. There was a significant difference between observed allelic associations in the gametes and the ones expected under random segregation of alleles for both male and female line. A χ^2-test revealed highly significant differences between the frequencies of coupled and recombined genotypes between male and female gametes (Table 3). We estimated the recombination fraction $c_{SpATT16/SpATT18}$ between loci SpATT16 and SpATT18 (Weir, 1996, p.230). For two loci (A, B), c_{AB} can vary between 0 (no recombination between loci = strict linkage) and 0.5 (random segregation of alleles from the two loci). The mean estimate for $c_{SpATT16/SpATT18}$ was 0.06 for the male line, and 0.23 for the female line, which means that 6% and, respectively 23% of gametes bear recombined two-loci genotypes.

TABLE 3

Frequencies of coupled and recombined two-loci haplotypes at SpATT18 and SpATT16, and the recombination fraction ($c_{SpATT18, SpATT16}$) estimated from them. The frequencies between male and female gametes are significantly different (χ^2 = 36.28 ; P < 0.01)

Genotypes	Male	Female
Coupled	320	230
Recombined	22	69
Total	342	299
$c_{SpATT18, SpATT16}$	0.06	0.23

DISCUSSION

Potential of the four microsatellites as genetic markers

The four microsatellite loci described here showed reliable and consistent amplification for *S. polychroa* and have been applied to population samples of different origins. They were highly polymorphic within local populations, with 15-20 alleles in standard population samples. SpATT12, SpATT18, and SpATT20 represent suitable markers for both parentage analysis and population genetic questions. Care has to be taken when using SpATT16 due to nonamplifying or undetectable alleles. Whether SpATT16 can be applied has to be decided for each study and population separately.

There was evidence from mother-offspring comparisons that some offspring samples contained remnants of maternal DNA from ingested yolk cells that was amplified during PCR but appeared much weaker than the true alleles. This has to be taken into account when analysing offspring sample banding patterns.

In general, the high polymorphism present in the diploid sexual study populations makes the four microsatellite loci ideal markers for individual identification, and identification of parents and offspring. Individuals collected from the same subpopulation showed sufficiently high polymorphism to allow successful parentage assignment even when several candidates for maternity and paternity were present (PONGRATZ, in prep.). In contrast to previously studied allozymes (PONGRATZ et al., 1998), the microsatellite loci can be used for studies of population differentiation on a very small scale. For polyploid, parthenogenetic populations they can provide a better resolution of the clonal structure (unpublished data), and they can also be extremely useful in the analysis of hybridization processes in mixed sexual/parthenogenetic populations.

Sex-specific linkage

We found significant differences in the recombination fraction between male and female line for one pair of loci. Sex differences in recombination have been documented in a variety of taxa by studying chiasma frequencies in spermatocytes and oocytes (review in BURT et al., 1991), but across species no conclusive relationship between sex and chiasma frequency could be observed (BURT et al., 1991). A higher chiasma frequency (which results in a higher recombination rate) in oocytes compared to spermatocytes was also observed in the planarian *Dendrocoelum lacteum* (JONES & CROFT, 1989). The higher recombination rate between loci SpATT16 and SpATT18 may indicate the same general trend in *S. polychroa*, although detailed recombination studies in humans revealed that the male-female recombination ratio can vary significantly over short regions (ROBINSON & LALANDE, 1990). Certain regions with higher female

recombination rate can occur, while other regions on the same chromosome show higher male recombination rates (ROBINSON & LALANDE, 1990; ZOGHBI et al., 1990). Therefore, a general conclusion regarding sex differences in chiasma frequencies and recombination rates, should not be drawn from the analysis of only two loci.

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Evolutionary conservation of the initial eye genetic pathway in planarians

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ABSTRACT. Eyes of all organisms share a common function, visual perception. In addition, the different types of eyes (camera-, mirror-, and compound) are present in different phyla and share the same visual pigment, rhodopsin, and the same initial genetic pathway triggered by the master control gene Pax-6. Although the developmental mechanisms are quite diverse, all data suggest that the different eye types found in metazoans derive from a common prototype and evolved in the different phyla by parallelism, intercalating new genes independently. In this manuscript, we describe the isolation and characterization of several genes that constitute the eye gene regulatory network in the planarian *Girardia tigrina* (Platyhelminthes; Turbellaria; Tricladida). Two Pax-6 genes, *GtPax6A* and *GtPax6B*, do not show an obvious correspondence to the two Pax-6 of Drosophila *ey* and *toy*. Two sine oculis genes *Gtsix-1* and *Gtsix-3* are closely related to the Six 1-2 and Six-3 families respectively. Furthermore, we demonstrate that the opsin gene *Gtops* shows greater similarity to mollusc opsins. *GtPax-6B* is expressed in both cell types of the planarian eye spots: the photoreceptor cells and the pigmented cells. In addition, *Gtsix-1* and the opsin gene *Gtops* are expressed in the photoreceptor cells. This expression pattern is present throughout the whole eye regeneration process and maintained in adults. *Gtops* double strand RNA injection does not inhibit eye regeneration but produces light insensitive eyes due to the absence of photopigment. The loss of function of *Gtsix-1* by dsRNA injection produces a non-eye phenotype in head regenerating blastemas, while the injected intact adult heads show a loss of the differentiated state of the photoreceptor cells through inhibition of opsin expression and the production of a blind phenotype. Our results on the prototypic eye spots of Platyhelminthes provide further important support for the idea of a universally conserved early eye genetic cascade in the Metazoa.

KEY WORDS: Platyhelminthes, planarian, homeobox, opsin, eye evolution, regeneration.

INTRODUCTION

Several studies indicate that the genetic program regulating eye development has been conserved in evolution. The gene regulatory network that controls the development of the *Drosophila* visual system is composed of several transcription factors and other nuclear proteins required for the specification of early eye morphogenesis (QUIRING et al., 1994; HALDER et al., 1995; SHEN & MARDON, 1997; PIGNONI et al., 1997). Pax-6 genes encode transcription factors that contain a paired domain and a homeodomain. Two duplicated Pax-6 genes have been described in *Drosophila*, *eyeless (ey*) and *twin of eyeless (toy*). Both genes and their homologs from other metazoans are capable of inducing ectopic eye development in *Drosophila*. Mutations in *eyeless* lead to defects in eye formation, suggesting that Pax-6 is the universal master control gene for eye morphogenesis (QUIRING et al., 1994). The study of other genes in the genetic cascade and their genetic interactions resulted in the identification of three genes in *Drosophila*, *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) that act downstream of *ey* (QUIRING et al., 1994; SHEN & MARDON, 1997). The *so* homologs, called *Six* genes, share a diverged homeodomain and N-terminal to the homeodomain, and another conserved region, the Six domain, which contributes to DNA-binding specificity (SERIAKU & O'TOUSA, 1994; CHEYETTE et al., 1994; OLIVER et al., 1995a; 1995b; KAWAKAMI et al., 1996). The Six genes are subdivided in three different families: Six1-2, Six3 and Six4. *eya* encodes a novel nuclear protein (BONINI et al., 1993) and shares a region of homology with the Eya vertebrate proteins, the Eya domain (XU et al., 1997; ZIMMERMAN et al., 1997). Eya and Six proteins are expressed in overlapping patterns including the eye primordia of vertebrates,

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whereas *eya* and *so* do so in *Drosophila* (OLIVER et al., 1995b; XU et al., 1997; SEIMIYA & GEHRING, 2000). These factors appear to act in a hierarchy in which *so* is directly regulated by *eyeless* (HALDER et al., 1998; NIIMI et al., 1999), the master control function. In turn, *so* requires *eyes absent* (*eya*) to induce ectopic eyes (PIGNONI et al., 1997). This genetic pathway has been established in *Drosophila* (reviewed in GEHRING & IKEO, 1999). Homologous proteins also regulate eye development in vertebrates, suggesting that this regulatory network is old, conserved in evolution, and has been adapted to the control of development of different visual systems found in both Protostomia and Deuterostomia clades (TREISSMAN, 1999).

Charles Darwin in "The Origin of species" discussed the question of eye evolution, and reasoned that such complex and perfect organs should have evolved from a simple prototypic eye; those primitive eyes can be found in planarians. The planarian eye spots consist of two cell types: a bipolar nerve cell with a rhabdomere as a photoreceptive structure and a cup-shaped structure composed of pigment cells (KISHIDA, 1967). During head regeneration, new eye spots are formed from precursor cells that probably differentiate into both cell types in a restricted area of the newly regenerated tissue or blastema.

Here we address the hypothesis that the eye genetic network is conserved in evolution, and, as a consequence, that *Girardia tigrina* eye development requires *Pax-6* and *sine oculis* homologs. We report the identification of two Pax-6 genes not closely related to *Drosophila ey* and *toy;* two *so* genes closely related to the *Drosophila so* and *optix* genes, which belong to *six 1-2* and *six 3* families respectively; and an *opsin* gene with high identity to that found in the Lophotrochozoa clade. Some of these genes are expressed in the eye primordia during regeneration and in the differentiated adult eyes. RNA interference (RNAi) experiments provide functional evidence that *Gtsix-1* is essential for maintenance of the differentiated state of photoreceptor cells, for opsin expression and for eye regeneration. Such results support the conservation of the early genetic pathway in the different eyes of metazoans.

MATERIAL AND METHODS

Gene isolation

A *GtPax6A* fragment was amplified by PCR from planarian cDNA (Smart PCR cDNA synthesis Kit, Clontech). The sense primer used (Px9), consisting of a degenerate sequence corresponding to amino acid sequence LEKEFER and the antisense primer used (Px10) consisting of a degenerated sequence corresponding to amino acid sequence QVWFSNR. The cycling program consisted of 35 cycles (94ºC, 30 sec; 45ºC, 30 sec; 72ºC, 30 sec). The identity of *GtPax6A* fragment was confirmed by sequencing. A lambda gt10 amplified cDNA library was screened with the 110 bp amplified fragment of *GtPax6A* according to GARCIA-FERNANDEZ et al., 1993. One phage was isolated containing an insert of 645 bp that spans from the homeodomain to the 3' end of the *GtPax6A* cDNA. This fragment was cloned in pBluescript (Stratagene) and the sequence was determined with Thermosequenase II dye terminator cycle sequencing Kit (Amersham). A partial fragment of 380 bp of *Gtops* was amplified using two specific primers based on *Schmidtea mediterranea* opsin partial fragment kindly provided by A. Sanchez and P. Newmark (Carnegie Institution, Baltimore). The amino acid sequence of the upstream primer used (op1) and the downstream primer used (op2), GFIGGLG and ELEMLK respectively.

Phylogenetic analysis of the Gtops opsin gene

The phylogenetic trees of opsin genes using the sequence between the 3rd and the 5th transmembrane domains were inferred by using the CLUSTALX package. Sequences were aligned with the software CLUSTALX, and refined alignment was done manually. The neighbor-joining method was used for phylogenetic tree construction. Sequences were obtained from the Swissprot and EMBL GenBank.

Whole-mount *in situ* hybridization

Intact animals and animals at different regenerative stages were used for whole-mount *in situ* hybridization according to UMESONO et al.,1997. The opsin clone op-170 corresponding to the last 170 bp of the *Gtops* fragment (GenBank accession no. AJ251660) was used to synthesize the DIG-labelled antisense probes (Boehringer Manheim).

Double-strand RNA (dsRNA) synthesis and microinjection

The Gtsix-1 clones so-5' and so-3'-2 (GenBank accession number AJ251661O), were used for dsRNA synthesis as described in SANCHEZ & NEWMARK 1999. Planarians were injected as described in PINEDA et al., 2000. At different stages of regeneration the injected animals were photographed, fixed and whole-mount *in situ* hybridizations for *Gtopsin* were performed.

RESULTS AND DISCUSSION

Isolation of eye developmental genes in Girardia tigrina

A large number of eye developmental genes have been isolated in *Girardia tigrina*. Initially, CALLAERTS et al., 1999 isolated the first Pax-6 homolog, which we now refer to as *GtPax-6B* since it shows the lowest similarity to the Pax-6 genes described in other Metazoa (Table 1).

TABLE 1

The number of different planarian eye network genes, together with the type of clones isolated and the level of amino acid identity to the most similar homeodomain and proteins. All sequences for comparison are taken from the EMBL and Swiss Prot databases. The function of some genes is also shown.

Planarian eye genes					
Class	**Pax-6**		**sine oculis**		**opsin**
	DtPax-6	*GtPax-6*	*Gtso*	*Gtsix-3*	*Gtops*
New proposed name	*GtPax-6B*	*GtPax-6A*	*Gtsix-1*	*Gtsix-3*	*Gtops*
Type of clone isolated	-PCR -cDNA -genomic	-PCR -cDNA	-PCR -cDNA -genomic	-PCR -cDNA	-PCR -cDNA
Identity	72% and 78% to *Drosophila* toy and eye homeodomains	92% and 90% to *Drosophila* toy and eye homeodomains	93% and 95% to mouse six1 and six-2 homeodomains	88% and 92% to *Drosophila* optix and mouse six3 homeodomains	66% to mollusc opsin protein
Expression and Function	photoreceptor and pigmented eye spot cells	Central Nervous System	Photoreceptor cells. It is essential for eye determination and differentiation	?	Photoreceptor cells. It is essential for eye light sensitivity

Studies by in situ hybridization on paraffin sections and electron microscopy of ultrathin sections revealed expression in the perinuclear area of both eye spot cell types, the photoreceptor cell and the pigmented cells. dsRNA injection of *GtPax6B* does not produce any clear eye phenotype. One explanation for these unexpected results could be the occurrence of gene redundancy. It was therefore of interest that more recently we were able to isolate a second Pax-6 homolog, which we name *GtPax-6A* as it shows a higher similarity to the Pax-6 family homeodomain (Table 1). No expression pattern nor functional data are yet available, but preliminary whole-mount in situ hybridization showed expression in the central nervous system. We anticipate that *GtPax6A* and *GtPax6B* are at least partially redundant in the determination of both eye cell types, and that, as a consequence, the production of any phenotype when the *GtPax6B* function has been disrupted, is prevented. Injection of dsRNA of both GtPax-6 in head regenerating organisms will clarify this point (work in progress).

A second type of eye genetic network genes isolated in planarians was the *sine oculis* genes. The first *so* planarian gene isolated was originally named *Gtso*. However, molecular comparative phylogenetic analysis places it in the Six-1, -2 family. Therefore, we changed the name from *Gtso* to *Gtsix-1* (Table 1). *Gtsix-1* is closely related to *Drosophila sine oculis* and C. elegans *Ceh-33* and *Ceh-34* and clusters in the family group with another branch in which the vertebrate representatives of Six-1 and Six-2 are situated. Sequence comparison of a second *sine oculis* gene from *Girardia tigrina* supports its orthology to the Six-3 family and for that reason we call it *Gtsix-3*. It is closely related to *Drosophila optix* and *C. elegans Ceh-32*. So far, no other Lophotrochozoa *sine oculis* genes have been isolated. *Gtsix-1* whole-mount *in situ* hybridization shows that it is expressed continuously in the rhabdomeric photoreceptor cells of the adult differentiated eye spots and during the different stages of eye regeneration (Pineda et al. 2000). Loss of function experiments by RNA double strand injections in regenerating animals completely inhibit eye regeneration, producing a non-eye phenotype (Fig. 1). RNA interference in adult heads leads to the gradual loss of the photoreceptor differentiated state, producing a blind phenotype at one week post-injection. These injected planarians do not show any phototropism, while the non-injected controls have a negative phototropism. The change in the differentiated state can be observed by analyzing the alterations in opsin

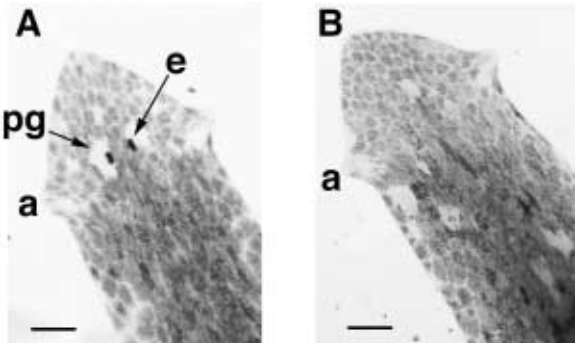

Fig. 1. – Inhibition of eye regenerative capacity by Gtsix-1 dsRNA. Three weeks head regenerating organisms viewed dorsally. (A) Bright field image shows the differentiated eyes in the regenerated head of a control organism. (B) Bright field image shows the absence of eye differentiation and periglobular unpigmented area in dsRNA Gtsix-1 injected at postblastema level after 3 weeks of regeneration. a, auricle; e, eye spot; pg, periglobular unpigmented area. (Bars= 400 μm).

expression by whole-mount *in situ* hybridization at different times post-injection and by studying eye morphology. Opsin expression decreases gradually during the first seven days post-injection (Fig. 2). A decrease of expression to zero can be explained as the result of a disruption of the eye gene regulatory network where, according to SHEN et al., 1997 and PIGNONI et al., 1997, sine oculis is located in the early eye genetic cascade. The gradual loss of opsin expression by Gtsix-1 RNAi could thus be due to an indirect effect. However, since Gtsix-1 is also expressed in the differentiated photoreceptor cells, it may also directly control opsin gene expression. Further analysis of the cis-regulatory region of the opsin gene will confirm if it is recognized by sine oculis, or by Pax-6 proteins, or both. We also checked Gtsix-1 function in photoreceptor cell maintenance by the histological analysis of *Gtsix-1* dsRNA injected organisms in comparison with controls. While the control eyes show a high density of photoreceptor cells with their rhabdomeric structures inside the eye cavity, the injected ones at 7d and 14d post-injection have a lower density of differentiated photoreceptor cells (work in progress).

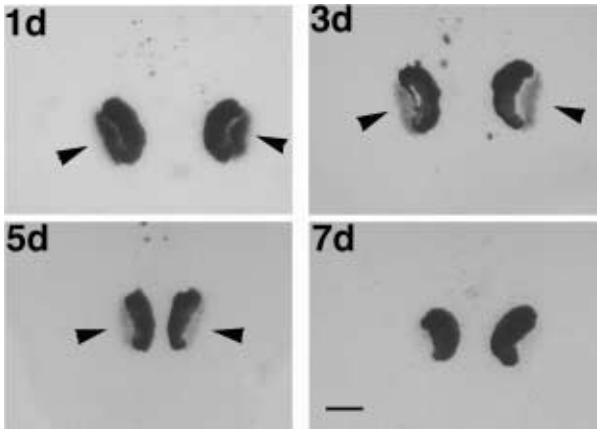

Fig. 2. – Dorsal view of Gtops gradually declining expression by whole mount *in situ* hybridization at different days post-injection of dsRNA of Gtsix-1. Internal black dots correspond to the pigmented cup shape of the eyes, while the external weak signal (arrow heads) corresponds to the blue signal from the whole mount in situ hybridization of opsin gene. Anterior at the top and upper left edge, the days after injection are indicated. (Bars: 200μm).

Another key gene shared by photoreceptors of Metazoa is the opsin gene, encoding the photoreceptor pigment present in all visual systems. Rhodopsin sequences analysed so far in vertebrates and invertebrates show a high degree of conservation, and all belong to the same family (for review see GEHRING & IKEO, 1999). Opsins are also present in bacteria, and have sensory functions. Despite a low overall sequence conservation, they show conserved structural functions like the seven transmembrane domains. The unicellular green algae *Chlamydomonas* develop at the base of the flagella a light sensitive organelle that contains a type of photopigment with limited sequence homology to invertebrate rhodopsins (DEININGER et al., 1995). The similarities observed in the photopigments have been used as another indication for the common origin of the visual system through a prototypic eye. We have isolated a *Girardia tigrina* opsin gene *Gtops* . Its amino acid sequence was compared and phylogenetic trees were constructed with opsin protein sequences of bacteria, algae, yeast and Metazoa. The neighbor-joining method was used for tree construction. We can observe the clustering of the two planarian opsin genes with the mollusc sequences, one of their Lophotrochozoa counterparts (Fig. 3). Such phylogenetic results are in agreement with other studies using different molecules such as ribosomal 18S and Hox genes

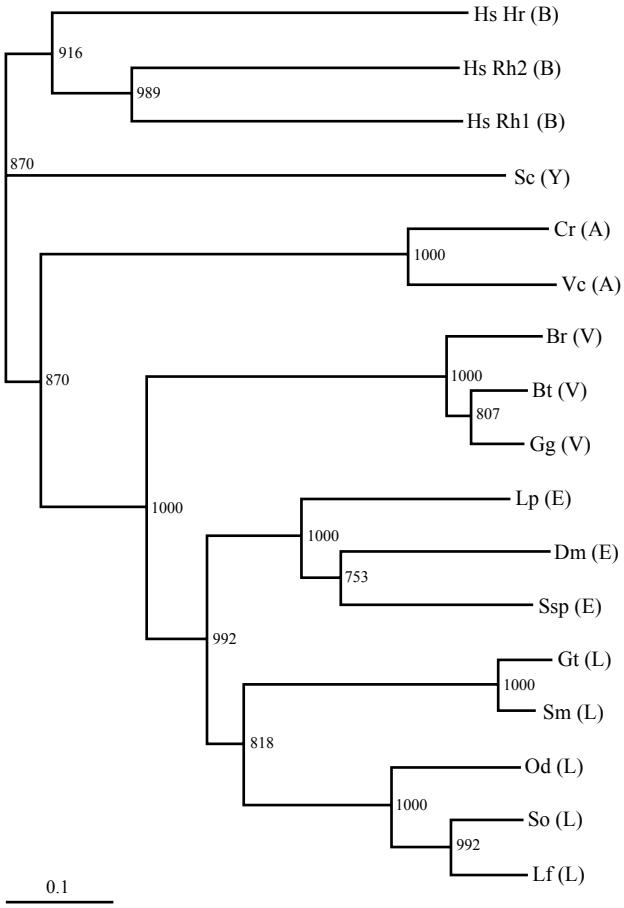

Fig. 3. – Phylogenetic unrooted tree of opsin proteins. Bootstrap values of 1000 runs are indicated as percentages at the nodes. The planarian Gtops protein clearly clusters with the other opsin proteins from the Lophotrochozoa clade. (B): Bacteria. Hs: *Halobacterium salinarium*. Hr: halorhodopsin. Rh1 and Rh2: sensory rhodopsin I and II. (Y): Yeast. Sc: *Saccharomyces cerevisiae*. (A): Algae. Cr: *Chlamydomonas reinhadtii* chlamyopsin. Vc: *Volvox carteri* volvoxopsin. (V): Vertebrate. Br: *Brachydario rerio* rhodopsin. Bt: *Bos taurus* rhodopsin. Gg: *Gallus gallus* rhodopsin. (E): Ecdysozoa. Dm: *Drosophila melanogaster* opsin1. Lp: *Limulus polyphemus* ocellar opsin2. Ssp: *Sphodromantis sp*. rhodopsin. (L): Lophotrochozoa. Gt: *Girardia tigrina* opsin. Lf: *Loligo forbesi* rhodopsin. Od: *Octopus dofleini* rhodopsin. Sm: *Schmidtea mediterranea* opsin. So; *Sepia officinalis*. Scale bar, genetic distance.

(CARRANZA et al., 1997; BAYASCAS et al., 1998). *Gtops* spatial expression was determined by whole-mount *in situ* hybridization of intact adults and regenerating pieces. In adults, *Gtops* was expressed continuously and uniformly in the photoreceptor cell bodies, whereas the rhabdomeric region of the photoreceptor cells was negative. A similar pattern of expression can be observed with *Gtsix-1*, but with a lower expression level. During the early stages of head regeneration, *Gtops* expression was detected in a group of differentiated photoreceptor cells close to the dorsal epidermis. This expression was maintained throughout regeneration (PINEDA et al., 2000). Opsin dsRNA injection induces a fast depletion of endogenous gene expression in the photoreceptor cells 24 hours post-injection, which eventually leads to the loss of phototactic behavior in the animal (SANCHEZ & NEWMARK, 1999; PINEDA et al., 2000).

Eye evolution: a common origin from a prototypic eye and an independent evolution by parallelism

The comparative embryological and morphological studies of metazoan eyes show different developing mechanisms and different morphologies suggesting an independent evolution of the different types of eyes (SALVINI-PLAWEN & MAYR 1961). However, molecular studies in the last decade have revealed the universality of rhodopsin as the visual pigment and the conservation in all studied Metazoa, including Platyhelminthes, of the early genetic cascade initiated by the gene Pax-6. Such molecular results suggest that all different eye types observed in Metazoa derive from a common prototypic eye and as a consequence have a monophyletic origin. Such prototypic eye can be found in some Platyhelminthes. The current work suggests that the development of the prototypic eye is controlled by a similar early genetic cascade. In molluscs we can observe a great variety of eyes in the mantle edge of Bivalvia (compound eyes, closed lens eyes with inverted retinal cells, reflecting mirror eye). Another eye type, the cephalopod eye, is similar in design to the vertebrate eye camera, but large embryological differences can be observed between the two (GEHRING 1996; HARRIS, 1997). The similarities in eye design in molluscs compared with the other metazoan eyes can be interpreted as evidence for a phenomenon of parallelism in the mechanisms by which the different Metazoa evolved their eyes from a common prototypic eye, using initially the same genetic network. The recruitment of different genes by intercalary evolution (GEHRING & IKEO, 1999) in the eye gene networks of the various evolutionary lines can lead to eyes with dramatically similar designs as a consequence of comparable developmental constraints, or, of course, to radically different final structures.

ACKNOWLEDGEMENTS

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Molecular aspects of cell proliferation and neurogenesis in planarians

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ABSTRACT : An *MCM2* gene (*DjMCM2*) which represents a suitable molecular marker for detecting proliferating neoblasts was isolated in planarians. Neoblasts are the only self-renewing cells in these organisms and may be able to differentiate into all cell types lost to physiological turnover or injury. To understand the molecular basis leading to neoblast commitment to a differentiative fate, some regulatory genes were cloned. Our interest was focused on the process of nerve cell differentiation. *DjXnp*, a planarian gene coding for a protein similar to Xnp/ATRX, a DNA helicase involved in mammalian brain development via chromatin structure modification, was obtained by RT-PCR. A similar strategy was also used to clone two different *Pax-6*-related planarian genes. Identification of factors involved at different levels in the control of gene expression during nerve cell differentiation could be of importance to understand the regulatory programs, which operate during neoblast differentiation in planarians.

KEY WORDS : Platyhelminthes., *Dugesia japonica*, neoblasts, cell proliferation, differentiation, MCM, Pax-6, XNP.

INTRODUCTION

A stable population of stem cells referred to as neoblasts is responsible for the renewal of all differentiated cell types in planarians. The presence of these cells is also crucial for the regenerative ability of these organisms. During regeneration, neoblasts increase in number by active cell proliferation and begin to accumulate beneath the wound epithelium giving rise to the regeneration blastema. Then, these cells differentiate into the various specialised cell types, and replace any missing structures by morphogenesis (BRØNDSTED, 1969 ; GREMIGNI, 1981 ; BAGUÑÀ, 1998).

Despite the obvious interest for the study of neoblasts, these cells are largely unexplored at the molecular level. Only recently the thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) has been successfully utilised to label stem cells in planarians (NEWMARK & SANCHEZ ALVARADO, 2000). At the same time, our analyses have demonstrated that the expression of *DjMCM2*, a member of the MCM2-7 family of DNA replication factors, can be used to specifically detect proliferating neoblasts (SALVETTI et al., 2000). These results provide new potential tools to improve our understanding of the cellular renewal system in these organisms.

The molecular mechanisms that regulate the differentiation program, by which neoblasts acquire distinct identities and specialised functions, are completely unknown. The central feature of cell differentiation is a change in gene expression. Three different regulation levels can be hypothesised for this process. As a first stage, an initial decondensation of the chromatin structure may be required in preparation for transcription. Subsequently, transcription factors can bind to specific sites on the chromatin to induce RNA synthesis. Finally, post-transcriptional regulatory mechanisms can be crucial for the modulation of the right amount of protein to be produced.

Besides a refinement of previous findings concerning the distribution of *DjMCM2*-expressing neoblasts (SALVETTI et al., 2000), the identification of some genes potentially involved in different processes of regulation during nerve cell differentiation is here reported : *DjXNP*, coding a putative protein similar to the mammalian Xnp/ATRX DNA helicase, and two *Pax-6*-related genes, *DjPax-6A* and *DjPax-6B*.

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MATERIAL AND METHODS

Animals

Planarians used in this work belong to the asexual strain GI of *Dugesia japonica* (Platyhelminthes, Tricladida) (ORII et al., 1993). Intact worms and regenerating fragments were kept in autoclaved stream water and maintained at 18°C in aquaria. Planarians were fed weekly with chicken liver and starved for one week before being used in experiments. Some specimens were amputated pre- and post-pharyngeally in order to induce bi-directional regeneration. Posterior regeneration was obtained by pre-pharyngeal amputation.

cDNA cloning and sequence analysis

DjMCM2 was cloned as described by SALVETTI et al. (2000).

A partial *DjXNP* cDNA fragment was amplified by RT-PCR using two degenerate primers corresponding to two conserved regions (RRIILTG and PPKHEYV) between human and mouse XNP/ATRX proteins. The sequences used for the comparison correspond to the EMBL/Genbank entries U72938 and AF026032. A similar procedure was utilised to isolate *DjPax-6B* (see Results). *DjPax-6A* was directly obtained from the sequence deposited in the EMBL/Genbank, using two specific primers.

Amplification products were cloned in pGEM-T Easy Vector (Promega). All clones were sequenced by automated fluorescent cycle sequencing (ABI). Similarity searches were performed using BLASTX; sequences were aligned with CLUSTAL W.

In situ hybridization experiments

Whole mount in situ hybridizations were carried out in intact and regenerating planarians according to the method described by AGATA et al., (1998). In situ hybridization on sections was performed as indicated by KOBAYASHI et al., (1998).

Synthesis of the digoxigenin-labelled sense and antisense RNA probes was performed according to standard protocols (Boehringer).

RESULTS AND DISCUSSION

DjMCM2

DjMCM2 is a putative member of a hexameric complex of proteins, MCM2-7, which are essential components of the prereplication chromatin, (for a recent review on MCM proteins, see TYE, 1999). We have isolated this gene in *D. japonica,* and demonstrated that it can be used as a molecular marker for specifically detecting proliferating neoblasts (SALVETTI et al., 2000). Proliferating neoblasts appear to be distributed in a non-uniform manner along the cephalo-caudal and dorso-ventral axes, being preferentially accumulated in dorso-lateral peripheral areas, with a minimum distribution at the cephalic level. This uneven distribution of the proliferative compartment in intact planarians has been further confirmed by using the in situ procedure directly on sections. This highly sensitive method has allowed us to investigate the distribution of *DjMCM2*-expressing cells in detail (Fig. 1).

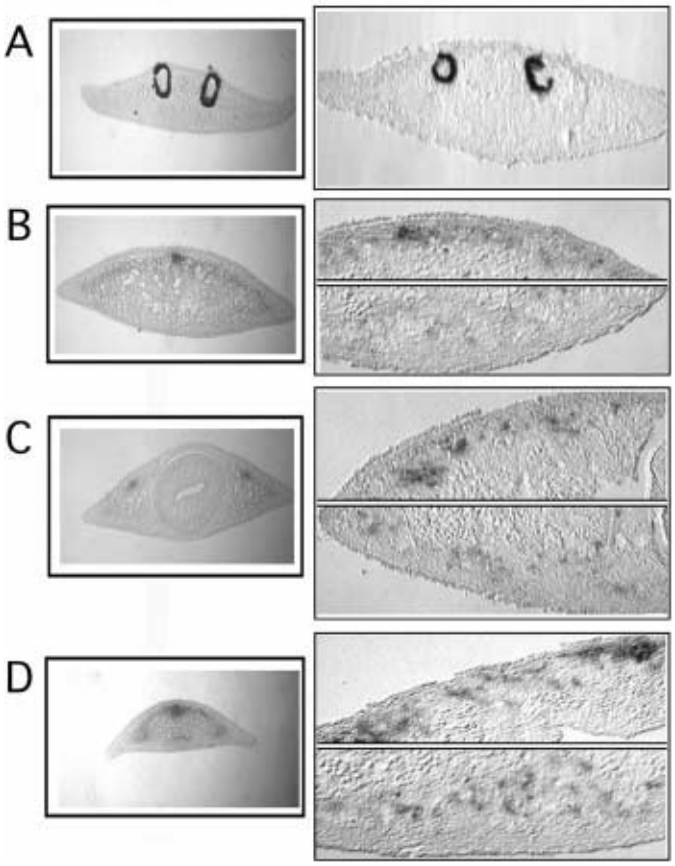

Fig. 1. – Expression of *DjMCM2* mRNA in an intact *Dugesia japonica*. (A-D) antero-posterior sequence of transverse sections. Left: wax sections after whole mount *in situ* hybridization. Right: *in situ* hybridized sections. In B-D the upper part is a magnification of the dorsal side, and the lower part is a magnification of the ventral side.

The analysis of *DjMCM2* expression pattern shows that a spatially regulated proliferative activity also characterises regeneration in planarians. In particular we were able to observe the presence of spatio-temporal changes in the *DjMCM2* RNA expression pattern in the stump, coordinated with the orientation of the cut. Moreover, during blastema growth, intensive cell proliferation was detected at the postblastema level, whereas no dividing cells were found in the blastema area (Fig. 2), where neoblasts are known to give rise to differentiated cells.

These observations support the hypothesis that a variety of intrinsic and/or extrinsic positional signals constitute a sort of cell niche (WATT & HOGAN, 2000), signalling

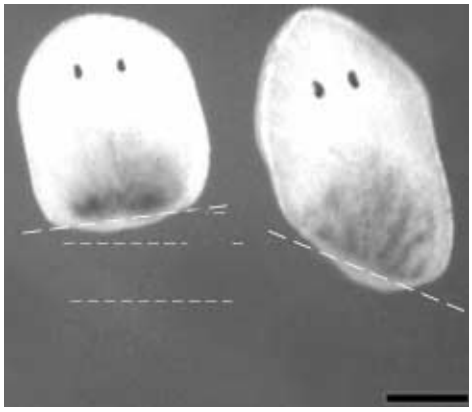

Fig. 2. – Expression of *DjMCM2* mRNA in posterior regeneration of *D. japonica*, visualized by whole mount *in situ* hybridization at 3 days after cutting. Blastema regions are devoid of hybridization signal. Dashed white lines indicate the limits of the blastema area. Scale bar = 500 μm.

neoblasts to divide or to undergo differentiation (AGATA & WATANABE, 1999). Identification of these factors will be an important step towards an understanding of the molecular mechanisms regulating neoblast behaviour.

DjXNP

Transition from inactive to active chromatin at specific chromosomal domains is probably one of the first steps required for cell differentiation. Regulatory factors and mechanisms involved in changes in the chromatin structure are until now, almost completely unknown. Recent reports indicate that putative DNA helicases, the XNP/ATRX proteins, appear to be involved in chromatin remodelling during nerve cell differentiation in mammals (GECZ et al., 1994; CARDOSO et al., 1998). These findings encouraged us to investigate the presence of similar molecules in planarians. We succeeded in isolating a cDNA fragment (*DjXNP*; Fig. 3A) which shows some similarity to the human and murine XNP/ATR-X genes (69%) (GECZ et al., 1994; STAYTON et al., 1994) and to the closest known non-mammalian relative, the nematode *xnp-1* gene (56%) (VILLARD et al., 1999).

We localised *DjXNP* transcripts in planarians by whole mount *in situ* hybridization. The hybridization signal had a widespread distribution throughout the parenchyma, and a significant amount of transcripts were found in proximity of the cerebral ganglia (Fig. 3B), which were identified by expression of the planarian synaptogmin gene (*DjSyt*: TAZAKI et al., 1999) (Fig. 3C).

During early regeneration, a preferential accumulation of *DjXNP* transcripts was observed in the parenchyma area, beneath the wound surface (Fig. 3D). Later on, these transcripts appeared preferentially localised in the nervous system presumptive territories (Fig. 3E).

The *DjXNP* expression pattern found in planarians essentially resembles that observed for *XNP/ATRX* transcripts in humans and rodents. In these organisms XNP/ATR-X transcripts are present in a variety of tissues,

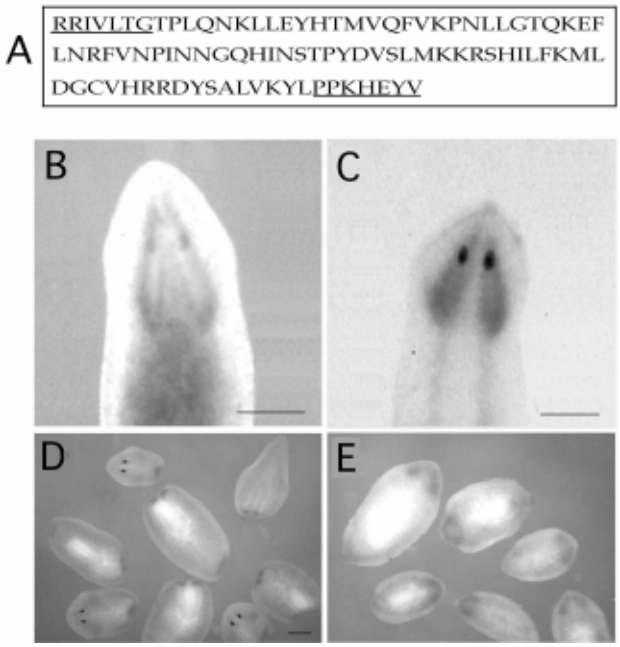

Fig. 3. – *DjXnp* characterization.(A) Deduced amino acid sequence of *D. japonica DjXnp* cDNA fragment. The regions corresponding to the primers are underlined. (B-E) Whole mount *in situ* hybridization. (B) Expression of *DjXnp* mRNA in an intact *D. japonica*. (C) Expression of *DjSyt* mRNA in an intact *D. japonica*. (D-E) Expression of *DjXnp* mRNA in regenerating *D. japonica*, at 3 days (D) and 7 days (E) after cutting. Scale bars = 500 μm.

and a preferential accumulation is found in developing brain (GECZ et al., 1994).

Although the identity of the *DjXNP*-expressing cells was not determined, these findings suggest that DjXNP may have a conserved function at the cellular level, playing a role *via* chromatin structure modification in differentiating neoblasts. In this case DjXNP could potentially regulate a subset of target genes specifically activated during early neuronal differentiation. The use of dsRNA-mediated genetic interference (SANCHEZ ALVARADO & NEWMARK, 1999) represents an essential tool for investigating this possibility.

DjPax-6A and *DjPax-6B*

A wide range of regulatory factors binding specific modules along active chromatin is known to play a key role in the control of gene expression in eukaryotes.

Pax-6-related genes could be used as informative molecules for an understanding of the regulatory cascade of the nervous and visual system, at the transcriptional level, during planarian regeneration. These molecules are sequence-specific transcription factors related to eye morphogenesis in a variety of animal phyla and are highly conserved during evolution. For example, the murine and human Pax-6 proteins are identical to each other and show extensive sequence similarity to the *Drosophila* Pax-6-related gene, *eyeless* (QUIRING et al., 1994; GEHRING & IKEO, 1999).

Fig. 4. – Comparison of the deduced amino acid sequence of *DjPax-6B* with those of *G. tigrina* (*GtPax-6*) and *D. japonica* (*DjPax-6A*). The amino acid sequences corresponding to the primers used, are boxed.

Up to now, two *Pax-6*-related genes have been identified in planarians: *GtPax-6* from *Girardia tigrina* (CALLAERTS et al., 1999) and *DjPax-6* from *Dugesia japonica* (Accession No. AB017632). These molecules are very similar in the two DNA-binding domains that typify the *Pax-6* gene class. However, we have observed that they share a much lower overall sequence identity than expected on the basis of the high conservation of *Pax-6* homologs. Accordingly, a homology search showed that *DjPax-6* had the highest overall sequence similarity ($p = e^{-107}$) with *twin of eyeless*, a second *Pax-6* gene, recently identified in *Drosophila* (CZERNY et al., 1999).

With the aim of contributing to the characterization of *Pax-6*-related genes in planarians, we looked for the presence of a second *Pax-6* gene in *D. japonica*. Two degenerate primers were used. The sense primer was designed taking advantage of the high sequence conservation between *DjPax-6* (here called *DjPax-6A*) and *GtPax-6* in the amino acid region QVSNGCV. On the other hand, the antisense primer corresponded to the *GtPax-6*-specific amino acid region TLFGYN (Fig. 4).

One of the cDNA fragments that we isolated, called *DjPax-6B*, was 520 bp long and contained an uninterrupted open reading frame, with 86% sequence identity to the *GtPax-6* corresponding region (Fig. 4).

Analysis of the expression pattern of *DjPax-6A* and *DjPax-6B,* and their functional characterisation, will contribute to an understanding of their role during nervous system regeneration. In particular, it will be possible to verify whether, similarly to the ones found in *Drosophila*, they act as key regulators in the genetic hierarchy controlling eye formation in planarians.

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Maintenance of A/P body regions in planarians by *tcen49*, a putative cystine-knot neurotrophin

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ABSTRACT. In freshwater planarians, the protein TCEN49 has been linked to the regional specification of the central body region, which includes the pharynx. Here we present the genomic and deduced amino acid sequence of the *tcen49*, show the expression of *tcen49* mRNA and compare the location of its protein in intact and regenerating organisms. The open reading frame encodes a secreted protein of 70 amino acids that shows no similarity to any other known protein, although it displays a cysteine pattern found in some members of the neurotrophic family. In intact adult planarians, transcripts are detected specifically in secretory cells in the central body region, whereas the protein is secreted from them to all the tissues in this region. Neither mRNA nor protein is detected in the anterior or posterior regions. During regeneration, the timing and dynamics of *tcen49* expression and TCEN49 location are always detected prior to any morphological evidence of the formation of the new central region, although the protein is not secreted until the pharynx starts its maturation. During anterior regeneration, *tcen49* is detected as early as day 1 of regeneration throughout the regenerate, which is different from posterior regenerates. Our results suggest (1) the presence of inhibitory mechanisms that regulate *tcen49* expression as well as the post-transcriptional regulation of its RNA and (2) that TCEN49 is not necessary to start regeneration but is involved in regional pattern maintenance.

KEY WORDS: Platyhelminthes, planarian, region-specific molecule, antero-posterior patterning, regionalisation, pattern maintenance, regeneration.

INTRODUCTION

For many organisms, including invertebrates and vertebrates, it has been shown that the establishment of the antero-posterior (A/P) polarity is preceded by the expression of different molecular regions, controlled by a number of genes that sequentially restrict different body regions. Once established, these regions have to be maintained. This becomes crucial in organisms that exhibit great morphological plasticity, as freshwater planarians do. The discovery and examination of an increasing number of genes involved in these phenomena help to give an integrated view of the mechanisms that govern the formation and maintenance of a defined body plan.

Freshwater planarians (Platyhelminthes, Tricladida) have attractive features that make them interesting for the analysis of the pattern-forming and -maintaining processes. These organisms are able to regenerate a whole organism from a small piece of the body, consistent with its original polarity (BRØNDSTED, 1969, for a historical review). Furthermore, planarians have the ability to grow and degrow depending on environmental conditions (food availability and temperature) (for a general review on regeneration, growth and degrowth, see BAGUÑÀ et al., 1990).

These features make the mechanisms used for the establishment, maintenance and re-specification of A/P polarity during regeneration especially intriguing. Although some *Hox* genes have already been identified in planarians (GARCIA-FERNÀNDEZ et al., 1993; ORII et al., 1995; BAYASCAS et al., 1997; ORII et al., 1999), the cellular and molecular mechanisms underlying the patterning of cells for a specific region along the A/P axis remain obscure. Particularly pertinent to these mechanisms is the protein TCEN49 from the planarian *Girardia tigrina*.

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TCEN49 is a region-specific secreted molecule present solely in the central body region. Its pattern of location and its dynamics during regeneration clearly suggest a role in the mechanisms that specify and/or maintain A/P body regions (BUENO et al., 1996).

In this paper we report the complete nucleotide and amino acid sequence of *tcen49*, a novel gene with a cysteine distribution resembling that of a cysteine-rich neurotrophic factor. The location of TCEN49 and the expression of its mRNA in intact and regenerating organisms suggest that TCEN49 is involved in the maintenance of A/P planarian body regions.

MATERIAL AND METHODS

Species, culture conditions and nomenclature

The freshwater planarians used belong to an asexual race of the species *Girardia tigrina* collected near the city of Barcelona. They were maintained in spring water in the dark at 4-6 ˚C and fed once a month with beef liver. The planarians chosen for the experiments were starved for at least 15 days before use. Organisms of 7-10 mm in length were cut at the levels described in BUENO et al. (1996) (see Figs 3 and 4) and the temperature was kept at 17±1˚C.We refer to the monoclonal antibody (MAb) as TCEN-49; to the protein as TCEN49; and to the gene as *tcen49*.

Isolation of *tcen49*

The MAb TCEN-49 (ROMERO et al., 1991) was used as a probe to screen a *G. tigrina* cDNA Lambda Uni-ZAP library (Stratagene) kindly provided by Dr. E. Castillo. The isolated *tcen49* cDNA (315 bp) was labelled by random-primer with [a^{32}P]dCTP (3000 Ci/mmol) (Amersham), and was then used as a probe to screen a *G. tigrina* genomic Lambda FIX II (Stratagene) library. Sequence analysis was carried out by using the BCM Search Launcher software. Southern blot analysis was performed by using 10 µg of *G. tigrina* DNA (GARCIA-FERNÀNDEZ et al., 1993) digested with *Eco*RI or *Hin*dIII (Promega) and hybridised with the same *tcen49* cDNA probe used in the genomic screening.

Generation of fusion protein, polyclonal antibodies, and western blot analysis

tcen49 was subcloned into the pGEX4T-3 vector (Pharmacia). The resulting glutathione S-transferase (GST) fusion protein (GST-TCEN49) was purified by using a Bulk GST Purification Module (Pharmacia), and thrombin digestion was done following Pharmacia´s instructions. Western-blot analysis of total protein extracts and the resulting thrombin-digested GST-TCEN49 was done under renaturing conditions as described in Bueno et al. (1996). Polyclonal antibodies to thrombin-digested GST-TCEN49 were obtained by sequential intraperitoneal injections in Balb/C mice according to standard procedures.

RNA in situ hybridisation and immunohistochemistry

Northern blot analysis was performed by using 15 µg of total RNA isolated from *G. tigrina* by the guanidinium thiocyanate method (Chirgwin et al., 1979). The filter was hybridised with the *tcen49* cDNA probe.

Whole-mount planarians were hybridised as described by Bueno et al. (1997a), using a 348 bp long-*tcen49* digoxygenin (DIG) labelled antisense riboprobe. Images were obtained by using a Zeiss Stemi SV 6 dissecting stereomicroscope (dark field) connected by a Sony video camera to a Macintosh Centris computer running Adobe Photoshop software. Cryosections were obtained in a *Clinicut Bright* cryostat and were hybridised at 60°C with the DIG labelled riboprobe, following standard procedures. The hybridisation was detected by using an alkaline phosphatase anti-DIG antibody and was developed with NBT/BCIP (Boheringer Manheim).

Immunostained paraffin sections were obtained as described in Bueno et al. (1996), using the Avidin-Biotin Complex method (ABC, peroxidase conjugated, Vector) to detect the primary antibody (TCEN-49).

RESULTS

Nucleotide and amino acid sequences

The *tcen49* genomic sequence was organised into two putative exons (9 and 201 nucleotides in length) and one intron (53 nucleotides in length), located at positions 504-556 (Figs 1A and 1B). Its nucleotide sequence revealed a short open reading frame encoding a deduced protein of 70 amino acids. This sequence revealed no significant similarity to any other reported protein. The VON HEIJNE method (1983) suggested a cleavage site between Ser 20 and Leu 21 (Figs 1A and 1B). The predicted mature polypeptide would have a *M*r of 5.3 kDa, in agreement with Western-blot analysis of the native secreted protein (BUENO et al., 1996). The distribution of the cysteine residues followed the pattern C-X(6)-C-X(3)-C-X(6)-C-X(3)-C-X(6)-C, where C is cysteine and X is any other amino acid, as for some members of the neurotrophic family (Fig. 1C). Moreover, these cysteines formed three disulphide bonds, as detected by cysteine methylation and mass spectrometry (work in progress).

To elucidate the genomic organisation of *tcen49,* we performed a Southern blot hybridisation analysis. As shown in Fig. 2A, three and five hybridisation bands were detected in the *Eco*RI and the *Hin*dIII digestions respectively. These results could be due to the polymorphism found in natural populations (Carranza, 1997), although we cannot discard the possibility that *tcen49* is present in

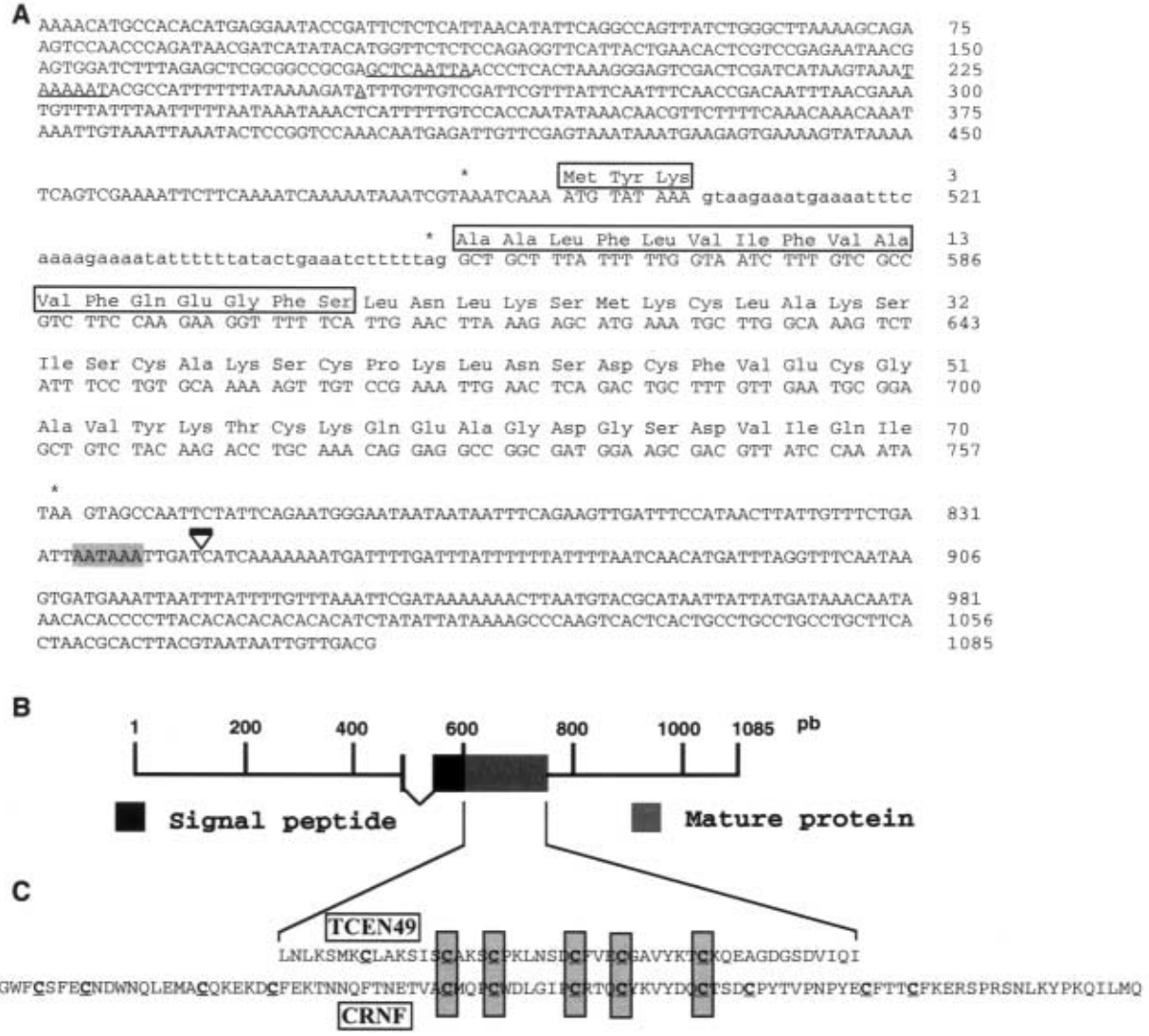

Fig. 1. – (A) DNA and deduced protein sequences of *tcen49*. The predicted signal peptide is shown in a box. Putative CAAT box, TATA box and transcription start site are underlined. A shaded box indicates the poly(A) signal sequence. The nucleotide and protein sequences are available in the GenBank Nucleotide Sequence Databases under the access number AF006956. (B) Diagrammatic structure of *tcen49*. The signal peptide is represented by black boxes; the mature protein by a grey box. Numbers at the top refer to the starting nucleotide of the sequence. (C) Comparison between TCEN49 and CRNF cysteine distribution.

more than one copy in the genome of *G.tigrina*. On Northern blots, the initially isolated 315 bp long cDNA detected a unique species of *tcen49* mRNA of approximately 550 nucleotides (Fig. 2B), which is consistent with the predicted size of the transcript (Fig. 1A).

To confirm that the sequence obtained corresponds to the planarian antigen recognised by the MAb TCEN-49, we performed a Western blot analysis of the fusion peptide generated from the *tcen49* cDNA with the MAb TCEN-49. As shown in Fig. 2C, TCEN-49 was able to recognise both the TCEN49 fusion protein and the thrombin-cleaved TCEN49 mature protein. We also produced a polyclonal antibody to the thrombin-cleaved TCEN49 mature protein, which exhibited the same pattern of immunostaining as the MAb TCEN-49 (Fig. 2D). From these experiments we can conclude that the *tcen49* gene of *G.tigrina* encodes the epitope recognised by the MAb TCEN-49.

Expression of *tcen49* and location of TCEN49

The results described below were obtained by analysing at least 10 organisms at each stage.

Intact adult planarians

tcen49 transcripts were detected in cells located within the parenchyma of the central-body region (Fig. 3A), except within the pharynx; no signal was observed in anterior and posterior regions, or using the *tcen49* sense riboprobe (negative control; Fig. 3B). In order to identify more accurately which cells express *tcen49* we performed RNA *in situ* hybridisation on planarian cryosections. *tcen49* expression was detected exclusively in cyanophilic-secretory cells of the central region of the body (Figs 3C and 3D). The area of immunochemical

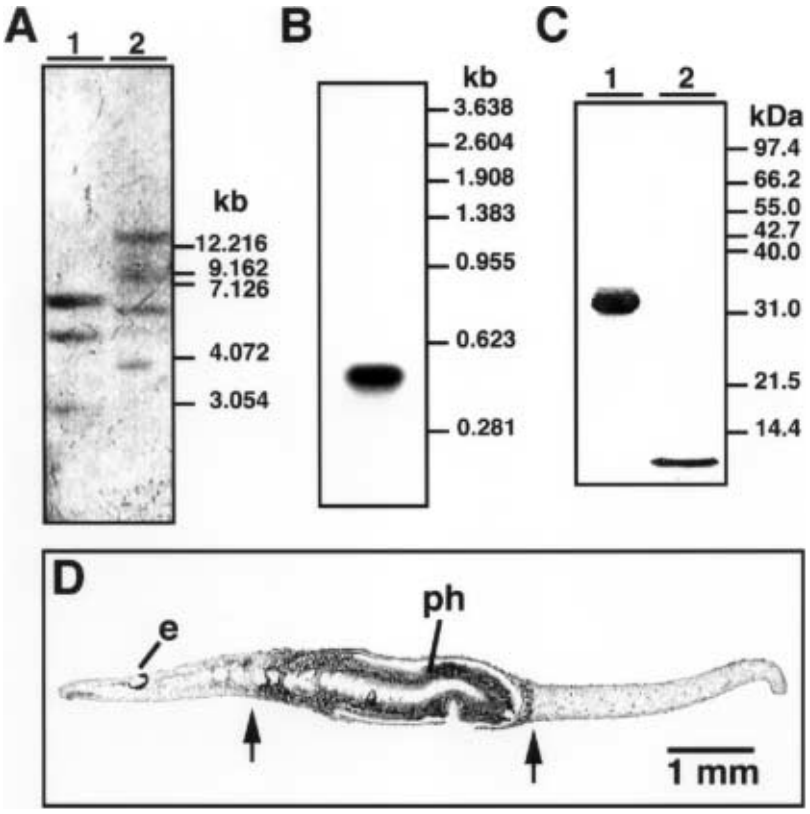

Fig. 2. – (A) Southern blot analysis of *tcen49*. Lane 1: EcoRI digestion; lane 2 HindIII digestion. (B) Northern blot analysis of *tcen49* mRNA. (C) Western blot immunodetection of the *in vivo* synthesised TCEN49 mature protein detected with the MAb TCEN-49. Lane 1: GST-TCEN49 fusion protein; lane 2: TCEN49 mature protein after thrombin digestion. (D) Sagittal section immunostained with the polyclonal antibody generated against the thrombin-digested GST-TCEN49 protein. Anterior is to the left, and dorsal to the top. Arrows indicate TCEN49 location boundaries. Abbreviations: e, eye; ph, pharynx.

location of the TCEN49 protein in intact adult organisms coincides with the area where *tcen49*-expressing cells are distributed and has been described elsewhere (Bueno et al., 1996; see Fig. 4 for a summary).

Regenerating organisms

During posterior regeneration in organisms cut at level A (levels of cutting are shown in Figs 3 & 4), the first hybridisation signal was observed at 6-7 days of regeneration. The signal was restricted to a condensed group of cells within the parenchyma, localised in the postblastema, in the area where the pharynx would form (Fig. 3F). The number of cells expressing *tcen49* mRNA increased as regeneration proceeded (not shown), forming a new central region with a new pharynx within it (the pharynx bud was detected at 7-8 days of regeneration). In organisms cut at level C (not shown in Fig. 3, but see Fig. 4 for a summarising scheme), *tcen49*-expressing cells were restricted to a group of cells in the postblastema as early as day 1 of regeneration, and were not detected in the growing blastema. From day 3-4, the number of *tcen49*-expressing cells increased, reestablishing a new central region.

During anterior regeneration in planarians cut at level C (Figs 3G, 3H and 3I), level D (Fig. 3J, 3K and 3L), and level E (not shown in Fig. 3, but see Fig. 4 for a summary), we detected *tcen49*-expressing cells as early as day 1 of regeneration throughout the regenerate, within and outside the region that usually expresses this gene in adult organisms (Figs 3G and 3J). From day 2-3 of regeneration, cells expressing *tcen49* became restricted again in the area where the pharynx was present (level C; Fig. 3H), or in the area where the pharynx would form (levels D and E; Fig. 3K) prior to any morphological evidence of its formation. *tcen49* expression was never detected within the blastema. From day 3, the number of positive cells increased and were sited exclusively around the old (level C; Fig. 3I) or new (level D and E; Fig. 3L) pharynx. Subsequently, each of these regenerates restored the adult proportions (Fig. 4) through an epimorphic-morphallactic process (Saló & Baguñà, 1984).

The area of immunochemical location of the TCEN49 protein coincides with the area of *tcen49*-expressing cells except during the transient stage of generalised transcription in anterior regenerates, and has been described elsewhere (Bueno et al., 1996; see Fig. 4 for a summary).

DISCUSSION

TCEN49, a molecule with no similarity to any reported protein

The data presented in this paper reveal that TCEN49 shows no significant similarity to any other known protein. However, the cysteine residues (see Fig. 1C) are distributed in a pattern also found in some neurotrophins, i.e. CRNF (cystein-rich neurotrophic factor) from the mollusc *Lymnaea stagnalis* (Fainzilber et al., 1996). Molecules belonging to this family have very diverse sequences but very similar structures, including a cystine-knot bonding in which these cysteines form three disulphide bonds. It is important to note that the cysteines from TCEN49 form three disulphide bonds, which is consistent with a cystine-knot bonding for TCEN49. TCEN49 crystallographic analysis (work in progress) could well confirm the extent of this.

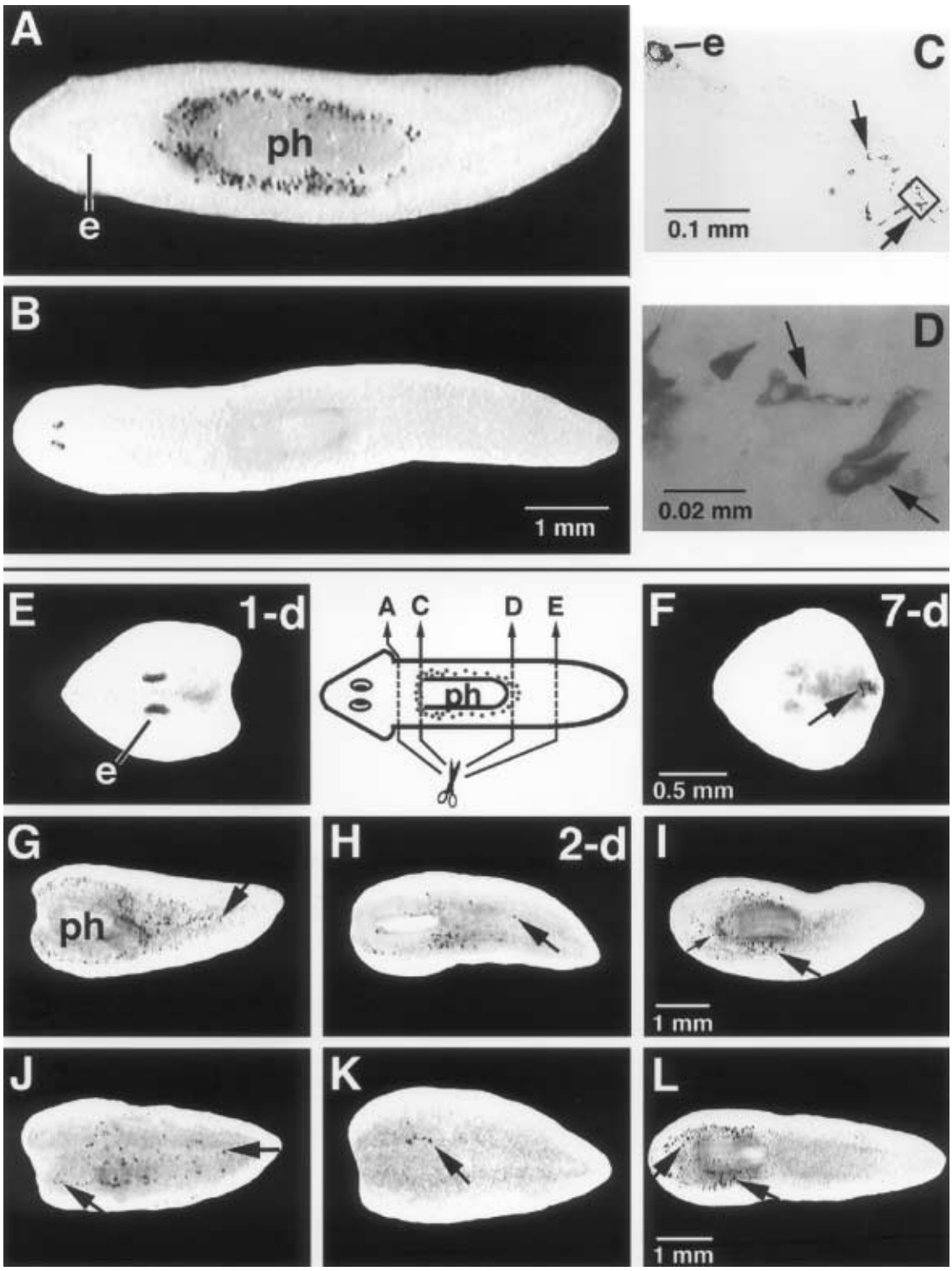

Fig. 3. – Expression of *tcen49* in intact adult planarians (A-D) and in regenerating organisms (E-L). Anterior is to the left. The ventral surface is shown (A, B). Whole-mount RNA in situ hybridisation with *tcen49* antisense riboprobe (A) or *tcen49* sense riboprobe (negative control; B). (C, D) Sagittal cryosections of intact adult planarians hybridised with *tcen49* antisense riboprobe. Arrows indicate some *tcen49*-expressing cells. (D) is the enlargement of the boxed region in (C). (E, F) organisms cut at level A; (G, H, I) organisms cut at level C; (J, K, L) organisms cut at level D. Days of regeneration indicated in the top-right corner apply for each column. Arrows indicate some of the parenchyma cells expressing *tcen49* mRNA. The diagrammatic scheme between (E) and (F) indicates the levels of regeneration analysed in this study. Spots indicate *tcen49*-expressing cells in an intact adult organism (compare with picture A). Abbreviations as in Fig. 2.

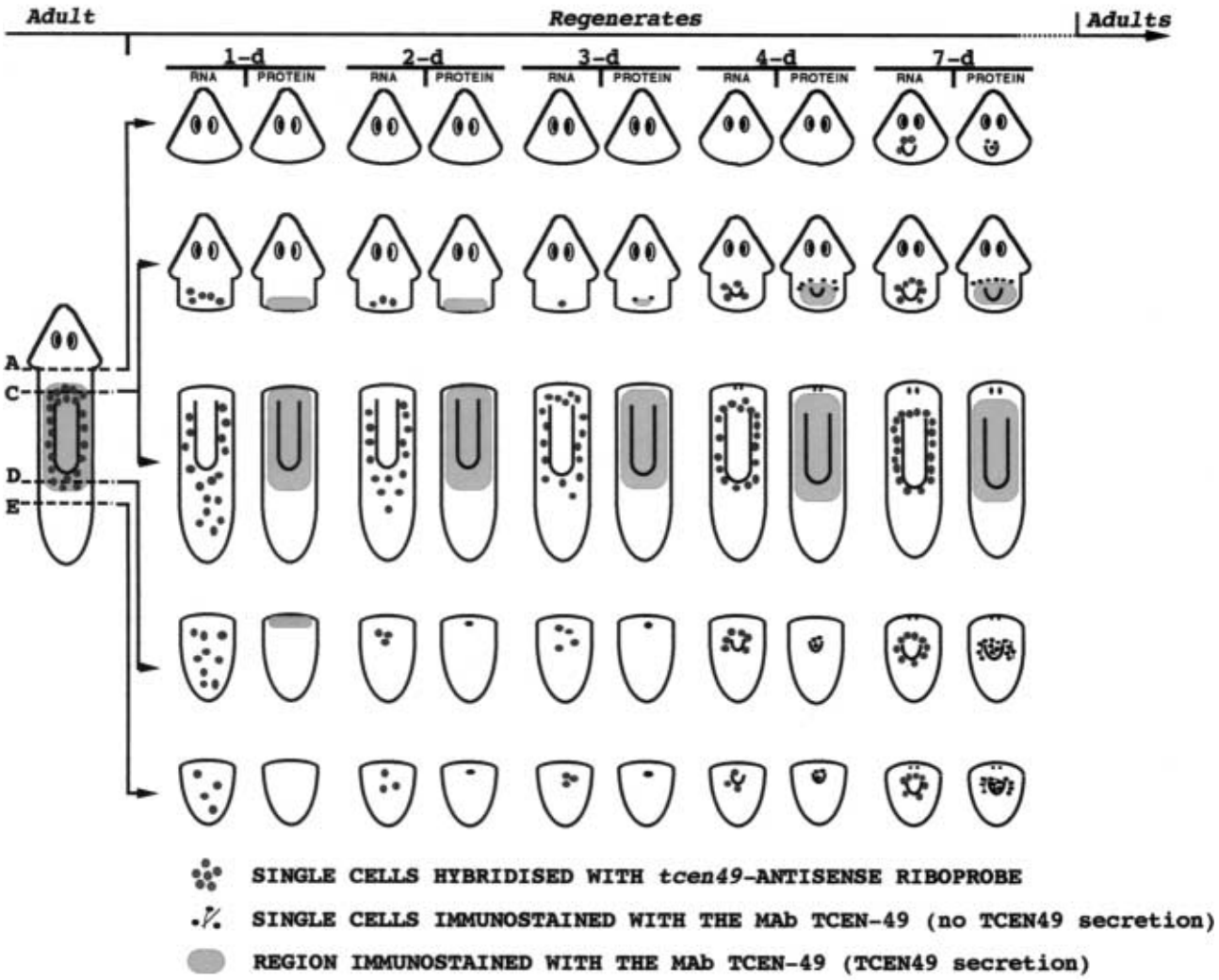

Fig. 4. – Summarising scheme of *tcen49* expression and TCEN49 localisation in adult and regenerating organisms.

tcen49 is differentially expressed in anterior *versus* posterior regeneration

Changes in *tcen49* mRNA expression in regenerates parallel changes reported for TCEN49 protein location (Bueno et al., 1996; see Fig. 4 for a summary) for both timing and dynamics. There is one interesting exception: during anterior regeneration in planarians cut at level C, D and E, *tcen49*-expression is transiently detected at day 1 of regeneration all along the regenerates. This contrasts with TCEN49 protein location at the same stages, which is never located outside the former central region or the new central-forming region. It also contrasts with posterior regenerates (levels A and C), which never undergo this stage of generalised *tcen49* mRNA expression. These results indicate that (1) *tcen49* behaves differentially in anterior *versus* posterior regeneration, and (2) during anterior regeneration there is a post-transcriptional regulation of *tcen49*.

Taken together, the results on *tcen49* expression lead to the clear differentiation of three molecular regions in planarians: (1) the anterior region, where *tcen49*/TCEN49 are never detected; (2) the central region, where *tcen49*/TCEN49 are always detected; and (3) the posterior region, where *tcen49*/TCEN49 are detected depending on the physiological conditions of the organism.

As the class II cyanophilic-secretory cells (the only cells that express *tcen49*) are present throughout the organism, several explanations may account for the head/tail differences in *tcen49* expression. (1) Differences in cell composition. Only a subtype of these cells is able to express *tcen49*, and it is present only in the central and posterior regions. This alternative implies the inhibition of *tcen49* expression in the posterior region of the adult. (2) No differences in cell composition. All class II cyanophilic-secretory cells are able to express *tcen49*, but *tcen49* expression is inhibited in adult organisms in the head and in the tail. This alternative implies a differential release of the inhibitory condition in the head from in the tail.

For both alternatives, generalised expression of untranslated *tcen49* mRNA at day 1 of anterior regeneration (lev-

els C, D and E) may be due to initial induction mechanisms initiated in response to the traumatic cutting (for general reviews on induction mechanisms, see BAGUÑÀ et al., 1990, and BAGUÑÀ, 1998), transiently releasing the inhibition of *tcen49* expression in the posterior region. Then, from day 2, *tcen49* expression would be inhibited in the newly forming anterior and posterior regions.

TCEN49 may be involved in A/P body region maintenance

It has been reported that the structures to be formed by the regenerative blastema and the postblastema close to the blastema are determined at 3-24 hours of regeneration, and that the determination of pharyngeal structures (such as the structure representing the central region) occurs at 12-36 hours of regeneration (for a general review, see BAGUÑÀ et al., 1994). Although the first TCEN49 location during regeneration occurs in the areas where the new central region will form between day 1 and 2 of regeneration before any morphological evidence of pharyngeal structures, it is not secreted until day 7-9 of regeneration. For this reason it may not function in the central region until the pharynx is completely regenerated and begins its maturation to become functional (BUENO et al., 1997b). All these data suggest that TCEN49 is not necessary for the initiation of regeneration, but rather is involved in the maturation and maintenance of this region. This is supported by preliminary RNA inhibition (RNAi) experiments performed on regenerating tails (level E) by injecting *tcen49* dsRNA, in which the organisms regenerate a new central region with a complete pharynx, but lyse at 9-12 days of regeneration.

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A molecular characterization of species and populations of *Dugesia gonocephala* s.l.

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ABSTRACT. We have employed a molecular approach to identify genomic characteristics in planarians belonging to *Dugesia gonocephala* s.l. In this paper we report the further characterisation of a repetitive DNA family (De1) that represents a useful molecular marker for establishing the genomic relationships among the species. We also describe the use of the molecular approach to obtain information about the genome organization of an asexual population of *D. gonocephala* s.l. from Montecristo Island, a natural park of the Tuscan Archipelago in the Tyrrhenian Sea. Specific DNA patterns have been individuated by using Southern blot analysis and the RAPD-PCR technique. These results, compared with those obtained in other species of *Dugesia gonocephala* s.l., indicate that some genomic differences characterise this geographically isolated planarian population. The possibility of a microspeciation event in the insular area is hypothesised.

KEY WORDS: Platyhelminthes, Tricladida, *Dugesia*, repetitive DNA, RAPD, phylogenetic relationships.

INTRODUCTION

Dugesia gonocephala s.l. includes a group of widely distributed planarian species, very similar in external features. Due to the important diagnostic value of the copulatory apparatus in the taxonomy of these organisms, the specific identification of the numerous asexual populations ascribable to *Dugesia gonocephala* s.l., is problematic. Karyotype information is usually of little help, because these planarians have chromosomes very similar in shape; aneuploidy and B-chromosomes can also be present.

Studies based on molecular analyses have recently allowed assigning asexual populations to a precise species by direct comparisons of genetic differentiation. Ribosomal DNA analysis results were particularly informative. In fact, both the chromosomal localization and the restriction pattern of 18S+28S rDNA (BATISTONI et al., 1999), as well as the sequence comparison of the internal transcribed spacer 1 (ITS1) region (BAGUÑÀ et al., 1999; BAGUÑÀ et al., 2001), revealed the presence of well-defined species-specific differences.

In addition, the characterisation of a highly repeated DNA family, conserved only in some species of the group (De1 family, originally isolated in *Dugesia etrusca* as a 1.4 kb *Hind*III fragment: BATISTONI et al., 1998), produced some information about their genomic relationships.

In this paper we report the cloning of a long fragment of a De1 unit. We also describe karyological and molecular characteristics of an asexual population of *D. gonocephala* s.l. from Montecristo Island.

MATERIAL AND METHODS

Animals and DNA extraction

Different species and populations belonging to *D. gonocephala* s.l. were used in this study. The precise locations where the animals were collected are given in BATISTONI et al. (1998).

Some specimens from Montecristo Island (Tuscan Archipelago, Tyrrhenian Sea, Italy) were kindly provided by Dr. M.A.L. Zuffi.

Genomic DNA was extracted from whole planarians as indicated by BATISTONI et al. (1998).

Karyological analysis

Chromosomes were prepared and analysed as described by DERI et al. (1999).

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Cloning of *Xba*I DNA fragments

*Xba*I-digested *D. etrusca* genomic DNA was electrophoresed on a 0.7% agarose gel. DNA fragments, at about 8kb, were recovered from the agarose with Qiaquick Gel Extraction kit (Qiagen) and cloned into the *Xba*I site of pGEM7Z(-) (Promega). On the basis of cross-hybridization with a previously cloned 1.4 kb *Hind*III repeat (p20 clone, BATISTONI et al., 1998), one of the obtained clones (p*Xba*I) was selected for the experiments and partially sequenced with the T7 DNA polymerase kit (Pharmacia).

Southern blot hybridizations

D. etrusca digested DNA was electrophoresed on a 0.7% agarose gel and blotted onto Hybond-N filters for hybridization experiments. Digoxigenin-labelled p20 and p*Xba*I inserts were used as probes. Hybridization conditions are described by BATISTONI et al. (1998).

RAPD analysis

RAPD procedure was essentially carried out according to WILLIAMS et al. (1990). Each PCR included about 25 ng of genomic DNA in a final volume of 20 µl, with 0.2 µM 10-mer oligonucleotide primer and 0.4 units of Taq DNA polymerase (Promega). An initial denaturation cycle (94°C, 4 min) was followed by 35 cycles (94°C, 1 min; 40°C, 1 min; 72°C, 2 min) and, finally, by an extension cycle (72°C, 4 min). Amplification products were electrophoresed on 1.5 % agarose gel; 1 kb DNA ladder (Gibco-BRL) was used as a molecular marker.

RESULTS AND DISCUSSION

Structure of De1 DNA elements

Our previous studies based on the presence/absence of a highly repeated DNA family (De1) dispersed throughout the genome of some *D. gonocephala* s.l. species, suggest that these sequences represent molecular markers that are important for making phylogenetic inferences (BATISTONI et al., 1998; 1999; BAGUÑÀ et al., 2000).

A Southern blot analysis of *D. etrusca*-digested DNA, using the previously cloned 1.4 kb *Hind*III De1 fragment as a probe (BATISTONI et al., 1998), indicated that this family is made up of long elements. *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I restriction enzymes produced single autoradiographic bands, between 8 and 12 kb (Fig. 1). The length of the De1 units should correspond to at least the longest band observed, i.e. about 12 kb.

In order to gain further insights into the structural characteristics and genomic organisation of these repeats, we have isolated a longer De1 fragment by *Xba*I digestion of *D. etrusca* genomic DNA. One of the clones obtained (p*Xba*I, containing an insert of about 8kb) was partially sequenced. A search within the EMBL/GenBank database revealed no significant similarity to any known sequence. In a terminal region of about 200 bp, p*Xba*I overlapped the *Hind*III De1 fragment (Fig. 2).

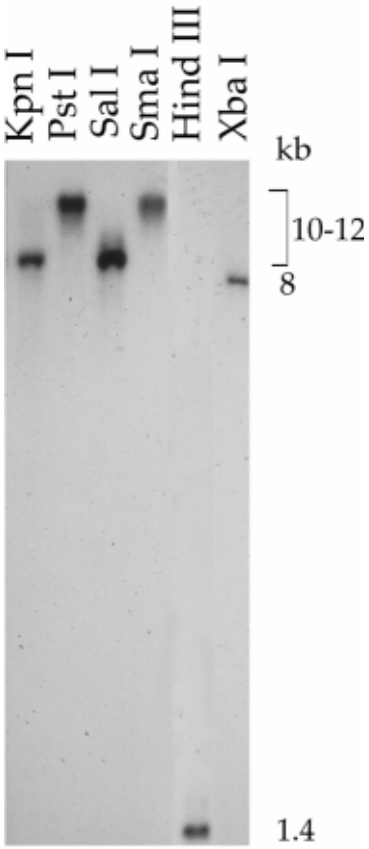

Fig. 1. – Southern blot hybridization of DIG-labelled p20 clone to *D. etrusca* genomic DNA, digested with *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Hind*III and *Xba*I restriction enzymes. *Hind*III lane shows the typical 1.4 kb band.

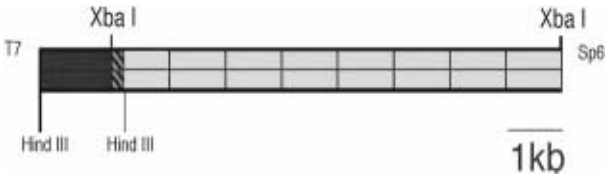

Fig. 2. – Schematic representation of De1 cloned DNA fragments. 8kb *Xba*I fragment (grey). 1.4 kb *Hind*III fragment (black). The overlapping region is shown.

The genomic organisation of De1 units was studied by analysis of the hybridization pattern revealed by p*Xba*I clone against blots of *D. etrusca* digests. The results obtained with *Hind*III, *Xba*I, *Cla*I, *Eco*RV and *Eco*RI are presented in Fig. 3. Complex patterns of hybridization bands of unrelated sizes could be observed in the different digests, suggesting that De1 elements are not organised as tandemly arranged sub-repetitions. These results are consistent with our previous observations (BATISTONI et al., 1998), and confirm that De1 DNA is made up of long repeats, scattered throughout the genome, and probably originated from transposable elements.

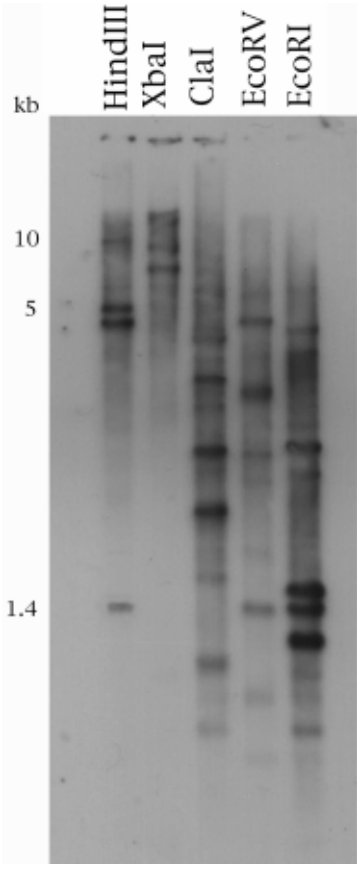

Fig. 3. – Southern blot hybridization of DIG-labelled p*Xba*I to *D. etrusca* genomic DNA, digested with *Hind*III, *Xba*I, *Cla*I, *EcoRV* and *EcoRI* restriction enzymes. The typical 1.4 kb band is present in the *Hind*III lane.

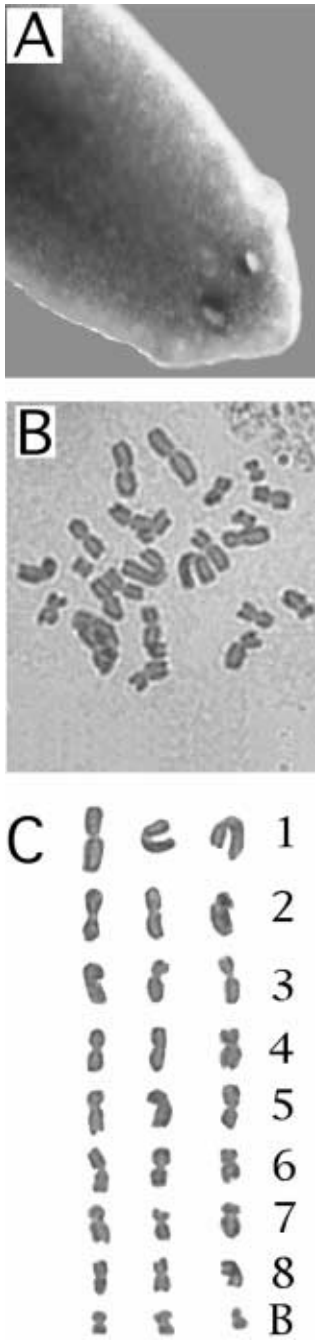

Fig. 4 (at the right). – *D. gonocephala* s.l. from Montecristo Island. (A) Dorsal view of a specimen. (B) Mitotic metaphase. (C) Triploid complement consisting of 24 chromosomes plus 3 B-chromosomes.

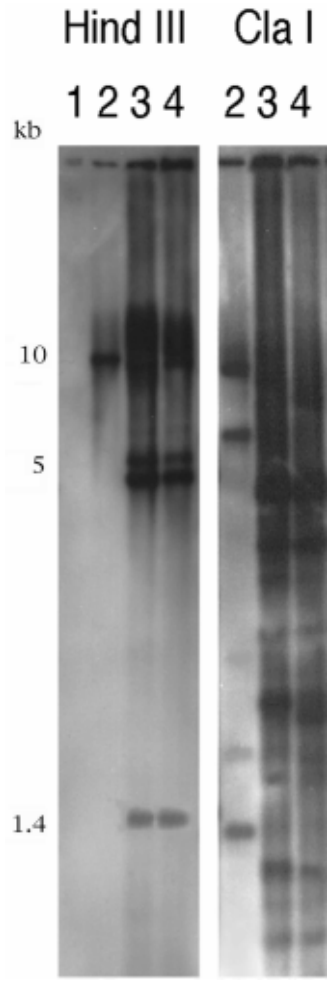

Fig. 5. – Southern blot hybridization of DIG-labelled p*Xba*I to genomic DNA from different planarians digested with *Hind*III and *Cla*I restriction enzymes. Lanes 1-4: *D. sicula*; Montecristo population, *D. etrusca* from Pisa, *D. etrusca* from Donoratico.

The use of molecular markers: the case of an asexual population of *D. gonocephala* s.l. from Montecristo Island

Some specimens of an asexual population of planarians ascribable to *D. gonocephala* s.l. (Fig 4A) were collected in a small stream on Montecristo Island. All examined specimens presented 24 standard chromosomes and 1-3 B-chromosomes, suggesting a triploid complement with respect to a haploid number n=8. However, such a complement can be more likely referred to an aneuploid condition, although numerically balanced (Fig. 4 B,C).

We used De1 DNA as a molecular marker in order to investigate the presence and the organisation of these sequences in the genome. Southern blot hybridization demonstrated that De1-like repeats are present in this population, but show differences in the restriction pattern with respect to those found in other planarians (Batistoni et al., 1998; 1999). Fig. 5 shows the hybridization pattern obtained with p*Xba*I clone in digests of planarians from Montecristo and two different populations of *D. etrusca*. No detectable signal can be observed in *D. sicula*, again confirming the specificity of De1 DNA for some species.

The genetic diversity of the specimens collected on Montecristo Island with respect to the other species that colonise both the Tyrrhenian islands and the Italian peninsula, suggests a possible specific identity of this population.

Geographic isolation probably plays a key role in speciation events of *D. gonocephala* s.l. (cf. Benazzi, 1982).

We can mention, as an example, the presence of at least eight well-identified species on the eastern Mediterranean, specifically Greece and the Greek islands (De Vries, 1984).

D. gonocephala s.l. is also well represented in the Western Mediterranean. A number of species from this geographical area was studied in detail over a long time by Benazzi and his school (cf. Mancino, 1998; Deri et al., 1999). In particular, the presence of three species was recorded on the Tuscan Archipelago: *D. ilvana* and *D. sicula*, from Elba Island (Lepori, 1948; Benazzi, 1950) and *D. benazzii* from Capraia Island (Benazzi, 1961).

RAPD analysis

In a preliminary attempt to develop a quick method to discriminate among different species and populations of *D. gonocephala* s.l., we applied a method based on PCR amplification, using single random primers of arbitrary nucleotide sequence (RAPD). This technique, often utilized as a valuable tool to determine genetic relationships in a variety of organisms, yielded interesting electrophoretic phenotypes also in planarians.

Results obtained with the primer 5'-TACCGACACC-3' showed substantial intraspecific variation in banding patterns, discriminating among different populations of *D. etrusca*. Peculiar RAPD profiles also differentiated planarians from Montecristo, as well as *D. sicula* and *D. benazzi* populations (Fig. 6).

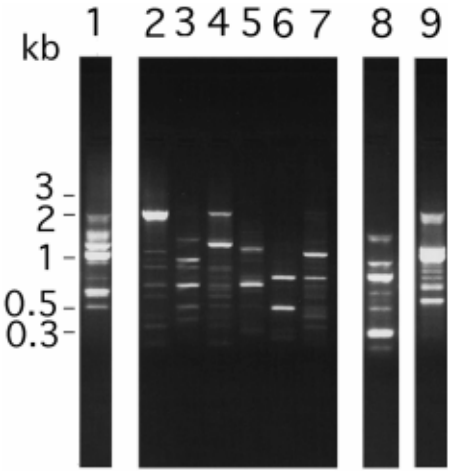

Fig. 6. – RAPD profiles of *D. gonocephala* s.l. planarians. 1: Montecristo population; 2-7: various populations of *D. etrusca* (2, Donoratico; 3, Torniella; 4, Rio di Calci; 5, Colli Berici; 6, Montemoro; 7, Revigliasco); 8: *D. sicula*; 9: *D. benazzii*.

The use of this technique with a larger number of primers should almost certainly open up the possibility of analysing genomic differences at the species, population and also individual levels. In turn this could represent a further tool for the investigation of evolutionary and biogeographical relationships in this planarian group.

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ABSTRACTS

The regeneration system of planarians

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Neoblasts, which are classically defined as prospective totipotent stem cells containing germ plasm-like granules, supporting planarian regeneration, now have been identified by expression of the DjvlgA and DjPTK3 genes, coding for a vasa-type ATP-dependent RNA helicase and a receptor-type tyrosin kinase, respectively. DjvlgA- and DjPTK3-positive cells are distributed in the mesenchymal space from head to tail, participating in formation of blastema and organ rudiments during regeneration.

In X-ray-irradiated planarians, which had lost regenerative capacity, the number of DjvlgA-expressing cells decreased drastically. When fragments containing neoblasts are transplanted into X-ray irradiated hosts, they can restore regenerative ability. We have shown propagation and migration of stem cells by chimeric analysis.

Interestingly, we found that neoblasts begin to transcribe tissue-specific genes in a position-dependent manner, while they are still in the mesenchymal space. This occurs prior to their migration to the organ rudiments or blastema, and at a time when they are not yet morphologically distinguishable as neoblasts. We speculate that the mRNAs transcribed in the stem cells may be trapped in a complex with RNA helicase(s), forming a "chromatoid body", and are not translated into protein until they migrate to the rudiment. After formation of the rudiments, these committed cells may receive a signal for organogenesis and then start to translate these mRNAs as well as to express pattern formation genes for organogenesis.

Results can be found in

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The *Schistosome* Genome Project (SGP) – a resource for studies of platyhelminth diversity, evolution and biology

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Since 1994, laboratories in both the Developed and Developing Worlds have collaborated to undertake genome analysis on the digenean *Schistosoma*. Although the rationale for the SGP is biomedical (to identify new targets for drug and vaccine development, to determine mechanisms of pathogenesis and drug resistance, and to analyse inter- and intra-specific variation), the biological and informatics resources generated by the SGP have considerable potential to reveal fundamental information on parasite biology, behaviour, diversity, and evolution, and thus to be of great value to workers in the wider platyhelminth field.

In the initial phase of the project, attention has focussed on: (a) Gene discovery. 13,000 expressed sequence tags (ESTs) representing 7,000 different genes (35% of the estimated gene content) have been generated for *S. mansoni*, and numerous stages of the life cycle examined. A smaller EST project for *S. japonicum* permits initial comparative studies. (b) Development of resources to facilitate physical mapping and genome sequencing. Markers have been mapped onto the karyotype to create a first generation chromosome map. A high quality, large fragment, genomic DNA library has been generated and is being used to develop a physical map of chromosome 3. (c) Informatics. WWW resources and a distributed database (SchistoDB) have been developed.

The quantity of data generated is now sufficient to allow meaningful "post genomics" / functional analysis. cDNA array and proteomics projects have recently been initiated to assign function to ESTs with no database homology, and to link the parasite's protein profile with its genome. Funds have also been secured for genomic sequencing. This will generate markers for the mapping project, provide additional gene discovery, and identify microsatellites, as well as reveal fundamental information on genome organisation.

The current status of the SGP was reviewed and specific applications of its biological and informatics resources for studies of wider platyhelminth biology were described.

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REGENERATION AND CELL BIOLOGY

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Early steps in the regeneration of the musculature in *Macrostomum* sp. (Macrostomorpha)

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ABSTRACT. *Macrostomum* sp., a microturbellarian from the Northern Adriatic, is able to regenerate only its posterior end after artificial surgery. Restoration of muscle tissue is one of the early events in regeneration. Morphallactic and epimorphotic processes occur simultaneously and consecutively. Shortly after surgery, rearrangement of muscles near the wound was observed. Six to eight hours later a faint transient network of muscle fibres was visible in the parenchyma at the site of the future blastema. One day after surgery an outgrowth of existing longitudinal fibres beneath the epidermis surrounded the developing blastema. At the caudal end of these fibres excessive forking was observed. New myocytes for circular musculature perpendicular to the growing longitudinal muscles were visible around the blastema. One week after surgery the adhesive plate and all muscles of the male copulatory organ were completely regenerated, after another week the regenerate had reached the length recorded prior to surgery.

KEY WORDS: muscle regeneration, Platyhelminthes, Macrostomorpha, epimorphosis, morphallaxis.

INTRODUCTION

Reorganisation and regeneration of lost musculature after artificial surgery as well as the restoration of the nervous system are some of the early processes in regeneration of Platyhelminthes (BAGUÑÀ, 1998; HORI et al., 1999). A layer of intact musculature at the regenerating end is necessary (SCHÜRMANN & PETER, 1998) for wound closure and subsequent blastema formation. While a large amount of literature on regeneration is available for the Tricladida, and regeneration of muscles has been studied in detail by electron microscopy (e.g. HORI, 1983; MORITA & BEST, 1984a,b) and with a monoclonal antibody (CEBRIÀ et al., 1997; CEBRIÀ & ROMERO, this volume), only few data are available on this topic for "microturbellarians" (see PALMBERG, 1990, 1991). We have traced the early regeneration of the musculature in *Macrostomum* sp. (a member of the *Macrostomum tuba* clade), which only regenerates its posterior end.

In this paper we have focused on distinguishing morphallactic and epimorphotic processes in regeneration of muscle tissue, that is the restoration of missing parts by pre-existing cells versus by proliferation and differentiation of stem cells, using fluorescence labelled Phalloidin (RIEGER & SALVENMOSER, 1991; and RIEGER et al., 1994).

MATERIAL AND METHODS

Animals were collected originally at Lignano (Northern Adriatic, Italy). Specimens used for this study were taken from laboratory cultures (details of cultures described in Rieger et al. 1988). Prior to experiments the animals were fed with diatoms for one week and starved for two days to minimise autofluorescence. Animals were cut in half and regeneration was observed of both the posterior and anterior parts (n=19). Surgery was carried out in artificial seawater. In order to avoid toxic effects, $MgCl_2$ was not used as an anaesthetic at this point. After the appropriate regeneration time worms were relaxed in seawater-isotonic $MgCl_2$ and fixed with 4% paraformaldehyde for one hour. Subsequently animals were rinsed several times in 0.1M phosphate buffered saline (PBS) and treated with 0.2 % Triton X-100 in PBS. The whole mount fluorescence technique was used, applying Phalloidin – Alexa (Molecular Probes, Oregon, USA, solution 1:70) to stain muscle F-actin filaments (RIEGER & SALVENMOSER, 1991; RIEGER et al., 1994), mounting in Vectashield (Vecta) and examination with a Polyvar epifluorescence microscope

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(Reichert, Austria) and a confocal laser scanning microscope (Zeiss, LSM 510 Germany).

RESULTS

Immediately after surgery constriction of the circular musculature occurred at the caudal end of the anterior regenerate (Fig. 1). The wound was closed after one to two hours. Phalloidin staining at this time showed severed and contracted longitudinal and diagonal muscles and constricted circular muscle fibres. After two to eight hours the regenerating end appeared round and morphallactic processes became visible: The ends of muscle fibres in the caudal-most circular muscle bands were bent in caudal or diagonal direction. Some of the dorsal longitudinal fibres of the body-wall musculature appeared to be rearranged and showed outgrowth in caudal and medial directions. Other fibres at the lateral sides in the animal were bent diagonally (Fig. 2). The growth process started later or was slower in the case of the ventral longitudinal fibres, or these fibres remained contracted for a longer period of time. Consequently, the wound appeared shifted to a more ventral position (Fig. 2). In addition, a network of delicate muscle fibres was observed after 8 hours in the region of the future blastema at this point. All muscles of the body-wall as well as some fibres from the gut musculature seemed to be involved in the formation of this network (Fig. 3).

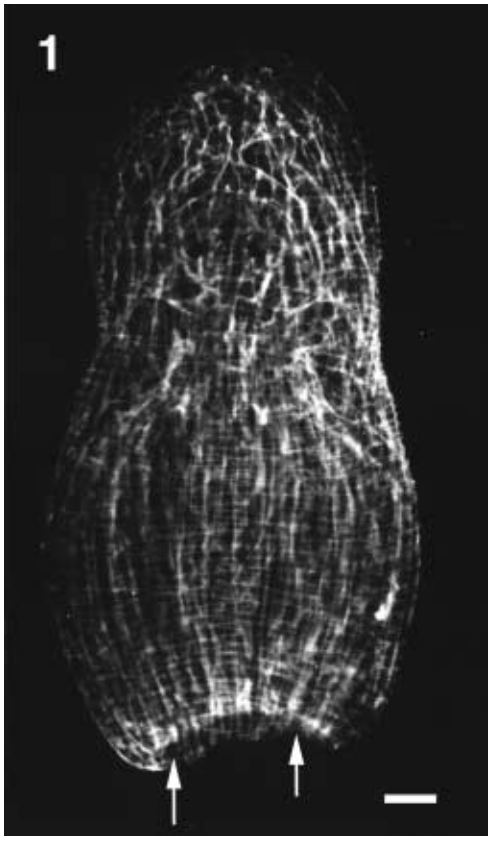

Fig. 1. – Phalloidin staining of *Macrostomum* sp., after dissection, dorsal view. Severed and contracted muscles are seen in the wound region. Scale bar: 25 µm.

In most animals the blastema became visible after 17 to 19 hours. Phalloidin staining after 30 (±6) hours of regeneration showed that the muscle network had completely disappeared. Instead, one could now observe caudal outgrowth and crossing of pre-existing longitudinal fibres, with extensive forking of these fibres at their connections to the basal matrix of the body wall (Fig. 4A). Faintly stained developing myocytes also became obvious, in perpendicular orientation to the longitudinal muscles (Fig. 4B). In contrast to the longitudinal musculature, circular fibres of the body-wall always developed from new myocytes. A few diagonal fibres could be seen at this time; they seemed to be derived from the longitudinal layer (Fig. 4B).

After two days, regeneration of the caudal end had started (Fig. 5 shows the stage after three days) and after seven days all anatomical elements of the caudal part appeared to be regenerated . The adhesive plate with its complex dorso-ventral musculature was rebuilt as well as the false seminal vesicle and the musculature of the male copulatory organ. After another week the regenerate had reached the same size as the posterior half in control animals.

The posterior fragments of the bisected animals did not survive, all of them died off within one to two weeks. We never found regenerated muscles in posterior fragments. Contraction of the circular muscles and wound closure were the only signs of regeneration. No further processes such as the formation of the network of muscles, outgrowth of longitudinal fibres etc., could be observed.

DISCUSSION

Wound closure is the first step in regeneration (see lit. in SÁNCHEZ ALVARADO, 2000). It is crucial for further survival to prevent exposure of the internal organs to the environment. In *Macrostomum* sp. wound closure is achieved by a contraction of circular musculature that leads to a reduction of the wound surface. Flattening of epithelial cells and rearrangements of junctional complexes occur within one to two hours (unpublished observation, D. Riedl). This phenomenon is also well known from freshwater triclads (BAGUÑÀ, 1998 and literature therein), from *Stenostomum leucops* and *Microstomum lineare* (PALMBERG, 1990, 1991) and is likely a common morphallactic process.

Two steps can be distinguished in the early muscle regeneration in *Macrostomum*. First, morphallactic processes take place whereby the musculature in the wound region is repaired and rearranged. Secondly, epimorphosis occurs in the form of differentiation of neoblasts into new muscle cells.

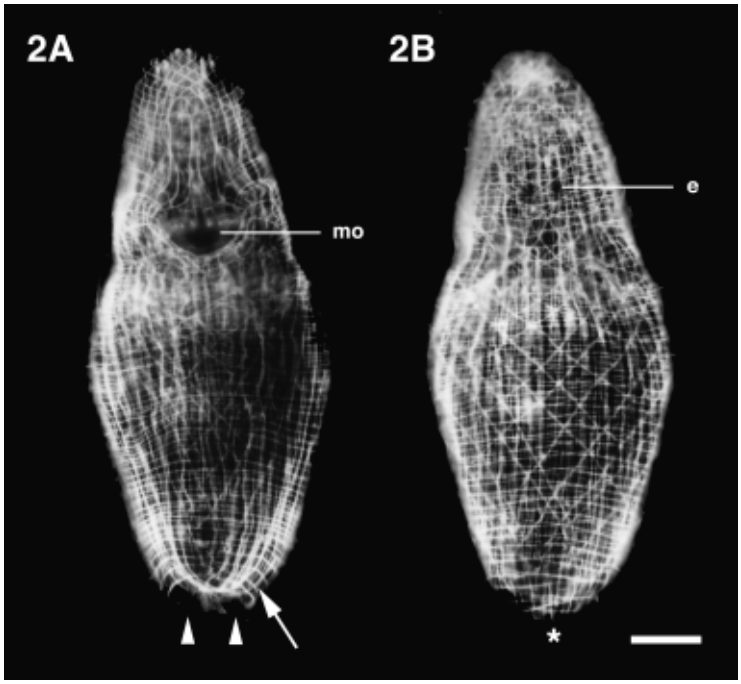

Fig. 2. – A: Ventral view of *Macrostomum* sp. after 2 h of regeneration. Wound is shifted slightly to ventral side (arrowheads). Some longitudinal fibres are bent medially (arrow). B: dorsal view of a specimen with intact body-wall musculature to the very caudal end (asterisk). Scale bar: 50 µm, mo = mouth opening, e = eye.

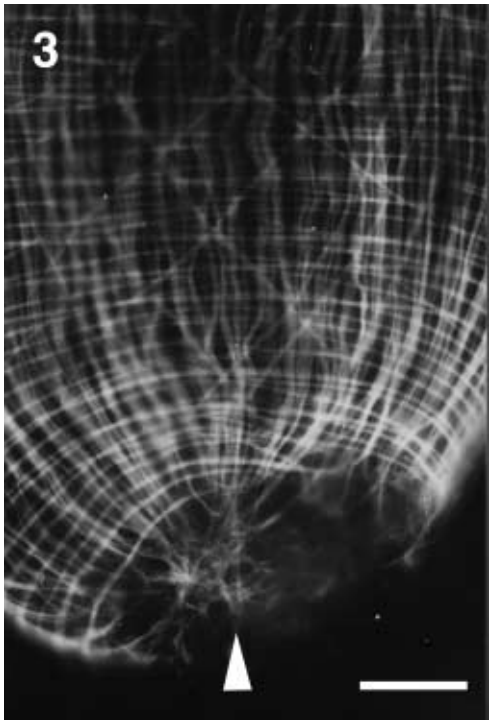

Fig. 3. – Phalloidin staining after 8 h of regeneration, ventral view. Note faint network of muscles in region of future blastema (arrowhead). Scale bar 15 µm.

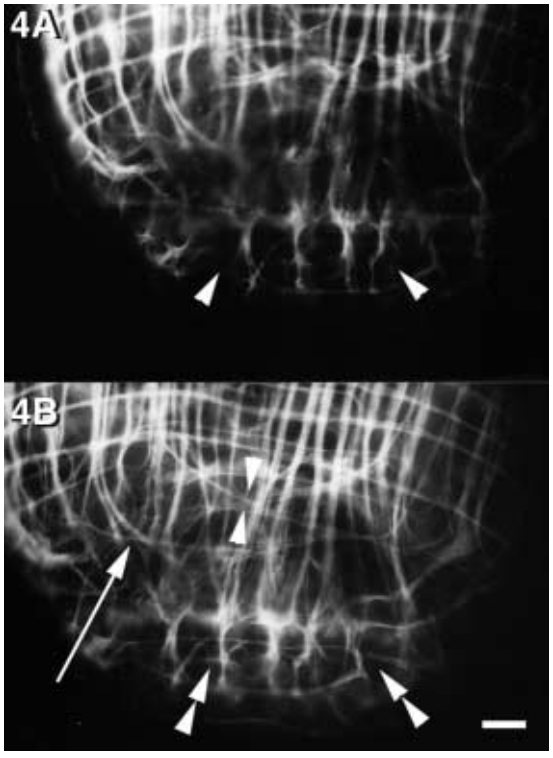

Fig. 4. – Ventral view of *Macrostomum* sp. after 24 h of regeneration. A: Outgrowth and forking of pre-existing longitudinal fibres (arrowhead). B: bending (arrow) and crossing (arrowhead) of longitudinal fibres. Note developing circular fibres (double arrowheads). Scale bar 15 µm.

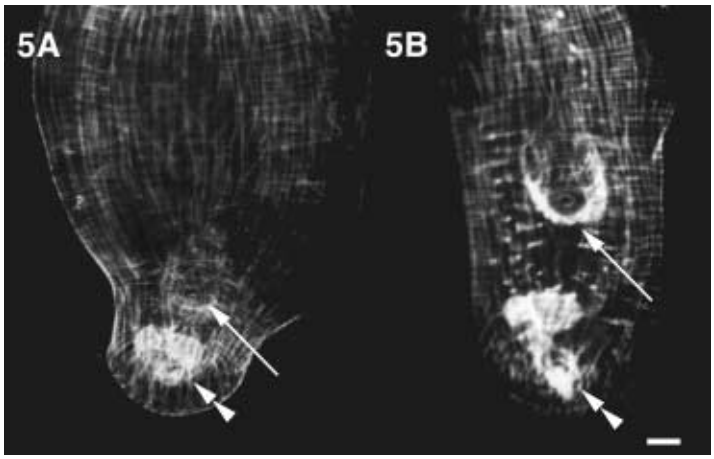

Fig. 5. – A: Phalloidin staining after 3 days of regeneration, ventral view. Note developing musculature of male copulatory organ (double arrowhead) and vagina (arrow) in the regenerating caudal adhesive plate. B: Caudal end with musculature of male and female copulatory system in mature control animal. Male copulatory organ (double arrowhead), vagina (arrow). Scale bar 25 µm.

The latter process is, however, different for circular and longitudinal muscles. While all fibres of the circular layer must be formed anew during regeneration, most fibres of the longitudinal layer result from outgrowth of pre-existing longitudinal fibres.

One hour after surgery the cut and contracted longitudinal fibres are clearly visible. During the following few hours outgrowth and bending of these pre-existing fibres in the body-wall musculature can be observed. The ventral displacement of the wound during restoration of the posterior end of *Macrostomum* sp. corresponds to the shift of the wound during restoration of the anterior end of planarians (CHANDEBOIS, 1980, CEBRIÀ, personal communication). This first step of regeneration does not require neoblasts; it is a purely morphallactic process.

The formation of the transient muscle network beneath the epidermis of the wound area, where the future blastema will be formed, may function in stabilizing the wound region. Its formation and later break-up are also morphallactic.

In *Macrostomum* a small blastema becomes visible after one day. In Platyhelminthes it is generally accepted that the blastema is formed both by undifferentiated neoblasts and by differentiating cells (HORI, 1983; MORITA & BEST, 1984a,b; SALO & BAGUÑÀ, 1984). At that point morphallactic and epimorphotic processes can be observed simultaneously also in the regeneration of the body-wall musculature.

The formation of myocytes in rings perpendicular to longitudinal muscles at the periphery of the blastema is the first sign of muscle cell differentiation from neoblasts. The ultrastructural features of neoblasts in *Macrostomum* and their role in growth and regeneration have been demonstrated (RIEGER et al., 1999; LADURNER et al., 1998; LADURNER et al., 2000). Neoblasts are concentrated in the blastemal area (unpublished observation D. Riedl, W. Salvenmoser). NEWMARK & SÁNCHEZ ALVARADO (2000) have reported the distribution of S-phase cells and migration and differentiation of neoblasts in whole-mounts of *Schmidtea mediterranea*. PALMBERG (1990, 1991) has shown, in *Microstomum lineare* and in *Stenostomum leucops* with tritiated thymidine and autoradiography, that not all muscle cells in the regeneration area were labelled. Taken together, these findings indicate the simultaneous occurrence of epimorphotic and morphallactic processes are common in early regeneration.

In *Macrostomum* sp. the nervous system also regenerates (unpublished results D. Riedl), and new muscle-nerve connections as well as specialized gap junctions (see RIEGER et al., 1991) must be formed to keep the musculature functioning. It was observed that as early as eight hours after surgery the main nerve cords had united and formed the caudal loop (data not shown). The involvement of neuroactive substances in activating neoblast differentiation during regeneration has been discussed previously (e.g. BAGUÑÀ et al., 1989). We assume that the lack of serotonergic neurons associated with the caudal portion of the central nervous system in the genus *Macrostomum* may in part be responsible for the incapacity of posterior fragments to regenerate anterior parts of the body (see LADURNER et al., 1997). The regeneration phenomenon cannot only be explained by the presence of neoblasts because S-phase cells are also present in the posterior fragment. Further investigations are necessary to elucidate this problem.

Later regeneration of the musculature in *Macrostomum* sp. is achieved primarily by epimorphosis, and the musculature of the male copulatory organ as well as the musculature in the adhesive plate must be formed from differentiating neoblasts. The growth of the regenerate to the final size is a process similar to cell maintenance (LADURNER et al., 2000, see also GREMIGNI, 1988 for differences between physiological and traumatic regeneration).

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Body-wall muscle restoration dynamics are different in dorsal and ventral blastemas during planarian anterior regeneration

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ABSTRACT. Planarians are simple, acoelomate, triploblastic organisms with a remarkable capacity of regeneration. In the last years, several specific cellular and molecular markers have been used to study this biological problem in these organisms. Here, we monitor body-wall musculature restoration during anterior regeneration through confocal microscopy and using a monoclonal antibody called TMUS-13, which recognizes the myosin heavy-chain of muscle cells. We have found differences in the dynamics of muscle pattern restoration between dorsal and ventral surfaces of the growing blastemas, especially during the first days of regeneration. Blastema contains old longitudinal fibers coming from the postblastema throughout all the regenerative process. These fibers could have a role in supporting the growing blastema and/or guiding the entry of different cell types from the postblastema region. New longitudinal fibers within the blastema seem to appear from outgrowing processes of the existing longitudinal fibers. On the other hand, new circular fibers appear *de novo* within the regenerative blastema. Finally, the original muscle pattern seems to be restored through intercalation of new muscle fibers throughout an initial muscle scaffold.

KEY WORDS: Platyhelminthes, planarian, body-wall muscle, regeneration, blastema.

INTRODUCTION

Pattern restoration during planarian regeneration remains, at present, obscure, mainly because of the lack of clear molecular approaches to solving this biological question (for a general review, see BAGUÑÀ (1998). In the last few years, however, several molecular studies, some using cell-type specific molecular markers, have been carried out (BUENO et al., 1997; BAYASCAS et al., 1998; SÁNCHEZ & NEWMARK, 1998; AGATA & WATANABE, 1999; KATO et al., 1999; KOBAYASHI et al., 1999). Recently, we started studying myocyte differentiation and body-wall muscle pattern restoration during planarian regeneration through immunostaining with a monoclonal antibody specific to planarian muscle cells. This antibody, called TMUS-13 (ROMERO et al., 1991; BUENO, 1994), recognizes the myosin heavy-chain protein from both mature muscle cells and differentiating myocytes (CEBRIÀ et al. 1997). Planarians have a well-developed body-wall musculature, which gives rise to a complex muscle network throughout the organism. This muscle network somehow makes up for the lack of a true skeletal system in these animals and supports all other kinds of cells. During regeneration muscle fibers perform an early function by closing the wound through a strong contraction of the body-wall (CHANDEBOIS, 1980). It is not clear, however, if these muscle fibers have a role in blastema formation and growth. Here, we describe how this muscle pattern is restored during anterior regeneration. In contrast with the results shown in a previous paper where we did not check for differences between dorsal and ventral blastemas (CEBRIÀ et al., 1997), we found differences in the dynamics of muscle restitution between dorsal and ventral surfaces of the blastema. These differences reflect the different patterns already observed in intact planarians and suggest that, at least ventrally, the blastema seems to contain, during the entire regenerative process, old muscle fibers coming from the postblastema (a narrow strip of old tissue close to the wound and where high proliferative activity is detected from the first hours of regeneration,

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Saló & Baguñà, 1984). The possible role of these existing muscle fibers as a support of the growing blastema and/or as a guide for the entry of myocytes or other cell types from the postblastema is discussed. Finally, we also discuss whether muscle pattern restoration is carried out through a distoproximal, proximodistal or intercalary sequence of events.

MATERIAL AND METHODS

Species and culture conditions

The planarians used belong to a diploid (2n=8) and asexual strain of the species *Schmidtea mediterranea* (Baguñà, 1973, Benazzi et al., 1975) collected in a small fountain in Barcelona (Catalunya, Spain). For anterior regeneration experiments, organisms 5-8 mm long were cut at a post-cephalic level and kept at 17±1ºC. All the organisms were starved for 15 days before use.

Whole-mount immunostaining with TMUS-13

Intact and regenerating organisms were treated with 2% HCl in distilled water for 30 s, which kills planarians instantly and leaves them completely flat. Then, they were fixed in Carnoy's solution (EtOH, chloroform and glacial acetic acid in proportions 6:3:1) for 2 hr at room temperature (RT). After fixation animals were washed in 75% MeOH in PBS for 5 min, bleached in 6% hydrogen peroxide (H_2O_2) in MeOH for 4-6 hr under light, and washed in MeOH 3 x 5 min. The animals were then rehydrated in a decreasing series of MeOH in PBST (PBS-0.3% Triton X-100), 5 min in each step. After a 5 min wash in PBST, animals were blocked in 0.25% bovine serum albumin (BSA) in PBST for 2 hr at RT and then incubated with the anti-myosin heavy chain monoclonal antibody TMUS-13 (Romero et al., 1991; Bueno, 1994, Cebrià et al., 1997) diluted 1/10 in 0.25% BSA in PBST for 16-24 hr at RT (shaking). The samples were then washed in 0.25% BSA in PBST for 6-12 hr (several changes of the medium) and TMUS-13 was detected with secondary goat-anti-mouse conjugated to fluorescein (GAM-FITC; Sigma) diluted 1:75 in 0.25% BSA in PBST for 16-20 hr in the dark. After washing in PBST for several hours and with several changes of the medium, specimens were mounted in Vectashield medium (Vector Laboratories, Inc.) and their fluorescence was detected with confocal microscopy.

Confocal microscopy

Confocal laser scanning microscopy (CLSM) was performed with a Leica TCS 4D (Leica Lasertechnik, Heidelberg) adapted to an inverted microscope (Leitz DMIRB). Images were taken using a x40 (NA 1.0) Leitz Plan Fluotar lens. Pictures shown correspond to 3D reconstruction from several collected images, each of them being the average of eight line scans at the standard scan rate. All the images are in the same pseudo-color (glow look-up table).

RESULTS

Cephalic body-wall muscle pattern is different in dorsal and ventral surfaces

As has been previously described, the body-wall muscle pattern of the most anterior region of planarians is different between dorsal and ventral surfaces (Cebrià et al., 1997). These differences are more evident for longitudinal muscle fibers. Dorsally, these longitudinal muscle fibers seem to converge upon a central zone close to the most anterior tip of the organism (Fig. 1A; arrows). In contrast, on the ventral surface the longitudinal fibers run in parallel as they reach the anterior border (Fig. 1B; arrows).

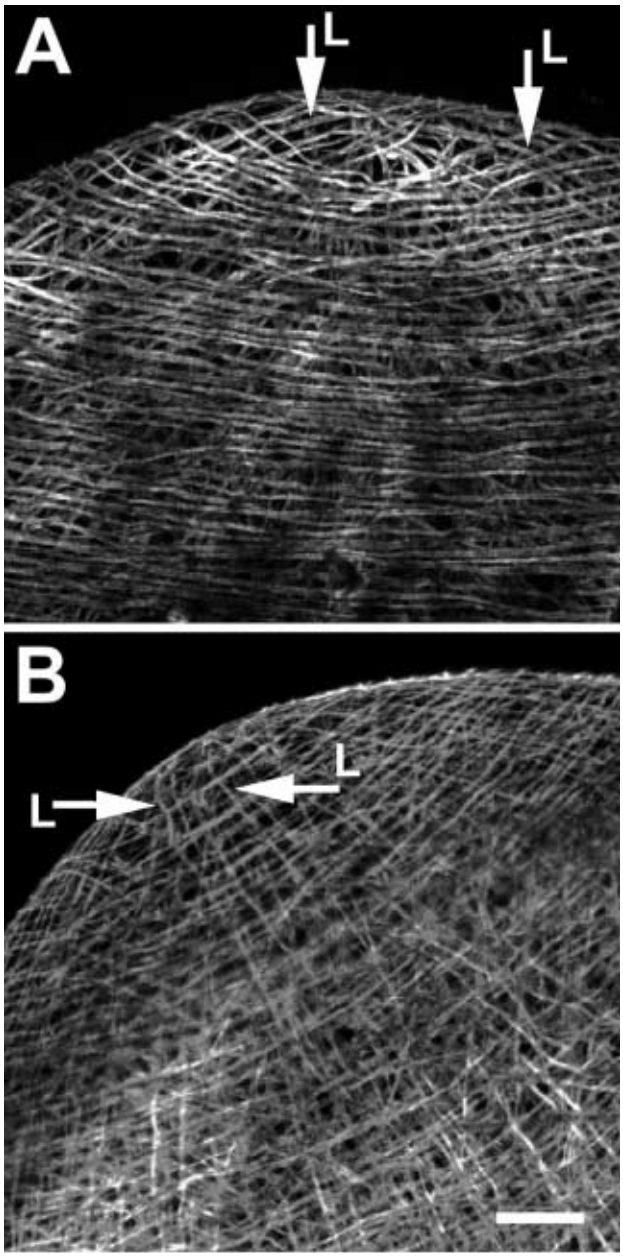

Fig. 1. – 3D projection from confocal microscopic images of body-wall musculature of the cephalic region immunostained with TMUS-13. (A) Dorsal view, with longitudinal fibers (arrows) converging to a central region, and (B) Ventral view, with longitudinal fibers (arrows) running in parallel. *L*, longitudinal fibers. *Scale bar* 50 μm.

Body-wall muscle pattern restoration during anterior regeneration

For anterior regeneration experiments animals were amputated at a post-cephalic level and the appearance of the new muscle pattern in the forming head was monitored with TMUS-13 and confocal microscopy. Fig. 2 shows how this muscle pattern is restored within the first 6 days of regeneration. At day 1 of regeneration the blastema is still rather small and difficult to distinguish. Dorsally, in the most anterior region and where the blastema is being formed, there appears a big "hole" lacking muscle fibers and delimited by rather disorganized old muscle fibers (Fig. 2A). This "hole" is neither a fixation artefact nor a blastema breaking, as it is really filled with many cells that are different from muscle cells. In contrast, the muscle fibers of the ventral surface keep a more organized pattern similar to that found in an intact organism (Fig. 2B). At day 2 of regeneration there is a significant increase in muscle fibers and differentiating myocytes within the dorsal "hole", although the pattern is still rather disorganized. It can be seen how existing longitudinal fibers enter the blastema (Fig. 2C; arrows). However, the blastema ventral musculature continues to be formed, apparently, by old existing fibers with a more organized pattern. This organization is seen more clearly in the longitudinal fibers, whereas circular fibers are absent within the blastema. In the most distal part of this blastema, the muscle pattern is partially lost, even when compared with a one-day regenerant. Distally, these ventral muscle fibers are arranged in a more relaxed pattern with wider distances between them (Fig. 2D). At day 3 of regeneration an incipient arrangement of the muscle fibers is seen on the dorsal surface of the blastema. These fibers

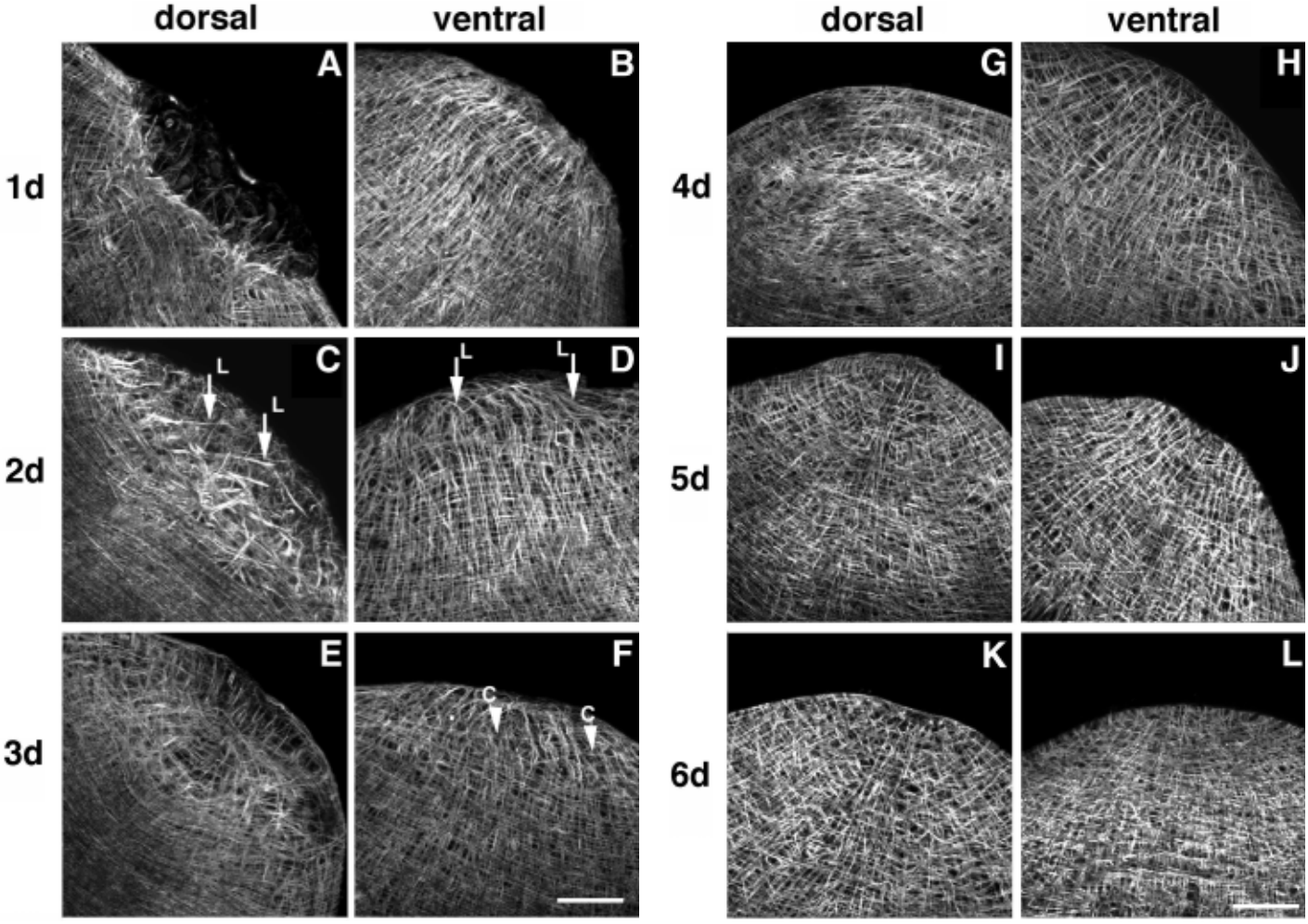

Fig. 2. – 3D projection from confocal microscopic images of regenerating heads immunostained with TMUS-13. Day 1 regenerant, dorsal (A) and ventral (B) views. Note a dorsal "hole" lacking muscle fibers. Day 2 regenerant, dorsal (C) and ventral (D) views. Existing dorsal longitudinal muscle fibers enter the blastema (arrows). Ventrally, the blastema only contains existing longitudinal fibers (arrows). Day 3 regenerant, dorsal (E) and ventral (F) views. Muscle fibers on the dorsal surface organize in a first outline of the definitive pattern, converging to a central region of the blastema. On the ventral surface of the blastema, new circular fibers appear (arrowheads). Day 4 regenerant, dorsal (G) and ventral (H) views. The muscle pattern in both dorsal and ventral surfaces is already restored. In the following days a more dense and compact muscle pattern appears (I-L). From this moment, cephalic muscle pattern can be considered restored. *L*, longitudinal fibers, *C*, circular fibers. Scale bar: 50 μm.

tend to converge to a central region of the blastema in a similar way to what occurs for an intact head (Fig. 2E). In the ventral surface, and in the distal part of the blastema, new thin circular fibers are formed (Fig. 2F; arrowheads). From the fourth day of regeneration the muscle pattern, both dorsal and ventral, is restored and in the following days the number of fibers increases, resulting in a more compact and dense pattern similar to the one found in intact organisms (Fig. 2G-L).

DISCUSSION

Biological significance of the differences described in dorsal and ventral blastemas

The differences observed between dorsal and ventral surfaces, mainly during the first days of regeneration, seem to reflect the differences found in an intact head which could be just indicating, at a structural level, how this muscle network is closed in the most anterior tip. We cannot exclude, however, that these differences are related to the way the epithelium heals the wound. One of the unsolved questions about planarian regeneration is how the regenerant fragments "know" which parts (anterior or posterior) are lacking and, consequently, have to be regenerated. CHANDEBOIS (1976; 1980) suggested that during anterior regeneration it is the dorsal epithelium which expands to close the wound. In contrast, the ventral epithelial cells would heal the wound in posterior regeneration. These different ways of wound closure would give the specific signals for anterior or posterior regeneration. If CHANDEBOIS' hypothesis is right, this dorsal region lacking muscle fibers could be related in some way to the expansion of the dorsal epithelial cells to close the wound. To corroborate this hypothesis, we should study how the muscle fibers are arranged at the caudal end of the organism as well as what happens during muscle pattern restoration in posterior regenerants. In fact, SALVENMOSER et al. (this volume) show how during posterior regeneration in the microturbellarian *Macrostomum* sp., the blastema is shifted ventrally and the differences between dorsal and ventral muscle patterns reverse the ones observed during planarian anterior regeneration.

Planarian regenerative blastema contains old muscle fibers from the postblastema

What we can say at present is that, from the beginning, the blastema seems to contain, at least ventrally, existing muscle fibers coming from the postblastema. These muscle fibers, mainly longitudinal, are detected within the blastema during the entire regenerative process, which suggests that they could play a role in supporting the growing blastema and/or in guiding the entry of myocytes from the postblastema. At day 1 of regeneration many myocytes appear in the postblastema, mostly on the ventral surface. As regeneration proceeds, they migrate into the blastema where they differentiate into new muscle fibers (unpubl. data). Therefore, the appearance of these myocytes close to the existing longitudinal muscle fibers suggests that the myocytes could use these fibers in their migration into the blastema. This association between myocytes and mature muscle fibers has also been described within the planarian pharynx (CEBRIÀ et al., 1999). Moreover, REITER et al. (1996) have shown how, during embryonic development of some platyhelminths, existing muscle fibers can guide the appearance of the new fibers.

Muscle pattern restoration: distoproximal, proximodistal or intercalary?

When considering muscle pattern restoration, one of the questions to answer is whether this restitution is carried out in a distoproximal or proximodistal sense; in this case, proximal is the region closest to the wound or postblastema, and distal is the tip of the growing blastema. We have to distinguish this question, which refers to the differentiation of the muscle pattern, from the distoproximal sequence of events through which the different regions (i.e. head, prepharyngeal or pharyngeal regions) are determined during regeneration (WOLFF et al., 1964; SALÓ, 1984). The results shown in Fig. 2 do not let us give a definitive answer to this question. At day 2 of regeneration the blastema contains many muscle fibers, but these are rather disorganized. At day 3, however, an incipient pattern can be seen dorsally, with new fibers converging to a central region of the blastema, as happens in an intact head. So, it seems that in few hours the fibers within the blastema organize themselves rapidly into a first outline of the definitive pattern, which will be completely restored from this initial muscle scaffold. Whereas new longitudinal muscle fibers within the blastema (especially on the dorsal surface) seem to appear from the outgrowth of existing longitudinal fibers coming from the postblastema, the circular fibers probably appear *de novo* within this blastema, as is suggested by comparison of dorsal and ventral blastemas between day 2 and day 3 of regeneration. At present we do not know how new circular fibers become oriented within the blastema, though it is possible that longitudinal fibers can be involved in this process. SALVENMOSER et al. (this volume) show how, during body-wall muscle regeneration in *Macrostomum* sp., the future circular fibers appear as myocytes perpendicularly oriented to the longitudinal fibers. The final restoration of the muscle pattern could then occur in proximodistal sequence, as is suggested more clearly for the appearance of the new muscle fibers within the blastema, or through an intercalary differentiation of the new fibers throughout all this muscle scaffold detected at day 3. A similar intercalary mechanism has been proposed for muscle pattern restoration during pharynx regeneration (ESPINOSA, unpubl.; BUENO et al., 1997) and cell-renewal (CEBRIÀ et al., 1999).

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Further observation on the early regenerates after fission in the planarian *Dugesia japonica*

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ABSTRACT. In addition to possessing remarkable regeneration powers, many species of planarians reproduce asexually by fission. Since fission takes place at the post-pharyngeal region in most cases, we amputated this body portion of intact animals and compared their regeneration process with that of fission fragments.

The fission fragments formed a well-defined blastema in the early regenerates, and the number of blastema-forming cells of the regenerates was larger than that of the amputated ones. When we compared the early regenerates from posterior fragments with those from anterior ones, it was clearly confirmed that posterior fragments invariably regenerated more rapidly. The present observations on the pre-fissioning planarians suggest that the rapid development of the early blastema is induced by fixed parenchyma cells at the preparatory parenchymal region for fissioning.

KEY WORDS: Platyhelminthes, planarian, fission, regeneration, differentiation.

INTRODUCTION

Triclads, especially the asexual strains of freshwater planarians, reproduce by fission (VOWINCKEL, 1970; GRASSO & BENAZZI, 1973; MORITA & BEST, 1984). Then each fission fragment regenerates the appropriate missing part to yield complete worms (BEST et al., 1969; LENDER, 1974; NENTWIG, 1978; BAGUÑÀ, 1998). By observing the fission fragments using light and electron microscopes, it is certainly possible to elucidate natural processes of regeneration more clearly. However, there is only little information about the regeneration of post-fissioned planarians (KENK, 1937; NENTWIG, 1978; MEAD, 1985; HORI & KISHIDA, 1998). It is uncertain if there is any similarity between the processes leading to formation of the missing body part in amputated animals and fission fragments. Observations on the fission fragments are expected to clarify details of cell behavior during regeneration because fissioning is never accompanied by mechanical tissue damage.

Our previous study has confirmed the occurrence of the preparatory stage before fissioning (HORI & KISHIDA, 1998). If any difference regarding the regeneration process is present between amputated animals and fission fragments, it is expected to appear as cell behavior within early regenerates. In this report, we offer information regarding some cytological changes occurring at the preparatory region, and then make comparison of early regenerates obtained from amputated animals and fission fragments.

MATERIAL AND METHODS

Asexual strains of the freshwater planarian *Dugesia japonica* were employed in this study. Worms were fed beef liver once a week. During all the experiments they were maintained in pond water at 18°C. Fissioning can be induced by increasing the level of some conditions such as temperature, population density, illumination, and by decapitation (CHILD, 1932; OKUGAWA & KAWAKATSU, 1956; BEST et al., 1969; VOWNICKEL, 1970; MORITA & BEST, 1984). To obtain a large number of fission fragments, well-fed animals (about 10 mm in length) were decapitated and placed in isolation (one animal/small dish). Then fissioning occurs most frequently at the post-pharyngeal region (BRØNDSTED, 1969; LENDER, 1974; NENTWIG & SCHAUBLE, 1974). For examining the preparatory zone of fissioning, we observed the post-pharyngeal portion of the animals three days after decapitation. Regenerates were obtained from two groups of the specimens; one was from fission fragments 20 and 24 hours

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after fissioning, and the other was from animals that had been amputated at their post-pharyngeal region 20 and 24 hours before. Pieces including the tail part are referred to as piece A, and those including the head part are referred to as piece P in this report.

All the specimens were fixed and prepared for light and electron microscopy as described in our previous report (HORI & KISHIDA, 1998).

RESULTS

Post-pharyngeal region

Comparisons were made between post-pharyngeal regions of the intact and decapitated animals. The intact region showed normal histological aspects in the epidermis and parenchymal tissues (Fig. 1a). Ultrastructurally, the parenchyma was occupied with various cells such as fixed parenchyma cells, regenerative or undifferentiated cells, gland cells, muscle fibers and protonephridial cells. These cells were arranged so intimately that the intercellular matrix was very small.

Some of the decapitated animals showed histological and cytological changes suggestive of preparation for fissioning. They occurred at the post-pharyngeal region as a lower dense zone between intestinal tracts (Fig. 1b). In the middle area, numerous fixed parenchyma cells were observed extending their cytoplasmic processes. Regenerative cells were located at the subepidermal region. Occasionally the epidermis was invaded by the fixed parenchyma cells so that some of the epidermal cells became degenerative.

Regeneration after fissioning

It was common to observe the extensive blastema in piece A of 20 h regenerates (Fig. 2a). Migrating regenerative cells were easily identified. Two or three layers of fixed parenchyma cells were built up in the subepidermal region (Fig. 4). Their cytoplasm had a large number of

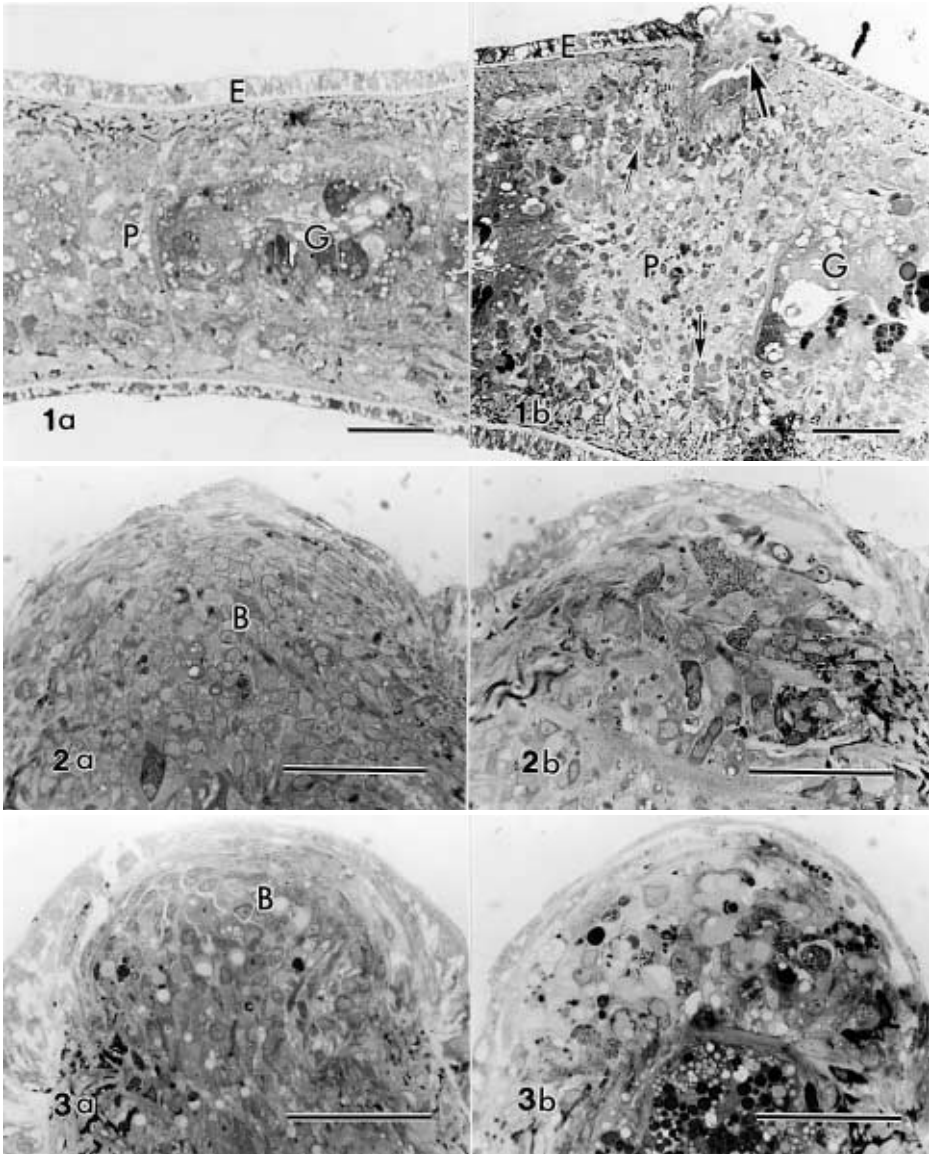

Fig. 1a. – Light micrograph of the post-pharyngeal region of the normal planarian. E; epidermis, P; parenchymal tissue, G; gastrodermis. Scale bar = 50 μm.

Fig. 1b. – Light micrograph of the post-pharyngeal region of the pre-fissioning planarian. Large arrow indicates the disorganization of the epidermis. Small arrows indicate an aggregation of regenerative cells. E; epidermis, P; parenchymal tissue, G; gastrodermis. Scale bar = 50 μm.

Fig. 2a. – Light micrograph of the regenerate, 20 h after fission; piece A. B; blastema. Scale bar = 50 μm.

Fig. 2b. – Light micrograph of the regenerates, 20 h after fission; piece P. Scale bar = 50 μm.

Fig. 3a. – Light micrograph of the regenerate, 20 h after amputation; piece A. B; blastema. Scale bar = 50 μm.

Fig. 3b. – Light micrograph of the regenerate, 20 h after amputation; piece P. Scale bar = 50 μm.

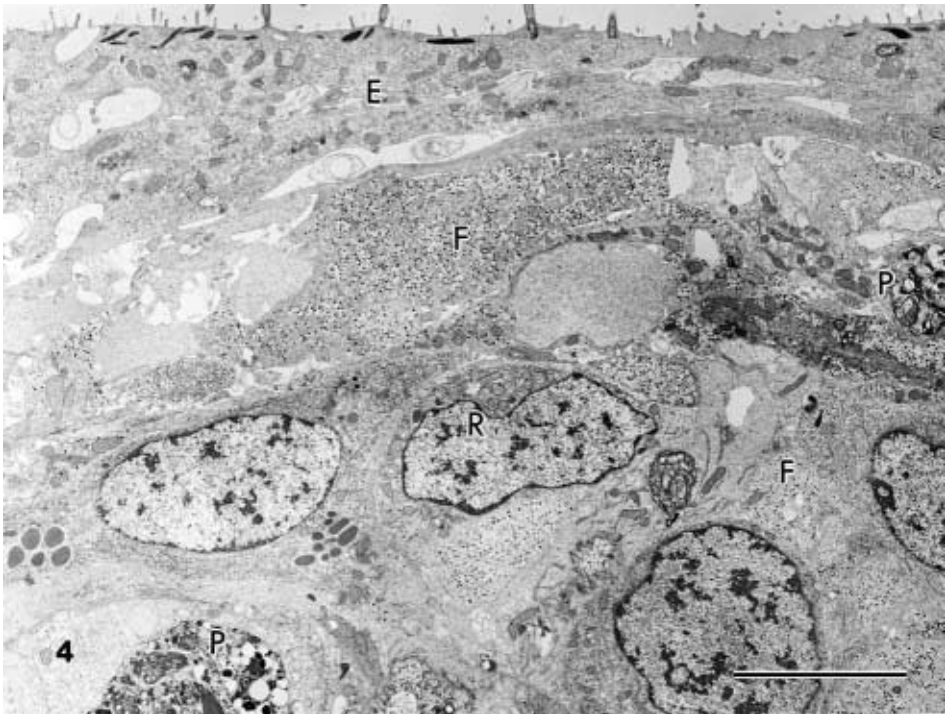

Fig. 4. – Electron micrograph of the regenerate, 24 h after fission. E; epidermal cell, F; fixed parenchymal cells, R; regenerative cell, P; phagosomes. Scale bar = 5 μm.

glycogen granules. Large phagosomes were occasionally observed containing cell debris. Regenerative cells appeared in the middle blastemal area in greater quantity than in the subepidermal region. In piece P, the blastema was not clearly formed (Fig. 2b). The wound zone consisted of several regenerative cells and differentiated cells from the uninjured tissue area. Occasionally amorphous substances were observed at the subepidermal extra cellular space.

Regeneration after amputation

A fairly extensive regeneration blastema was formed in piece A (Fig. 3a). It was mainly composed of regenerative cells. Fixed parenchyma cells extended their cytoplasmic processes among these regenerative cells and participated in phagocytosing cell debris. The regeneration blastema was poorly formed in piece P (Fig. 3b). Only a few regenerative cells were observed in the regenerate. Intercellular space at the wound region was dilated and filled with amorphous substances.

Quantitative data

In order to compare development of the blastema from the regenerates, the cell number per 100 μm^2 of the blastema was calculated on the light micrographs. The data are summarized in Table 1. It is of interest to note that cell density of the blastemal zones was much higher in piece A than piece P. It was also obvious that the blastema developed more rapidly in the fission fragments than in the amputated animals.

TABLE 1

Number of cells per 100 μm^2 of the blastema

REGENERATES		20hr	24hr
FISSION	Piece A	1.14	1.52
	Piece P	0.63	0.87
AMPUTATION	Piece A	1.01	1.20
	Piece P	0.40	0.80

DISCUSSION

Preparation for fissioning

No morphologically differentiated plane of fracture is evident before the onset of fission (Best et al., 1969; Moraczewski, 1977). However, histologically the first recognizable indication of the actual fission is evident at the post-pharyngeal region (Pedersen, 1958; Peter, 1995; Hori & Kishida, 1998). In *Microstomum lineare*, it appears as an occurrence of nerve fibers, muscle fibers and activation of cell reproduction (Reuter & Palmberg, 1983). The present observation shows the portion having low histological staining intensity at the post-pharyngeal region of the decapitated animals. The observation suggests that preparation for fissioning occurs both in the epidermis and the parenchymal tissue. Aggregation of the regenerative cells in the subepidermal region seems to provide for the forthcoming regeneration.

Regeneration after fissioning

Some authors have reported that fission fragments form no blastema because the regeneration after fissioning could be carried out not by epimorphosis but by morphallaxis (KENK, 1937; NENTWIG, 1978). In the present study, however, each fission fragment formed a well-defined blastema though the spatial patterns of cell distribution are not identical between early regenerates of fission fragments and amputated animals. In particular, development of the blastema is crucial in piece A.

The organization of structurally unique fixed parenchyma cells in the pre-fissioning zone is required for a rapid formation of the blastema. At the onset of fissioning, these cells are first organized into functional cell layers beneath the degenerating epidermis. Glycogen granules in their cytoplasm are possibly used for energetic support of regeneration (MORITA, 1995). In the case of regeneration after artificial amputation, it takes a length of time to recover from the mechanical damage. MORITA (1991; 1995) has observed that the fixed parenchyma cells or reticular cells arrive at the wound surface very rapidly, and then phagocytose cell debris of degenerative cells in the decapitated planarians. The present study suggests another role of these cells in the regeneration of fission fragments.

During epitheliogenesis of the wound epidermis, we have observed some extent of amorphous substances at the subepidermal extracellular space (HORI, 1979). These substances seem to be the precursor of the basement membrane. Localized deposit of amorphous substances at the extracellular space indicates rapid reorganization of the epidermal basement membrane in the fission fragments.

The difference in cell density within the blastema between pieces A and P is due to some factors such as the difference of tissue organization with and without a pharynx, cell numbers of pre-existing undifferentiated cells, and positional pattern of nerve cords.

Nerve cords are often observed penetrating the blastemal area during regeneration (NENTWIG, 1978). It is believed that the nervous system of turbellarians could play a regulatory role in cell behavior and proliferation (BEST et al., 1969; LENDER, 1974; PIGON et al., 1974; REUTER and PALMBERG, 1983; MORITA & BEST, 1984; BAUTZ & SCHILT, 1986; BAGUÑÀ et al., 1989). However, it is unknown how the nervous system relates to the positional difference in cellular activity for the regeneration. Understanding of the control mechanism of the nervous system is expected to elucidate the difference of regeneration between pieces A and P.

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Planarian cell culture: a comparative review of methods and an improved protocol for primary cultures of neoblasts

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ABSTRACT. To develop an improved method for preparing and cultivating planarian cells, several protocols published previously were compared with each other. Cells, and in particular neoblasts, proved remarkably resistant to hyposmotic conditions. However, survival periods depended critically on the content in nutrients and on osmotic conditions. Starting from an optimized method to disintegrate planarian tissues and prepare purified neoblast fractions, different media and additives were tried. Hyposmotic media and layers of extracellular matrix components enhanced the adhesion of neoblasts and favoured the formation of transient processes. Proteins in the medium supported long-term survival of neoblasts that retained a spherical shape. Eventually, an isosmotic medium was devised that supported the survival of neoblasts with a viability of 46% on day 31 of primary cultures. With light microscopical techniques, no signs of differentiation were observed in these cultures. Mitoses were detected until the second day of cultivation. In contrast, cultures of total cells still displayed mitoses after 7 days of cultivation. Some guidelines are proposed for future research directed towards establishing permanent neoblast lines.

KEY WORDS: cell culture, culture media, extracellular matrix, neoblasts, osmotic effects, planarians, primary cultures, *Schmidtea (Dugesia) polychroa*, Platyhelminthes.

INTRODUCTION

The first attempt at planarian cell culture to be published originated during an initial boom in modern regeneration science and was undertaken in Ch. M. Child´s laboratory in Chicago (MURRAY, 1927). No report of any permanent platyhelminth cell line has been published up till now. The importance of the only proliferative cell type, the neoblast, in regeneration of turbellarians – especially in connection with the molecular data reported in recent times (for reviews, see BAGUÑÀ, 1998; BAGUÑÀ et al., 1994) and the experimental potential a permanent neoblast line would offer – should stimulate intensification of the efforts to establish such a cell line.

When devising planarian cell culture media, authors started from solutions used for amphibians (BETCHAKU, 1967), embryonic chick tissue (MURRAY, 1927), mammals (BETCHAKU, 1967; FRANQUINET, 1973) or a combination of recipes for vertebrate and snail cells (TESHIROGI & TOHYA, 1988). The importance of nutrients and osmotic conditions was realized early (MURRAY, 1927). Relatively later, analytical data from planarians have been used as a basis for deriving culture media. TESHIROGI & TOHYA (1988) added amino acids to the medium in the concentrations determined from planarian extracts. To guarantee isosmotic conditions, osmotic values of planarian tissues were taken into consideration by SCHÜRMANN & PETER (1993). A survey of the great variety of media is given in Table 1. The exact composition of the extracellular environment is, however, still unknown. Preparing an extract of the extracellular matrix that is free from any contamination originating from damaged cells is extremely difficult because of the parenchymal structure of triclad turbellarians. This fact contrasts sharply with the ease of obtaining and analyzing body fluids from vertebrates resulting, in the end, in an elaborate inventory of culture media and conditions.

The techniques to isolate cells from planarians may be grouped into a few categories. As it is not possible to dissect a triclad completely into its organs and neoblasts are widely scattered throughout the parenchyma, whole animals or defined body regions of them have to be the source

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for any cell preparation. Tiny fragments were obtained by simple cutting and kept either in tissue culture medium (FRANQUINET, 1973; SEILERN-ASPANG, 1960, among others) or cultivated under conditions leading to the accumulation of certain cell types, primarily neoblasts (BETCHAKU, 1967, 1970). To dissociate cells from planarian tissue, BETCHAKU (1967) chose moderately hyposmotic conditions (80 mOsmol/l, compared to 125-128 mOsmol/l for planarian tissues). Selective adhesion to glass and resistance to the osmotic conditions led to the accumulation of neoblasts within 1-2 days in this case. FRANQUINET (1981) introduced the dissociation of planarian tissues into single cells by disintegration in a Dounce homogenizer. To study DNA and RNA synthesis and other processes, cell suspensions were cultivated for up to 7 days without separating or enriching any cell type. In order to prepare large quantities of neoblasts within a reasonable time, we have combined the disintegration in a Dounce homogenizer first with preferential adhesion to glass and plastic dishes and prolonged survival of neoblasts under hyposmotic conditions (SCHÜRMANN & PETER, 1988) and later with a fractionation of cells by centrifugation in a Percoll density-gradient (SCHÜRMANN & PETER, 1993; SCHÜRMANN et al., 1998).

MATERIAL AND METHODS

Cells were prepared from *Schmidtea (Dugesia) polychroa* (Schmidt 1862). The laboratory cultures were originally derived from a population living in the river Amper (Bavaria, Germany) and kept in the dark at 19 ºC. The karyotype corresponded to the triplohexaploid "biotype B" (BENAZZI & BENAZZI LENTATI, 1976). The planarians were fed with tubificid worms at intervals of 7 days, starved a week and kept in 0.02% neomycin sulfate in culture water for at least 24 h prior to disintegration. Artificial pond water (MC CONNELL, 1967) was used for the planarian cultures. The reagents were purchased from Sigma, with a few exceptions cited in the following text. Cell culture grade was chosen, where available. To test their reproducibility and gain experience for developing an improved method, several protocols of previous publications were tried: those of BETCHAKU (1967), FRANQUINET (1981) in the original and in a modified form (SCHÜRMANN et al., 1998), FRANQUINET et al. (1985) and TESHIROGI & TOHYA (1988).

Osmolarities were determined by freezing point depression of media or planarian extracts with an Advanced Laboratory wide-range osmometer, model 3W2. For this purpose, planarians were disintegrated in distilled water by ultrasound (Branson Sonifier, model 250) and the dilution was taken into account in the calculations.

The disintegration method used to obtain cell suspensions was the same as described previously for the isolation and fractionation of neoblasts (SCHÜRMANN et al., 1998). A modification of this method is presented in this volume (BEHENSKY et al., 2001). For the primary cell cultures, however, any enzyme addition was omitted. To reduce viscosity caused by mucus, only "Digest-Eur" from Eurobio, Paris, was added to give a final dilution of 1% of the "10X" stock solution. This corresponded to a concentration of 0.00063% 2,3-dihydroxy-1,4-dithiolbutane with a little physiological saline. The confusion that arose in this context from different publications originating from Franquinet´s laboratory will be discussed later. Digest-Eur was present during all purification, washing and centrifugation steps, but was not contained in any of the culture media. All manipulations, beginning from disintegration, were done under sterile precautions. A laminar flow bench was used where appropriate.

For total cell cultures, the initial cell density was $6x10^5$ cells per ml. Neoblasts with a purity of roughly 90% were collected from a discontinuous four-step gradient (SCHÜRMANN, 1993; SCHÜRMANN et al., 1998) at the density boundary 1.05/1.07 and cultivated under sterile conditions in different media at 18 °C, in the dark except during the short observation and handling periods. Each Petri dish made of polystyrene (Greiner, Kremsmünster), with a diameter of 35 mm, was filled with 1 ml of a suspension containing $8x10^5$ cells. After 1 h, the cells had sedimented and the cultures were filled up with 1 ml of fresh medium. Media were changed every third day. A cooled incubator (Heraeus Cytoperm 8088, with 95% air + 5% CO_2) was used only for the cultures with the medium devised by TESHIROGI & TOHYA (1988). The cultures were viewed and photographed through an inverted microscope with phase contrast optics (Reichert Biovert). Control preparations stained with azure A – eosin B (PEDERSEN, 1959) confirmed the diagnosis of neoblasts based on their nucleocytoplasmic ratio observed with phase contrast illumination. To test viability, the stain exclusion method with nigrosin (50 µg/ml) was used (KALTENBACH et al., 1958). In several cases, the fluorescent LIVE/DEAD viability/cytotoxicity kit (L-7013) from Molecular Probes was applied (BELETSKY & UMANSKY, 1990; POOT et al., 1997). To determine mitotic indices, colcemide (100 or 60 µg/ml culture medium) was added and the arrested metaphases were stained with DAPI (Hoechst) and counted after 5 or 4 h.

Eventually, an isosmotic medium (Table 1) was developed, starting from the recipe given by TESHIROGI & TOHYA (1988). The main modifications consisted in adding the biological buffer Hepes and its sodium salt instead of $NaHCO_3$ and CO_2, and in the replacement of the amino acid mix by commercially available stock solutions. The calcium concentration was chosen to meet the requirement of 1 mM for maintaining RNA and DNA synthesis (MARTELLY, 1984). This medium had an osmolarity of 126 mOsmol/l and a pH of 7.40. One litre was prepared from 7,208.8 mg Hepes (free acid), 3,514.1 mg Hepes (sodium salt), 985.4 mg NaCl, 800.1 mg $NaHCO_3$, 26.4 mg KCl, 150.0 mg $CaCl_2.2H_2O$, 90.1 mg $MgSO_4.7H_2O$, 0.3 mg $MnCl_2.4H_2O$, 68.5 mg KH_2PO_4, 150.0 mg sodium pyruvate, 300.0 mg D-glucose, 50.0 mg D-trehalose, 49.0 mg L-glutamine, 0.3 mg d-biotin, 100.0 mg neomycin sulfate, 2.5 mg tricine, 2.0 mg phenol red, 10.00 ml BMS (Biochrom KG, Berlin), 2.00 ml MEM essential amino

acid solution (50x), 5.00 ml MEM non-essential amino acid solution (100x) and 3.00 ml MEM vitamine solution (100x). These components were dissolved in highly pure sterile water (double distilled quality) from a Millipore plant to give a final volume of 1 l. BMS is a standardized supplement containing compounds from fetal calf serum and bovine serum albumin; 1 l contains 15.00 g of protein. The MEM additives were obtained from GIBCO-BRL.

RESULTS

Reproducibility of methods published previously

BETCHAKU´S (1967) original method resulted in the accumulation of neoblasts he described. Both glass and polystyrene dishes were applicable. Cells adhered better to glass. For a successful isolation of neoblasts, it was essential that the tiny tissue fragments adhered to the bottom of the dish. Frequent gentle shaking increased the yield of neoblasts. When the method described by FRANQUINET (1981) was reproduced in strict compliance with the published protocol, cells could not be cultivated successfully and showed fragmented nuclei after one day. This was evidently due to the presence of the cationic detergent benzalkonium chloride. Viability tests (LIVE / DEAD test) with cells filtered through meshes down to 15 µm gauze showed no viable cells upon exposure to 1% benzalkonium for 1.5 h, and far less than 1% viable cells when the concentration was 0.033%. Cilia of epithelial cells ceased to beat at the latter concentration (SCHÜRMANN, 1993). As discussed below, Digest-Eur has evidently been taken erroneously for benzalkonium, resulting in a misleading description of the method. With benzalkonium chloride in the medium, neoblasts adhered readily to the culture dish and were firmly attached after 20 min. However, this might at best serve for enriching these cells immediately before fixation. Also Digest-Eur favours adhesion of neoblasts, although to a lesser extent. After 45 min, this cell type adheres preferentially to glass and plastic surfaces.

Influence of different culture media and matrices on the behaviour of neoblasts

Neoblasts isolated and purified by the new combination of methods mentioned above (BEHENSKY et al., 2001; SCHÜRMANN et al., 1998) adopted a spherical shape within a few hours from isolation (Fig. 1). Cells varied in size, depending on the osmotic values, with diameters from 11.0-13.2 µm (mean: 12.2 µm) in a hypotonic medium (TESHIROGI & TOHYA, 1988), from 10.3-13.0 µm (mean: 11.7 µm) in the newly devised isosmotic medium with 126 mOsmol/l (see above under materials and methods) and from 9.4-12.0 µm (mean: 10.7 µm) under strongly hyperosmotic conditions of 345 mOsmol/l (FRANQUINET, 1981). The cytoplasmic rim around the nucleus was, on the average, from 0.65-0.85µm wide, depending on the osmotic conditions. Cells mostly died on the first day in the hyperosmotic medium, but could be cultivated for weeks in the other media. Neoblasts adhered to glass or polystyrene dishes to a percentage of 80-90% two hours after seeding. Adhesion was far less firm than for mammalian cells and than for the neoblasts isolated after BETCHAKU (1967). The primary and secondary processes described by this author (Fig. 2) were observed within

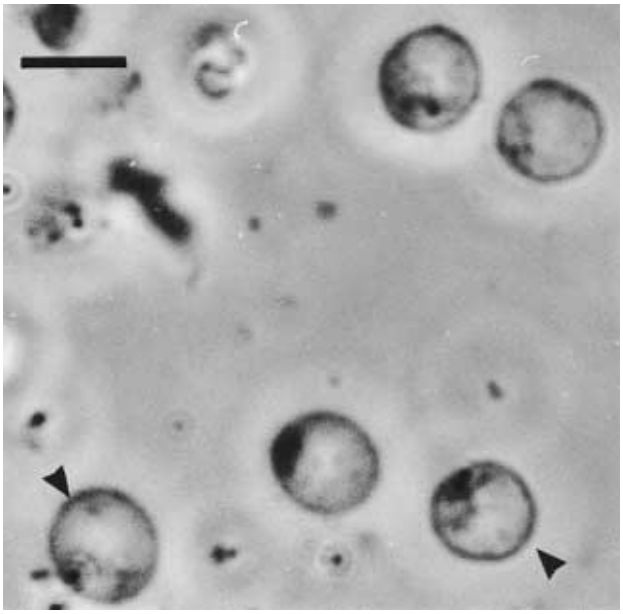

Fig. 1. – Neoblasts in hyposmotic saline (5/8-concentrated Holtfreter´s solution, 80 mOsmol/l, after BETCHAKU, 1967), cultivated for 7 h. Phase contrast optics, inverted microscope. The dark cytoplasmic rims (arrowheads) are clearly visible. Bar: 10µm.

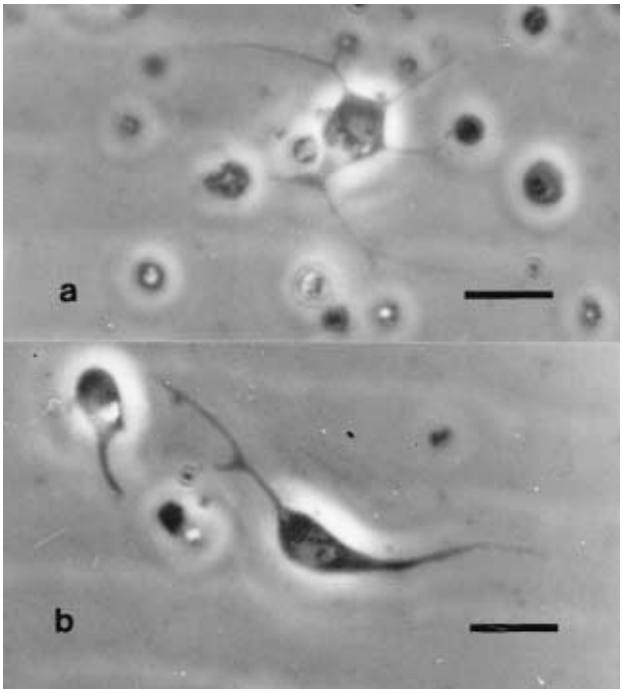

Fig. 2. – Neoblasts with processes formed in hypotonic medium (the same as for Fig. 1) in 46 h old culture: a) without protein, b) with 3% BMS in medium. Optics as for Fig.1. Bars: 20 µm.

24 h in roughly 10% of the cells under hypotonic conditions and in about 5% in the isosmotic medium. Multiple processes were observed. Lowering the osmotic value resulted in longer processes. All these were not permanent, but degenerated gradually from 48-72 h from the start of cultivation. Layers of collagen I from the rat favoured the formation of processes in the isosmotic medium (Fig. 3). In the hypotonic medium "DHM" with 80 mOsmol/l (see Table 1 and BETCHAKU, 1967), processes from 40-70 µm in length could be found on collagen layers.

The majority of cells retained the spherical shape. Attachment was enhanced up to 95% of the cells when the surface of the Petri dish was coated with extracellular

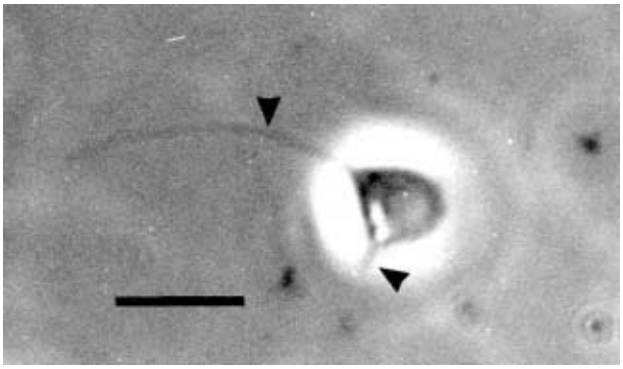

Fig. 3. – Neoblast cultivated in the new isosmotic medium on a layer of collagen I from rat tail for 26 h, with processes (arrowheads). Optics as for Fig. 1. Bar: 20 µm.

TABLE 1

Comparison of selected planarian cell culture media, with concentrations of major constituents

Medium:	**MPM**	**DHM**	**BPM**	**FMM**	**FM73**	**FM81**	**FM85**	**SPM**	**TTP**	**IPM**
mOsmol/l	30-150*)	80	69*)	160	175*)	345	155	139	85	126
pH	–	7.3	7.3	8.0	7.2-7.3	7.25	7.25	7.25	7.4-7.6	7.40
Inorganic ions [mM/l]										
Na^+	11.6-15.5	38.93	32.50	65.90	14.00	140.44	53.37	61.23	41.34	42.39
K^+	.22 -.30	.42	.34	2.13	6.71	10.50	7.40	7.22	.35	.89
Mg^{++}	+	–	–	1.07	.65	1.59	.70	1.05	.37	.38
Ca^{++}	.15-.20	.57	.45	.47	2.72	4.66	1.00	.42	.48	1.03
HCO_3^-	.20-.26	1.49	1.19	–	2.02	18.84	7.20	17.89	9.52	9.63
$H_2PO_4^- + HPO_4^{--}$	–	–	–	.62	–	.97	.48	.62	–	.51
SO_4^{--}	–	–	–	.67	.65	1.59	.70	1.05	.37	.37
Cl^-	12-16	39.00	31.20	67.85	24.13	139.96	55.00	50.33	33.13	20.34
Buffers [mM/l]:										
Hepes, anion + free acid	–	–	–	–	–	8.34	20.00	8.34	–	43.75
Tricine	–	–	–	30.00	–	–	–	–	.014	–
CO_2: 5% in atmosphere	–	–	–	–	–	–	–	–	+	–
Nutrients [mM/l]:										
Acetate	–	–	–	–	–	.34	–	.34	–	–
Pyruvate	–	–	1.36	.40	–	–	–	–	–	1.36
L-Glutamine	–	–	–	–	–	1.33	–	.34	.33	.34
D- Glucose	5.5-55.5	–	1.66	1.40	110.99	5.55	10.00	1.85	1.66	1.83
D- Trehalose	–	–	–	–	–	–	–	–	.15	.15
Amino acids	–	–	+	+	–	+	+	+	+	+
Vitamins	–	–	+	+	–	+	+	+	+	+
Antibiotics	–	–	N	PS	P	PS	PS	PS	–	N
Mammalian serum	0-25%	–	.3%	+/-	5%	–	–	–	1%	–
BMS	–	–	–	–	–	–	–	–	–	1%

Comments and references:

Solute compositions of culture media have been calculated from the original recipes. The constituents (ions, nutrients, vitamins etc.) added with the sera are not included in the concentrations listed. Osmolarities and pH values were taken from the original descriptions or measured, with three exceptions (*) that were estimated by calculations from the solute concentrations. The pH indicator phenol red is contained in all media except MPM, DHM and BPM. All media except MPM are prepared with distilled water.

+ indicates the presence, - the absence of a constituent.

N: neomycin sulfate, P: penicillin, S: streptomycin.

MPM: Murray´s planarian medium (MURRAY, 1927). Pretreated well water served as the solvent, with minor components and trace elements (Al^{+++}, Fe^{+++}, Mg^{++}, Mn^{++} and NO_3^-, among others). A wide range of concentrations and different variants, including the addition of some amino acids and peptone (1g/l), were tried. Dilutions without sheep serum had estimated osmolarities up to 88 mOsmol/l.

DHM: Dilute Holtfreter´s medium (5/8 of the original concentration for amphibian tissues), introduced by BETCHAKU (1967).

BPM: Betchaku´s planarian medium (BETCHAKU, 1967). Minor constituents: individual mixture of amino acids, vitamins and choline.

FMM: Fukushima´s and Matsuda´s medium (FUKUSHIMA & MATSUDA, 1991). Different modifications were tried. The composition optimized for maximal leucine incorporation is given in the table. This medium is prepared from the mammalian F–12K medium, that contains a cocktail of vitamins and amino acids, nucleosides, nucleotides, lipoic acid and minor amounts of Cu^{++}, Fe^{++} and Zn^{++}. Various additives, among them vertebrate sera and planarian extracts, were tested.

FM73: FRANQUINET´s (1973) medium. In addition to the constituents listed above, this medium contains hydrolyzed lactalbumin (500 mg/l) and yeast extract (250 mg/l) as sources for amino acids and vitamins.

FM81: FRANQUINET´s (1981) medium (see also FRANQUINET & MARTELLY, 1981) prepared from Gibco´s medium 199 with Hank´s salts (1/3) and Eagle´s MEM (2/3), including a combination of amino acids and vitamins. See text for the problems with this medium. In addition to the components listed above, a trace amount of Fe^{++}, polyvinylpyrrolidone (500 mg/l) and Tween 80 (66.67 mg/l) are present.

FM85: Franquinet´s medium 85 (FRANQUINET et al., 1985) contains polyvinylpyrrolidone (12 µM/l) in addition to the compounds listed above. Alternatively, calcium concentrations of 1 nM, 10 µM and 1mM per l were applied.

SPM: Schürmann´s planarian medium (SCHÜRMANN et al., 1998), prepared from Gibco´s medium 199 and Eagle´s MEM similar to FM81, but only with the vitamins and amino acids and without the salts contained in the MEM. See text for details. Also polyvinylpyrrolidone (500 mg/l) and Tween 80 are contained in this medium.

TTP: Tohya´s and Teshirogi´s planarian medium (TOHYA & TESHIROGI, 1988). This is the only medium requiring a CO_2-incubator. An amino acid mixture resembling the amino acid composition of planarian extracts (but without arginine), a vitamin combination adapted from other media, choline chloride and Mn^{++} (1.5 µM/l) are contained in addition to the components listed in the table. The osmolarity was measured after the addition of fetal calf serum. The original paper states an osmolality of 80 mOsm.

IPM: Isotonic planarian medium is the new culture medium presented in this paper. In addition to the components given above, it contains Mn^{++} (1.5 µM/l), d-biotin (.30 mg/l) and vitamins plus amino acids from the concentrated solutions for Eagle´s MEM (Gibco). Basal medium supplement (BMS), a standardized serum derivative, is used instead of other sera. Based on the analysis provided by the supplier, its ionic and other major components have been included in the above concentration listing. Details for preparing this medium are given in the text.

matrix components: collagen I from the rat acted similarly to a homologous planarian matrix preparation (PASCOLINI et al., 1992) and to other matrix layers (SCHÜRMANN, 1993). Protein in solution enhanced survival and reduced the attachment of neoblasts. In media with 5-10% fetal calf serum, cells did not adhere, retained their typical neoblast appearance to a considerable extent and still formed monolayers even after 30 days (Fig. 4). Aggregates formed in Petri dishes coated with fibronectin from the rat and even more in cultures without matrix coatings when the calcium concentration was elevated from 1 mM to 2 mM. Such aggregates tended to detach from the substratum with increasing cultivation periods.

Viability and longevity of cells in different culture media

Osmolarities of 125-128 mOsmol/l were found for *Schmidtea polychroa*. PRUSCH (1976) reported 126 mOsmol/l for *Dugesia dorotocephala*. For devising the isosmotic medium, isotonicity was assumed between the cells and the extracellular space. 345 mOsmol/l were measured for FRANQUINET´s (1981) medium.

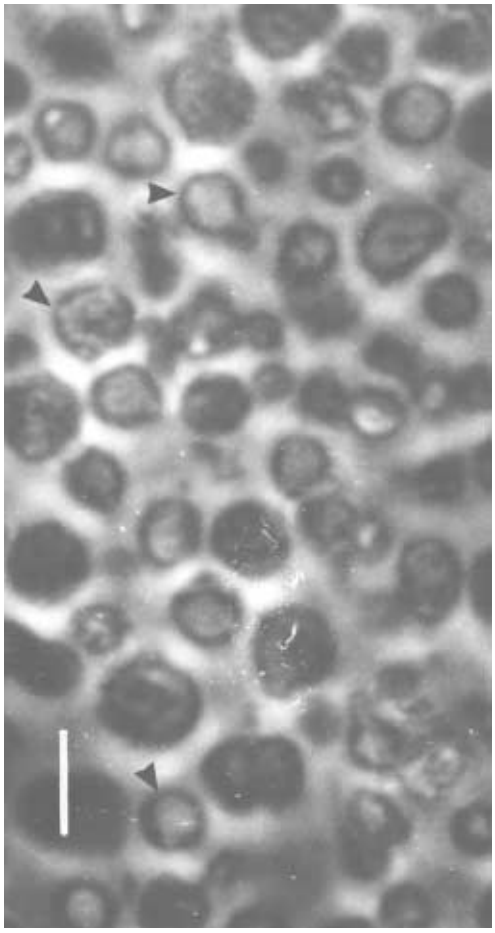

Fig. 4. – Neoblasts cultivated in the new isosmotic medium after 30 days in a Petri dish without matrix coating and with 3% BMS in the medium. Several cells show the characteristic narrow cytoplasmic rim (arrow heads), while others appear to be degenerating. Optics as for Fig. 1. Bar: 10 µm.

The initial viability of neoblasts differed little between the media tested in long term cultures. During extended cultivation periods, however, the new isosmotic medium proved superior and resulted in 46% of viable neoblasts after 31 days (Fig. 5). The cells cultivated in this medium

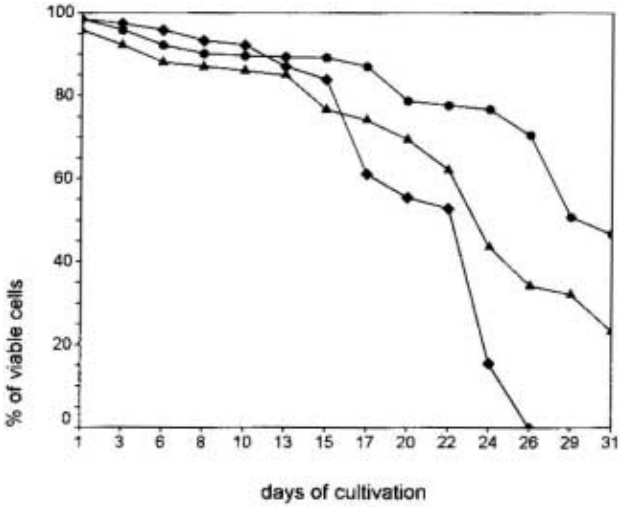

Fig. 5. – Viability of neoblast cultures from *Schmidtea polychroa* in different media, screened with the nigrosin stain exclusion test.
- ● Isosmotic medium (126 mOsmol/l) described in this paper
- ◆ Hyperosmotic medium: 154 mOsmol/l (FRANQUINET et al., 1985)
- ▲ Hyposmotic medium: 85 mOsmol/l (TESHIROGI & TOHYA, 1988)

showed no signs of differentiation when viewed through a light microscope. The same medium also supported the formation and survival of restitution bodies (PETER, 1995).The initially similar viabilities in media differing in composition were confirmed in a pilot count using the LIVE / DEAD kit to test neoblasts having been exposed for 1.5 h to the various media. The same percentage – 78% – of neoblasts was viable in a medium with 85 mOsmol/l (TESHIROGI & TOHYA, 1988) and in the new medium with 126 mOsmol/l. In contrast, hypertonic media as well as strongly hypotonic solutions heavily reduced viability: 10% Locke´s medium (30 mOsmol/l), recommended by MURRAY (1927) as an optimal medium, resulted in only 57% of viable cells. After one day, the majority of these cells had disintegrated, the culture dishes being covered with fragments and debris. The medium with 345 mOsmol/l (FRANQUINET, 1981) lowered viability only to 70%, but cells degenerated rapidly in this medium and could not be observed for more than two days. As will be discussed below, the cited osmotic value seems to result from an erroneous description in the published protocol.

In neoblast cultures with the isosmotic medium, mitoses could be observed during the first day of cultivation, with a fraction of 1.0% of the cells in metaphase. A slight increase to 1.2% was observed upon addition of 0.1 mM serotonin. When total cell suspensions were cultivated in a nearly isosmotic medium (139 mOsmol/l; see SCHÜRMANN et al., 1998) derived from the composition given by FRANQUINET (1981), metaphases were found at a rather constant rate slightly above or below 1% from the beginning of primary cultures until 7 days. This result confirms the original findings.

DISCUSSION

Planarian cells seem to be relatively resistant against hypotonic conditions. Survival seems, however, reduced in the respective media. The amitoses reported by MURRAY (1927) were obviously observed in dying cells. On the other hand, the same author reports good survival when nutrients such as 25% of horse serum were added. When estimating osmolarities, the lowest value is roughly 30 mOsmol/l for 1/10 LOCKE´s solution and 105-150 mOsmol/l for the medium containing serum. One difficulty in evaluating conditions and results in this and other older publications is that the establishment of hanging drop cultures, with a relatively high cell or tissue density in the media and generally without changes of the medium, implied restricted resources as well as poorly defined and hardly reproducible media compositions due to compounds diffusing into the solution from damaged tissues or cells.

Some irritation has arisen from the papers published by FRANQUINET and co-authors. The strongly hyperosmotic medium probably resulted from confusion of complete Eagle´s minimum essential medium (MEM) with the amino acids and vitamins contained in this medium. Eurobio Laboratories, Paris, have been the source for the culture media. Whereas MEM is listed in the catalogue in several variants, all with complete salts, amino acid and vitamin stock solutions for MEM are also offered separately. FRANQUINET (1981) states that the medium used previously (FRANQUINET, 1973, 1976) had been modified to yield the new recipe. This medium had an estimated osmolarity of about 175 mOsmol/l. If Hank´s medium 199 was diluted threefold and the amino acids and vitamins of MEM plus the additional salts listed separately by FRANQUINET (1981) were added, 139 mOsmol/l were measured (Table 1; see also SCHÜRMANN et al., 1998). The mitoses reported for 7 days in Franquinet´s paper and the related composition of a recipe published later (FRANQUINET et al., 1985), with an osmolarity of 155 mOsmol/l, favour the interpretation given above.

Digest-Eur has apparently been mistaken for benzalkonium chloride. In the catalogue issued by Eurobio, Digest-Eur is introduced as a mucolytic agent for clinical use in filtrating sputum, setting up bacterial cultures and, instead of trypsin, to detach cultivated cells from surfaces. It is described as a "concentrate 10X of 2,3-dihydroxy – 1,4-dithiolbutane for a rapid digestion and the fluidification of expectoration products" and delivered in 10 ml flasks. The chemical composition refers to dithiothreitol and / or

dithioerythritol, and the action is evidently based on the reduction of disulfide bridges formed by cysteine residues in glycoproteins. The kit offered contains, in addition, a solution of "Benzalkonium 1p. 3000" (=0.033%) in quantities of 100 or 500 ml to establish cultures of acid-resistant bacilles. In the pertinent papers, the term "benzalkonium" (FRANQUINET, 1981) changes to "benzalkonium (Digest-Eur, Eurobio)" in FRANQUINET & MARTELLY (1981) and later to "Digest-Eur" (FRANQUINET et al., 1985). The interpretation presented here is confirmed by the fact that the senior author discussed the discrepancies with Dr. Martelly, one of Franquinet´s co-workers, during a meeting in Graz in 1989. She remembered small vials as source for the "Digest-Eur" and somewhat later kindly sent a package of exactly those 10 ml vials with "10X Digest-Eur" that had been left over from the former experiments. FUKUSHIMA & MATSUDA (1991) report, indeed, correctly on the highly toxic effect of benzalkonium chloride. But apparently neither benzalkonium chloride nor strongly hyperosmotic conditions were really applied by FRANQUINET´s group.

With the exception of BETCHAKU (1967, 1970), previous attempts to cultivate planarian cells have used total cell suspensions. Under these conditions, the following survival periods were found: 3-15 days (MURRAY, 1927), 14 days (FRANQUINET, 1976), 2-3 weeks (TESHIROGI & TOHYA, 1988) and 4 days (FUKUSHIMA & MATSUDA, 1991). Purified neoblast fractions were cultivated only by BETCHAKU (1967), who arrived at a survival of 4 days at maximum. In no case has a standardized viability test been applied. The 14 weeks reported by SCHÜRMANN & PETER (1988) for primary cultures of neoblasts in the hypotonic (80 mOsmol/l) diluted Holtfreter´s medium (see BETCHAKU, 1967) refer to aggregates for which neither viability nor cell structure has been tested. Therefore, the 31 days reported here for primary cultures of purified neoblast fractions with a final viability of 46% in the isosmotic medium is the longest survival period found for neoblasts up till now, without differentiated cells and without microscopically visible signs of differentiation.

Further experiments with homologous matrix coatings should offer the possibility to simulate *in vivo* conditions on a less complex level by combining selected growth factors, tissue extracts and mitotically inactive cells with primary cultures of neoblasts, with the objective to study differentiation or stimulate proliferation *in vitro*. To promote mitoses, additional factors and / or the presence of differentiated planarian cells seem necessary (cf. BAGUÑÀ et al., 1989). The addition of X-irradiated planarian cells, possibly separated from the cultivated neoblasts by filters, and the use of homologous extracellular matrix components could bring improvements in this respect. Neoblasts form a heterogeneous pool (BAGUÑÀ, 1998). Selecting the true stem or progenitor cells among them should enhance the fraction of cells proliferating *in vitro*. For this purpose, additional criteria such as ultrastructural characters (RIEGER et al., 1999) and cytochemical data (BEHENSKY et al., 2001) may provide valuable tools to enrich special proliferative cells by appropriate separation techniques. Mere isotonicity might not be sufficient for optimal conditions. The available ion analyses refer solely to whole animals (PRUSCH, 1976). Determining the extracellular concentrations of inorganic ions and other components, for example by fluorimetric measurements following injections of appropriate dyes, may well supply essential data for a further improvement of culture conditions that might eventually result in an established neoblast line.

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Quantitative analysis of turbellarian cell suspensions by fluorescent staining with acridine orange, and video microscopy

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ABSTRACT. A combination of methods has been developed for analyzing cell suspensions from turbellarians with respect to cytochemical and morphometric parameters and with special emphasis on the characterization of neoblasts. Tissues were disintegrated by mechanical and enzymatic means. Neoblasts were separated and / or fractionated by different centrifugation protocols in Percoll density-gradients. Staining with acridine orange under conditions denaturing RNA but leaving intact double stranded DNA yielded green fluorescence of DNA and red phosphorescence of RNA. Both light emissions were documented and quantitative image analyses were performed. By computing the integral intensities of green and red light, quantitative measures were gained for DNA and RNA contents. By correlating these light emissions to each other or to cell size, histograms characteristic for given neoblast pools were obtained. The protocol described should facilitate monitoring the heterogeneity of the neoblast compartment as well as studying cellular dynamics during growth, regeneration and other physiological processes.

KEY WORDS: acridine orange, cell suspensions, disintegration of tissues, DNA, *Dugesia tahitiensis*, fluorescence microscopy, image analysis, *Macrostomum*, neoblast fractions, planarians, RNA, video microscopy, Platyhelminthes.

INTRODUCTION

Turbellarians are generally assumed to consist of two compartments from a cell biological point of view: a functional compartment made up of differentiated cells incapable of dividing and a proliferative compartment (BAGUÑÀ, 1998; BAGUÑÀ et al., 1994). In triclad planarians, the latter undergoes considerable variation depending on size, nutritional state and need for regeneration (BAGUÑÀ & ROMERO, 1981). It is built up by a relatively uniform cell type, mostly termed neoblast (BAGUÑÀ, 1981). A high nucleocytoplasmic ratio that may be estimated with phase contrast optics and a strongly basophilic cytoplasm with mitochondria, densely packed ribosomes but no further organelles are the main characteristics of the neoblast. There are various indications for the heterogeneity of this cell population that comprises true stem cells, progenitor cells and early stages of differentiating cells (for a review, see BAGUÑÀ et al., 1994). The number of methods available for analyzing this heterogeneity is rather limited. Their uniform appearance is an obstacle to distinguishing subtypes and finding the true stem cells among neoblasts. Among other criteria, cell kinetic data (BAGUÑÀ et al., 1989), behaviour in density-gradient centrifugation combined with morphological characters (SCHÜRMANN et al., 1998) and ultrastructural traits (HORI, 1992, 1997; RIEGER et al., 1999) have been used for substantiating this heterogeneity. Recent developments include, in addition, the application of bromodeoxyuridine (BrdU) labeling (LADURNER et al., 2000; NEWMARK & SÁNCHEZ ALVARADO, 2000) to follow cell proliferation and monitoring of *vasa* (*vas*)-related transcripts for distinguishing somatic stem cells from germ cells and differentiated somatic cells (SHIBATA et al., 1999). Whereas BrdU should turn out as a label universally applicable, the *vasa*-related genes refer to chromatoid bodies. These are documented well for triclads, but are absent, for example, in the Macrostomidae (RIEGER et al., 1999). It is still an open question if the corresponding gene is expressed in this case.

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A quantitative and relatively rapid cytochemical approach should greatly assist in subtyping neoblasts and monitoring variations within the neoblast pool. In a planarian, DNA amounts surpass the contents equivalent to the G1 phase only in these cells, and their ribosomal RNA forms a prominent fraction. Therefore, correlating DNA and RNA contents with each other and with cell size (volume) and other morphometric parameters such as circularity, elongation factor and circumference could form a basis for characterizing and monitoring the neoblast compartment. For this purpose, fluorescent staining of cell suspensions from *Dugesia tahitiensis* Gourbault 1977 has been combined with quantitative image analysis. Neoblasts from *Macrostomum* n. sp. were analyzed by the same methods.

MATERIAL AND METHODS

Animals

Specimens of *Dugesia tahitiensis* Gourbault 1977 (Turbellaria: Tricladida Paludicola) were taken from a laboratory stock derived from the original collection made for the description of this endemic and purely asexual species (GOURBAULT, 1977). *Macrostomum* n. sp. (Turbellaria: Macrostomorpha), belonging to the *M. tuba* group, was supplied by Prof. Dr. Reinhard Rieger, Innsbruck, who reared a laboratory stock from animals collected in the Adriatic sea near Lignano, Italy (RIEGER et al., 1999). Reagents were obtained from Sigma with a few exceptions cited in the following text. Whenever available, tissue culture grade reagents were applied.

Disintegration (maceration) of tissues

For a high yield of cell suspensions with a low percentage of aggregates (these impede monitoring individual cells in image analysis), former techniques applying a combined mechanical and enzymatic tissue disintegration (SCHÜRMANN et al., 1998) were modified with respect to the enzyme mix. Whole animals starved for one week and kept in 0.02% neomycin sulfate in culture water for at least 24 h were disintegrated in Dounce homogenizers with loosely fitting pestles. Tolerances of 50-70 µm (25-35 µm on each side) were found suitable for *D. tahitiensis* and *Macrostomum* n. sp. Disintegration was performed with ten strokes of the pestle in an ice bath. Isotonic media were applied for both species (see SCHÜRMANN & PETER, 2001, for *Dugesia*). In any case, glucose (10 mM) was added to support the survival of the cells. For *Dugesia*, a first incubation with DNAse (460 U/ml; Sigma DN-25) and hyaluronidase (2100 U/ml; Sigma type V, H-6254) lasted 10 min at 18 ºC. Then, collagenase (600 U/ml, Sigma type IV, C-5138) and its essential cofactor calcium chloride (0.6 mM in the assay) were added and the tissue was resuspended in the homogenizer using the pestle as described above. A further incubation (30 min, 18 ºC) was followed by the addition of EDTA (disodium salt, concentration of 2 mM in the assay), resuspension of cells in the homogenizer as above and an incubation period of 10 min at 18 ºC. DNAse hydrolyzed DNA from damaged cells that would otherwise clump the cells together. By the combined treatment with hyaluronidase and collagenase, the extracellular matrix was degraded sufficiently to avoid the formation of aggregates. For *Macrostomum* n. sp., a medium approximately isotonic to sea-water proved best. It consisted of 250 mM KCl, 250 mM NaCl, 10 mM HEPES buffer (pH 7.4), 2 mM EDTA (disodium salt), 10 mM glucose and 460 U/ml DNAse. Finally, the cell suspension was either washed (see below) and stained for fluorescence microscopy or filtered through a series of meshes with 40, 30, 20 and 15 µm mesh size for separating smaller cells, in particular neoblasts. For cells from *Macrostomum* n. sp., an 8µm net was added. In any case, debris was eliminated by one or several washing steps (centrifuge for 10 min at 500xg and 4 ºC and resuspend) before staining. All media for centrifugation contained 2 mM EDTA and 10 mM glucose. A change of medium was achieved by analogous centrifugation steps. Finally, the cell suspensions were diluted with the last washing medium to yield the desired cell density. By this method, cells with a high viability were obtained. Roughly 80% of the cells filtered through the last net were scored as live (green versus red fluorescence for dead cells) in the LIVE/DEAD Kit L-7013 of Molecular Probes (BELETSKY & UMANSKY, 1990; POOT, 1997). In a pilot experiment, maceration as described by BAGUÑÀ & ROMERO (1981) was combined with fluorescent staining. As cell membranes were permeabilized by this procedure, gradient centrifugation was not applicable.

Gradient centrifugation

Either total cell suspensions or neoblast sub-fractions were used for further analysis. To separate and fractionate neoblasts, one of the protocols described for centrifugation in Percoll gradients (SCHÜRMANN et al., 1998) or a newly developed *in situ* gradient may be used. To run this gradient, 2 ml of the final cell suspension ($2x10^6$ cells per ml or less) were mixed with 8 ml of Percoll solution (Pharmacia) to yield a final density of 1.08. Centrifugation (15-30 min, 32,000xg, 4 ºC) using an angle rotor (Beckman JA-21, 40º) resulted in the formation of a continuous gradient (1.02-1.10) and the simultaneous isopycnic separation of cells according to their buoyant densities. Fractions were collected after perforating the centrifuge tubes with a hypodermic needle, and the Percoll was removed by one to three washing steps (centrifugation at 500xg for 10 min at 4 ºC) with fivefold dilution with medium in each step.

Acridine orange staining

For fluorescent staining, acridine orange (Molecular Probes, high purity grade A-1301) was chosen as a dye intercalating into dsDNA, thereby showing enhanced

green fluorescence (λ_{max}=522 nm) upon excitation at a λ_{max}=502 nm and forming aggregates with polyanions such as ssRNA that emit red light (λ_{max}=638 nm) by phosphorescence. Under proper conditions, DNA is kept in the double stranded form, whereas double stranded RNA regions are denatured to single strands (DARZYNKIEWICZ & KAPUSCINSKI, 1990; WATSON, 1991). Several staining protocols, including such with previous fixation of the cells, were compared with each other (ROMEIS, 1989; TRAGANOS et al., 1977). A two-step equilibrium method with acridine orange present during observation and measurement proved superior, as photobleaching was avoided and the bright fluorescence persisted for at least 12 h when the slides were stored at 4 °C in a moist chamber. The treatment consisted in a detergent pretreatment (0.07% Triton- X100 in the assay at pH 3.0) followed by staining at pH 3.8 with a final dye concentration of approximately 40 µM, in the presence of EDTA and NaCl to selectively denature RNA (TRAGANOS et al., 1977, p. 47). Staining was performed in suspension. Thereafter, slides were prepared with the aid of a cytocentrifuge (Cytorotor in a Heraeus Megafuge 1.0) and viewed with a Leitz Aristoplan microscope equipped with epifluorescence, using the dual band filter XF 53 (Omega Opticals), with bandpasses from about 540-560 nm and 620-640 nm.

Microscopy and image analyses

For documentation and quantitative analysis, the images captured from the microscope by a 3CCD video camera (Hamamatsu C5810) were stored in a PC connected online to the camera. The variable integration time of the camera was set to yield maximal red and green intensities well below the maximum of 255 and thus lying within the linear range of the grey scale. With the image analysis software LUCIA 3.52a (Laboratory Imaging, Prague), red and green light intensities were integrated on a pixel basis and related to cell and nuclear sectional areas. These areas are projections of the largest extension of a cell and comprise all fluorescent (phosphorescent) light emitted from the whole cell or nucleus, respectively. They represent, therefore, the cell volume. The intensities of the red and green light emitted are proportional to the amount of nucleic acids and thus a relative measure for the DNA and RNA contents.

RESULTS AND DISCUSSION

Neoblasts were discerned by their narrow cytoplasmic rim appearing red, whereas the green fluorescence of nuclear DNA superimposed on the red light emitted by nuclear and overlying cytoplasmic RNA, resulting in a bright yellow. Neoblasts from both *Dugesia tahitiensis* and *Macrostomum* n. sp. (Fig. 1) showed the same staining patterns. Controls with the classical azure A – eosin B stain (PEDERSEN, 1959) resulted in similar pictures, with RNA stained intensely blue, whereas DNA was not stained under the conditions applied. Different cell sizes, distributions and intensities of red and green colour could be distinguished visually (Fig. 2) and characterized by quantitative video microscopy. Various staining patterns were obtained from somatic cells, either differentiated or in the course of differentiation (Fig. 3). These cells differed in their appearance from neoblasts. As they approached a spherical form upon isolation, they could not be classified with certainty.

A pilot study proved the same staining protocol to be applicable to macerated planarian tissues (BAGUÑÀ & ROMERO, 1981). Cells retained their characteristic shape in

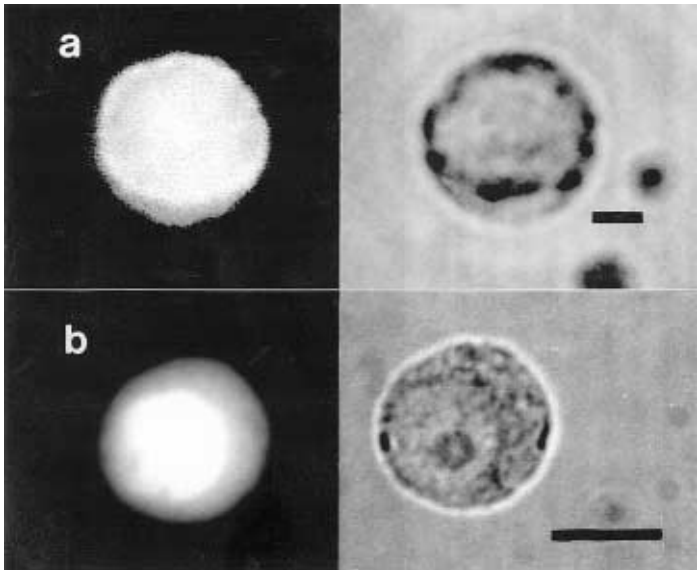

Fig. 1. – Neoblasts from a) *Dugesia tahitiensis*, b) *Macrostomum* n. sp., stained with acridine orange. The same cell is represented by its fluorescent image (left) and viewed with phase contrast optics at the same magnification (right); nuclear and cell borders in the phase contrast images coincide with the respective delineations seen through the fluorescence microscope. Scale bars: 3 µm.

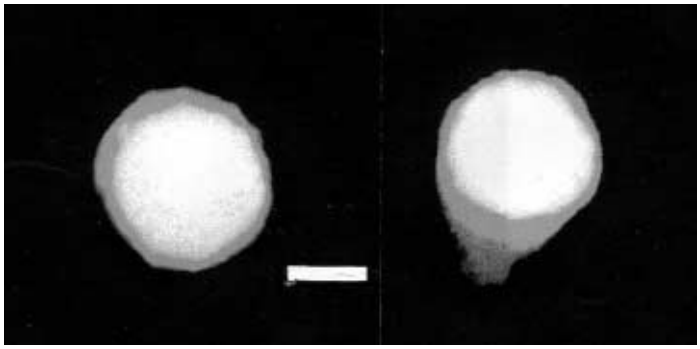

Fig. 2. – Two neoblasts from *Dugesia tahitiensis* differing in nucleocytoplasmic ratio, stained with acridine orange. The dark rim corresponds to the red area in the coloured image. Scale bar: 5 µm.

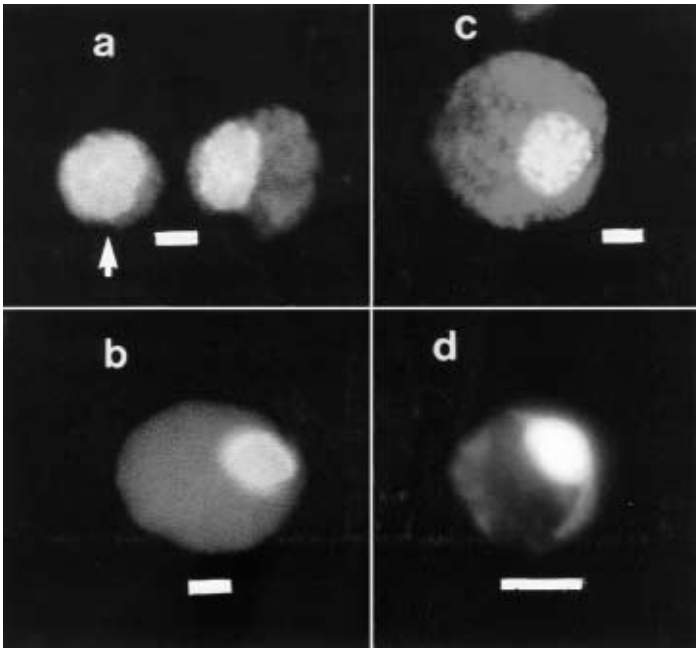

Fig 3. – Selected differentiation stages of turbellarian cells stained with acridine orange:
a) large neoblast (arrow) and differentiating cell of *Dugesia tahitiensis*;
b) differentiating cell of *Dugesia tahitiensis*, possibly similar to the "striped cell" described by BAGUÑÀ & ROMERO (1981);
c) differentiated cell of *Dugesia tahitiensis*, d) differentiated cell of *Macrostomum* n. sp. Figs (c) and (d) may represent gland or parenchymal cells.
Without characters other than size and staining pattern, all these classifications must remain tentative at present. Scale bars: 5 µm.

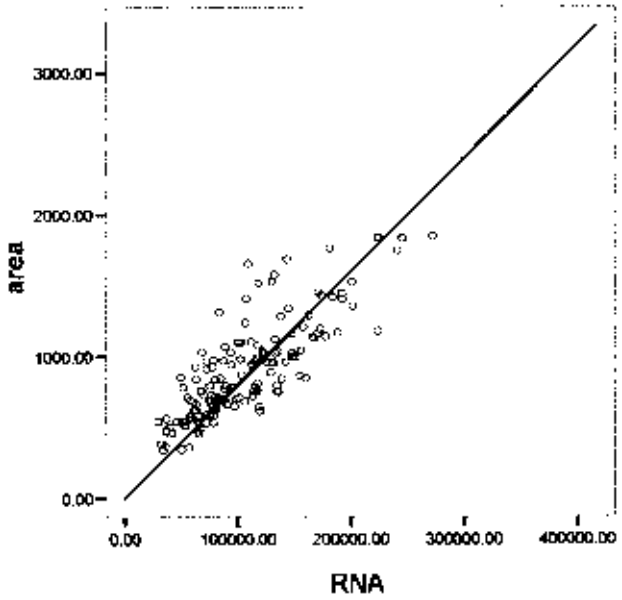

Fig. 5. – Scatter plot with linear regression of RNA contents of neoblasts from *Dugesia tahitiensis* (same disintegration as for Fig. 4) given in units analogous to those for DNA and calculated in a similar way versus total areas of cells in pixels (representing total cell volume).

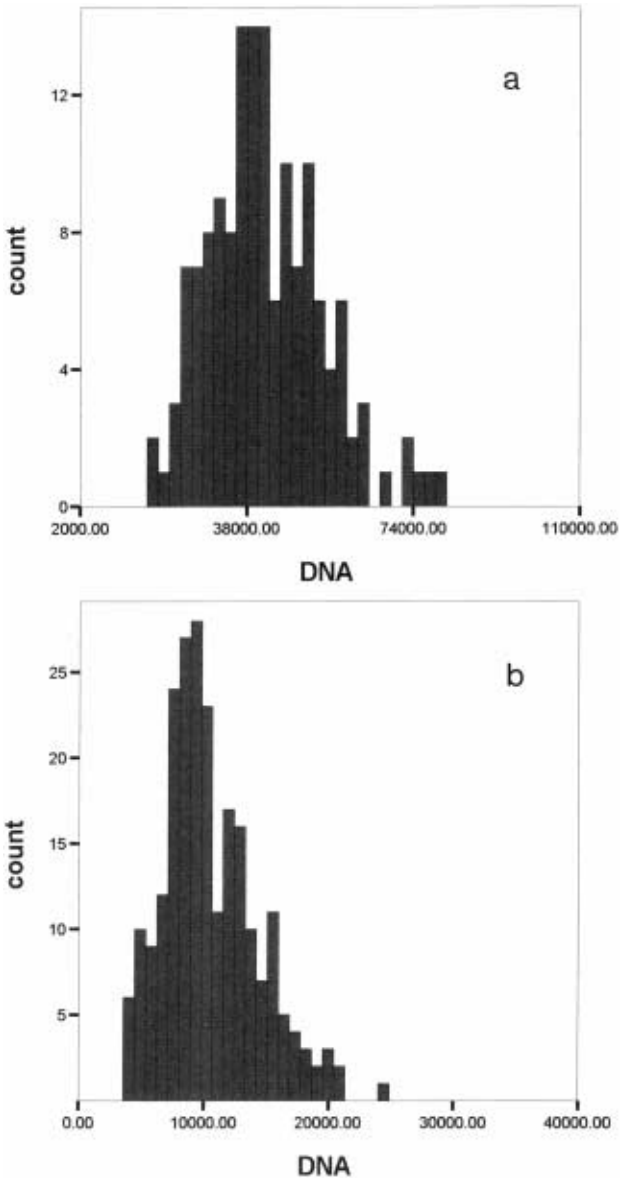

Fig. 4. – Histograms representing distributions of DNA contents for neoblasts of
a) *Dugesia tahitiensis* and b) *Macrostomum* n. sp. Abscissae: total green fluorescence as a measure for DNA contents, expressed in relative units calculated by multiplying the mean green values with the respective area in pixels. 5 specimens of *Dugesia* and approximately 100 individuals of *Macrostomum* were disintegrated and processed to yield these histograms.

this case. This may, in future, serve as a bridge for a reliable classification of cells different from neoblasts, solely on the basis of size and staining pattern. As gradient centrifugation requires intact cell membranes, the respective protocol was, however, not applied further in the present study.

First studies showed similar distributions of DNA contents (Fig. 4) for *Dugesia tahitiensis* and for *Macrostomum*, the latter with a lower DNA content, in accordance with the smaller neoblast size. These distributions met the expectations for proliferating cells: as cells in G1-phase contain half the DNA amount of G2 and mitotic cells, with those in S-phase ranging in between, a continuous distribution of DNA contents over a twofold range should be found. In fact, this range was somewhat larger. These findings are in agreement with related results for lymphocytes (see, for example, Watson, 1991) and may be explained by the influence that the accessibility of DNA exerts on staining intensity. This accessibility depends, in turn, on the cell cycle phase. Interestingly, RNA contents appear to increase proportionally with cell size (Fig. 5). The image analysis program package allows us to produce classifications based on fluorescence intensities and morphometric parameters, and to indicate fluorescence intensities in profiles drawn through cells.

Some caution in fine tuning the staining conditions has to be observed when analyzing cells different from neoblasts, as other polyanions may bind to acridine orange. Especially lysosomes may accumulate this dye (Darzynkiewicz & Kapuscinski, 1990). By constructing plots and histograms based on the analytical results, neoblast populations may be characterized and cellular dynamics monitored, as for example during regeneration and asexual reproduction of *Dugesia tahitiensis* or during growth and regeneration in *Macrostomum*. As differentiated cells possess a DNA amount corresponding to the G1 phase and are larger than neoblasts, they should easily be discriminated by image analysis. In a similar way, various lymphocyte populations have been monitored by flow cytometry (Watson, 1991: 246-259).

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Production of intestine-specific monoclonal antibody and interspecific cross-reaction in triclads and polyclads

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ABSTRACT. We have produced a monoclonal antibody (mAb②2B6) specific to intestinal and epidermal cells of *Dugesia japonica*. This mAb②2B6 also reacted with the secretory granules of a kind of sub-epidermal gland cells, epithelial cells and gland cells in the pharynx. The results of the interspecific cross-reaction tests with four other freshwater triclads and four polyclads showed that this mAb②2B6 can be used as a marker, specific to only intestinal cells in both *Planocera multitentaculata* and *Planocera reticulata* of polyclads.

Immuno-electron microscopical research revealed that the cytoplasm of phagocytic cells and the spherical large granules in granular clubs reacted strongly to this mAb in the intestine. Also, mAb positive material seemed to be absorbed by pinocytosis from phagocytic cells into the granular clubs. These observations support the theory that the granular clubs are protein reserve cells, not gland cells for intraluminal digestion.

KEY WORDS: intestine, monoclonal antibody, interspecific cross-reaction, Platyhelminthes.

INTRODUCTION

The intestinal cells of triclads and polyclads consist of two kinds of cells, the phagocytic cell and the granular club. Although some intestine-specific monoclonal antibodies have been produced in freshwater triclads (SHIRAKAWA et al., 1991; ROMERO et al., 1991; SHINOZAWA et al., 1995), the function of the granular club remains obscure. In other orders of Turbellaria, mAb has not been produced. To further clarify the function of intestinal cells in Turbellaria, we tried to produce another intestine-specific monoclonal antibody and examined the localization of this antigen. The interspecific cross-reactions for four freshwater triclads and four polyclads were also examined.

MATERIAL AND METHODS

Animals

Specimens of *Dugesia japonica* Ichikawa & Kawakatsu, 1964 were of the GI strain given by Himeji Institute of Technology. *Dugesia ryukyuensis* Kawakatsu, 1986 was of the OH strain collected from Okinawa Island and strained in Hirosaki University. *Phagocata vivida* (Ijima & Kaburaki, 1916) and *Seidlia auriculata* Ijima & Kaburaki, 1916 were collected from Mt. Iwaki, near Hirosaki. *Bdellocephala brunnea* Ijima et Kaburaki, 1916 was collected from a spring in Hirosaki. *Notoplana humilis* (Stimpson, 1857) and *Pseudostylochus intermedius* Kato, 1939 were collected from the Natsudomari Peninsula, Aomori Pref. *Planocera reticulata* (Stimpson, 1855) and *Planocera multitentaculata* Kato, 1944 were collected from Fukaura, Aomori Pref.

Immunotechnique

The monoclonal antibody was produced by injecting dissociated cells of regenerating *D. japonica* three days after cutting, as antigens, into the mice of the BALB/c strain. The injected dissociated cells were small cells obtained after filtration with a stainless steel mesh of 200 μm, a nylon mesh of 30 μm and a nylon mesh of 20 μm in order of pore size. The procedure for immunization, production of mAb and assay for antibody followed that described in a previous paper (SHIRAKAWA et al., 1991). The ABC method (HSU et al., 1981) was applied to paraffin sections of planarian tissues (fixed in

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6% formalin in a one-quarter strength PBS) to examine the interspecific cross-reaction. After the immunostaining, a counterstaining with Meyer's Haematoxylin was performed. In negative controls, the primary antibody was replaced by mouse myeloma ascites or cloning medium S-Clone SF-B (Sankou Junyaku Co.).

Post-embedding staining using colloidal gold for immuno-electron microscopy followed FAULK & TAYLOR (1971). Specimens were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 2 h at 4°C. After being washed in buffer, they were dehydrated in graded ethanols and embedded in LR-White. Thin sections on nickel grids were rinsed with PBS and 1% BSA for 30 min. The sections were incubated for 3-4 h with primary antibody, washed with PBS, then incubated for 2 h with gold-labelled goat anti-mouse antibody IgG+M 15 nm (Zymed) diluted 1:30 in 0.1% BSA-PBS. The sections washed with PBS and distilled water were finally stained with uranyl acetate and lead citrate and observed with a JEM-1210 electron microscope. In negative controls, the primary antibody was replaced by mouse myeloma ascites diluted 1:1000 in PBS.

RESULTS

We have produced a monoclonal antibody, mAb②2B6, specific to intestinal and epidermal cells of *D. japonica*. The isotype was found to be an IgG1 (light chain is *k*) with a mouse-monoclonal isotyping kit (Amersham Co.).

This mAb②2B6 also reacted with a kind of sub-epidermal gland cells, epithelial cells and gland cells in the pharynx of *D. japonica*. The results of the interspecific cross-reaction tests with four other freshwater triclads and four polyclads are shown in Table 1. This antibody did not show an obvious positive reaction in any of the intestines of the other four freshwater triclads, though it reacted with the epidermis of each one. Furthermore this antibody also showed a reaction with the nervous system of *S. auriculata* and *B. brunnea*. The eyes, in particular, showed a strong positive reaction. This antibody also showed positive reaction with the intestinal cells of three kinds of polyclads. Particularly in *P. multitentaculata*, only intestinal cells reacted strongly and negative control showed no reaction (Fig. 1).

Immuno-electron microscopical observation in *D. japonica* showed that the cytoplasm of phagocytic cells and the spherical granules in granular clubs reacted strongly to this mAb in intestine. mAb positive vacuoles scattered in the cytoplasm of phagocytic cells seemed to be absorbed by pinocytosis from phagocytic cells into the granular clubs (Fig. 2). In phagocytic cells, not only these vacuoles but also the matrix of the cytoplasm showed a strong reaction. In granular clubs, a strong reaction was

TABLE 1

Cross reaction tests of mAb②2B6 prepared against *D. japonica* for freshwater triclads and polyclads.

	int.	epi.	s.g.	n.t.	pha.	par.
Tricladida						
Dugesiidae						
Dugesia japonica	++	++	++	-	+	-
Dugesia ryukyuensis	±*	+	+	-	+	-
Planariidae						
Phagocata vivida	-	+	-	-	-	-
Seidlia auriculata	-	+**	+	+	+	-
Dendrocoelidae						
Bdellocephala brunnea	±*	+	+	++	+	-
Polycladida						
Leptoplanidae						
Notoplana humilis	-	-	-	-	-	-
Planoceridae						
Planocera reticulata	+	-	-	-	-	-
Planocera multitentaculata	++	-	-	-	-	-
Diplosoleniidae						
Pseudostylochus intermedius	+	+	+	-	+	+

int., intestine; epi., epidermis; s.g., sub-epidermal gland cell; n.t., nervous tissue; pha., pharynx; par., parenchyma

* Only the surface of intestinal cells showed a weak reaction.

** Only ventral epidermis of head region showed a positive reaction.

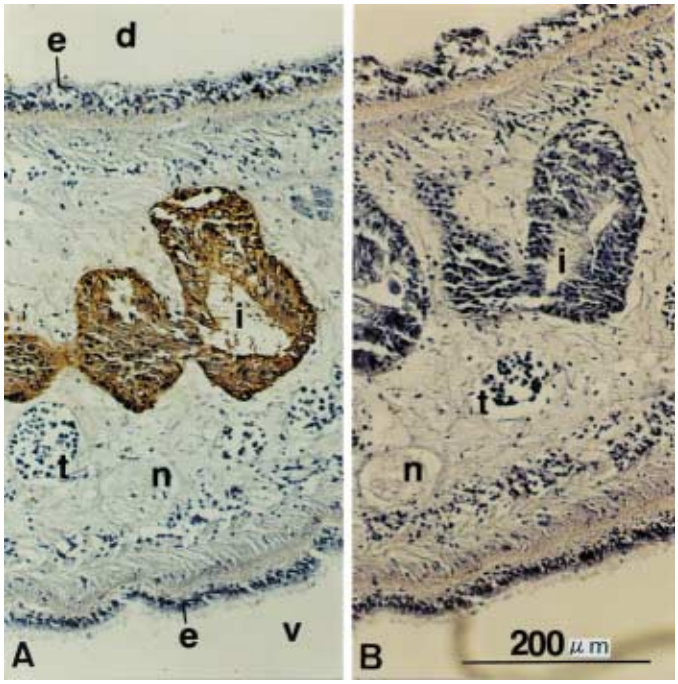

Fig. 1. – Cross-reaction test of mAb②2B6 against freshwater triclad *D. japonica* for polyclad *P. multitentaculata*.
A, Intestine (i) showing intense positive reaction (brown) for this mAb by ABC method. B, Negative control. No reaction is seen. d, dorsal; e, epidermis; n, nerve; t, testis; v, ventral. A and B at the same magnification.

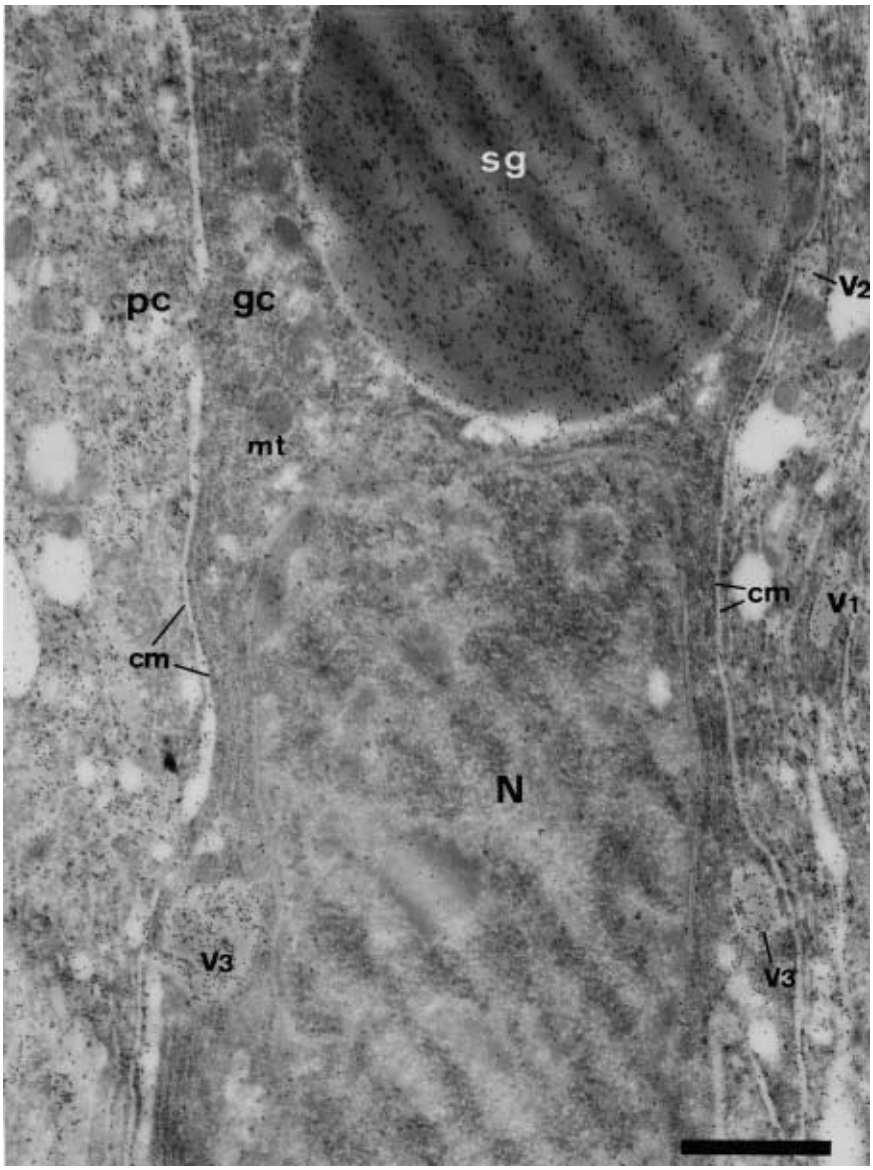

Fig. 2. Immuno-electron micrograph of intestinal cells of *D. japonica* by colloidal gold labeling.
The cytoplasm of phagocytic cell (pc) and spherical large granule (sg) in granular club (gc) react strongly to this mAb. This picture also shows the process of the mAb positive material being absorbed by pinocytosis. Vacuole 1 (V1) is still in phagocytic cell. The limited membrane of vacuole 2 (V2) is fused with the cell membranes (cm) and the contents are being absorbed into the granular club. Vacuoles 3 (V3) have been already absorbed into the granular club (gc). N, nucleus; mt, mitochondria. Bar shows 1 μm.

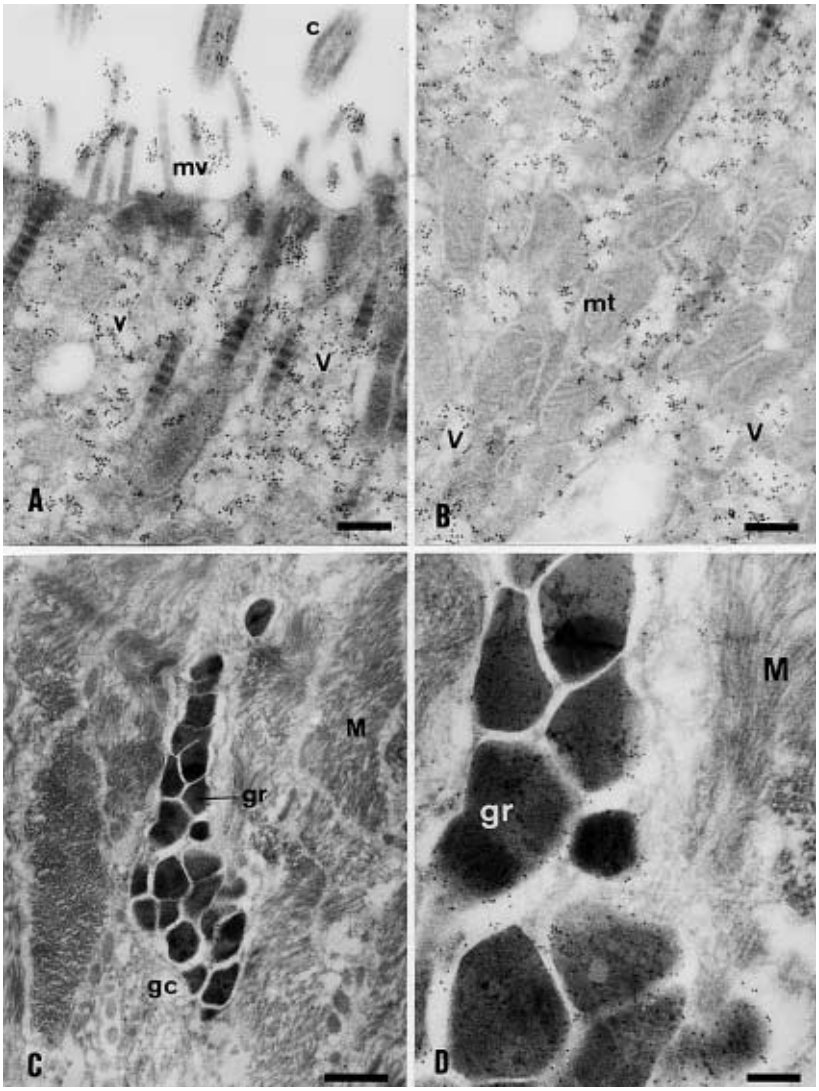

Fig. 3. Immuno-electron micrographs of mAb positive-reaction cells of *D. japonica*. A, upper part of epidermal cell. B, middle part of epidermal cell. C, sub-epidermal gland cell (gc) and muscle cells (M). D, enlarged secretory granules (gr). Gold particles were found on the microvilli (mv), cilia (c) and vacuoles (v) in epidermal cell and on the secretory granules (gr) in the sub-epidermal gland cell. mt, mitochondria. Bars of A, B and D show 250 nm. Bar of C shows 1 μm.

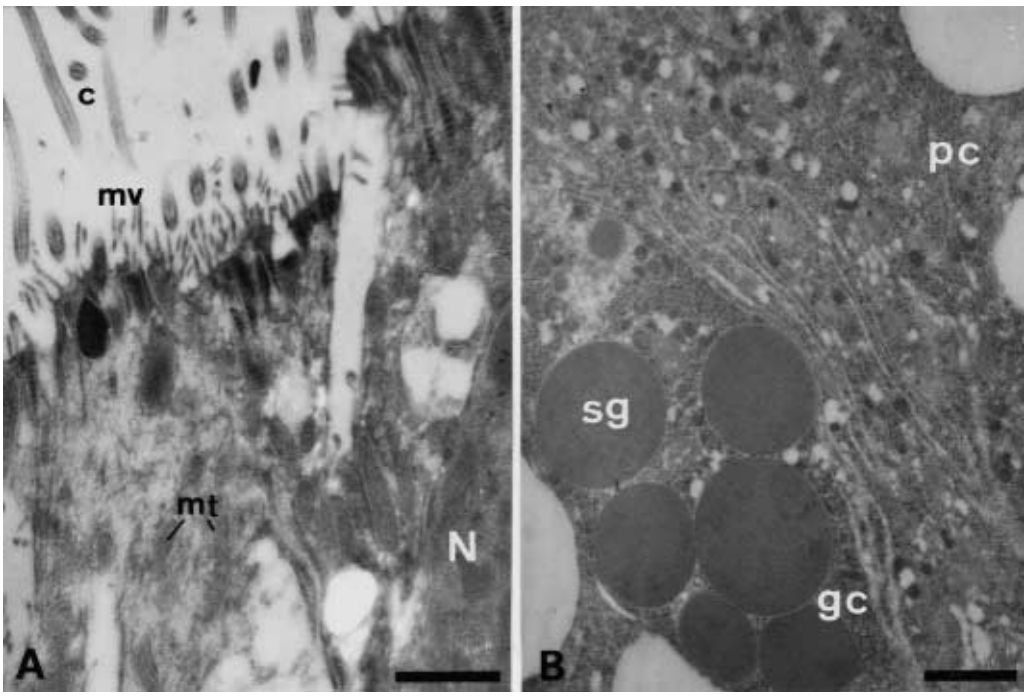

Fig. 4. Immuno-electron micrographs of negative controls. A, epidermis. B, intestine. Few gold particles were found on the both tissues. c, cilium; gc, granular club; mt, mitochondria; mv, microvilli; N, nucleus; pc, phagocytic cell; sg, spherical granule. Bar of A shows 1 μm. Bar of B shows 10 μm.

observed in the vacuoles absorbed by pinocytosis in addition to the spherical large granules (Fig. 2).

In epidermal cells, microvilli, cilia and vacuoles regarded as epitheliosomes (RIEGER et al., 1991) positioned in the upper half of the cells, showed positive reaction to this mAb (Fig. 3, A, B). In sub-epidermal gland cells, secretory granules reacted strongly to this mAb (Fig. 3, C, D).

In negative controls, few gold particles were found on the epidermal cells and two kinds of cells in intestine (Fig. 4).

DISCUSSION

mAb③12B-D reported by SIRAKAWA et al., (1991) reacted strongly and specifically to the gastrodermal and epidermal cells of *Phagocata vivida*. The isotype was found to be an IgG3 and the localization of the antigen was in lipid droplets in phagocytic cells (unpublished data). Thus it was found that mAb②2B6 is different from mAb③12B-D. The localizations of other intestine-specific mbs have not been examined.

The antigens of this mAb②2B6 were distributed in the epidermal cells of the four species examined, though only the ventral epidermis of the head region showed a positive reaction in *S. auriculata*. This results show that a common antigen (epitope) of freshwater triclads is included in the epitheliosomes scattered throughout the epidermis of each species, and that the antigenic determinant is also included in the cells of glands, nervous tissue and the pharynx in some species. The positive reaction of microvilli and cilia on the epidermal cells would be due to the emission of the antigenic determinant from epidermal cells and sub-epidermal gland cells. As the cells injected as the antigen were small cells filtered with nylon mesh of 20μm in pore size, we think that most of the cells were epidermal cells. The results of cross-reaction tests may also support our speculation. It is interesting that the antigenic determinant, including in the epidermal cells of *D. japonica*, also exists in the intestinal cells of polyclads, though *N. humilis* does not have the same antigen. In both *P. multitentaculata* and *P. reticulata*, as this mAb reacts only with intestinal cells, mAb②2B6 can be used as a useful antibody to examine the function of the intestine, and it can also be used as a marker to monitor cellular behavior. In *P. intermedius*, many tissues except nervous tissue reacted to this mAb. We can not use this mAb as a cell marker in this species, because we could not get a single band in western blot analysis of this species (data not shown). In seven other species, this mAb showed no reaction to the parenchyma, so it may be used as a non-mesenchymal cell marker.

The granular clubs in the intestine are filled with spherical granules. The function of this cell remains obscure, and two theories have been presented. One theory is that the cells are protein reserve cells (WILLIER et al., 1925; and others). The other theory is that they are gland cells secreting enzymes for extracellular digestion (JENNINGS, 1974; and others). Our immuno-electron microscopical observation supports the theory that the granular clubs are protein reserve cells. Because the spherical granules and the vacuoles absorbed by pinocytosis from phagocytic cells showed strong positive reaction to the mAb②2B6, it is a possibility that the spherical granules act as a reserve for the protein transported from phagocytic cells.

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SHORT NOTES

Gradients and regeneration: the case of TNEX59 in the planarian *Girardia tigrina* (Platyhelminthes, Tricladida)

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During development, multicellular organisms determine and then differentiate the distinct regions that constitute the body's architecture. These regions are established and controlled by a number of molecules, including nuclear factors, that drive the organism from the egg to its final shape. We studied the molecules involved in the regionalisation of the freshwater planarian body (Platyhelminthes, Turbellaria, Tricladida). These organisms are known for their great power of regeneration and their ability to grow or degrow depending on environmental conditions (1). Using a monoclonal antibody (MAb) from a planarian-specific MAbs library (2), we identified TNEX59, a molecule detected in a regional fashion. Here we present the preliminary results on its localition in intact and regenerating organisms.

The specimens used in this study belong to an asexual race of *Girardia tigrina*. The MAbs used were obtained following standard procedures (3) with some modifications (4). Immunohistochemistry on sagittal paraffin sections was performed as described in (4), and Adobe Photoshop was run to determine pixel intensity of nuclear staining.

TNEX59 localisation in intact organisms

TNEX59 is a nuclear protein detected mostly in mesenchymal cells in a distribution gradient, with a higher proportion of nuclei with the faintest signal located in the central body region (Fig. 1A). However, a few cells located within the epidermis are stained with the MAb-recognising TNEX59.

To explore the gradient of TNEX59, the stained nuclei of sagittal sections were analysed. Five areas along the antero-posterior (A/P) axis were selected (Fig. 1A), and staining of their nuclei was quantified relative to the darker nuclei of each area.

Stained nuclei were classified in three categories, according to the intensity of staining: Type I nuclei (mild dark), Type II nuclei (dark) and Type III nuclei (deep dark). The plot of the percentage of nuclei for each staining category and for each area analysed shows that (Fig. 1B): (a) the most central areas have more Type I nuclei (mild dark); (b) the most anterior (head) and posterior (tail) areas have more Type III (deep dark) nuclei; and (c) Type II nuclei are equally distributed along the A/P body axis.

It can be deduced from these data that the gradient is due to the distribution of Type I and III nuclei along the A/P body axis. Two hypotheses can account for this gradient: (a) different categories of nuclei correspond to different cell types, and the gradient depends on the distribution of these cell types along the A/P body axis; (b) The gradient is independent of the cell types and depends merely on the respective position of mesodermal cells along the A/P body axis. Immunostaining on pla-

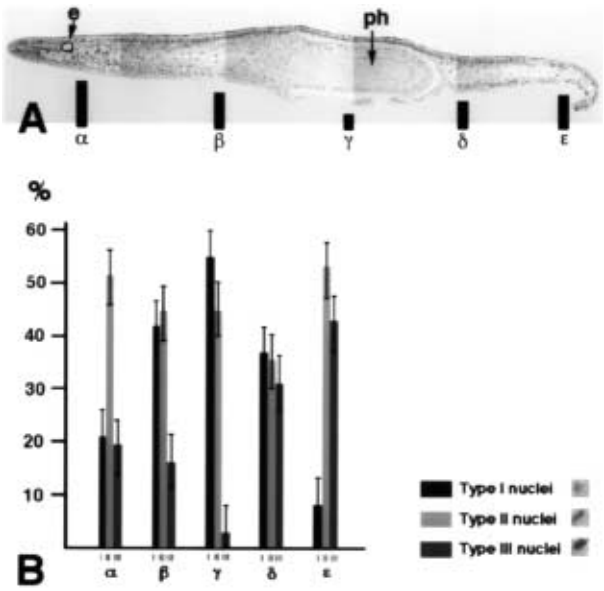

Fig. 1. – A). Intact adult planarian immunostained with TNEX59. Sagittal section. Anterior is to the left, and dorsal to the top. Real size: 8mm. Areas of nuclei quantification are marked. B) Plot of the percentage of nuclei for each staining category and for each area analysed. Several sections of three specimens were examined. Error bars represent SD (SD = 7.5). Abbreviations: e, eye; ph, pharynx.

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narian macerated cells (work in progress) would help to answer this question.

TNEX59 localisation and dynamics in regenerating organisms

The dynamics of TNEX59 localisation during regeneration were analysed in regenerating tails kept at 17±1ºC (organisms cut at postpharyngeal level expected to regenerate new central and anterior regions). In brief:

1st day of regeneration

TNEX 59 is located in the nuclei of blastema cells early in regeneration, as soon as the blastema can be identified, and the pattern of expression is re-established, although transiently (Figs 2A,B). Surprisingly, most nuclei of epithelial cells express TNEX59, in contrast with intact adult organisms.

3rd day of regeneration

TNEX59 expression fades in most nuclei of epithelial cells, as in intact adult organisms. The accumulation of highly stained morphologically undifferentiated cell nuclei in the central area that will generate the pharynx primordium (5) attenuates the gradient.

5th day of regeneration

The nuclei of pharynx primordium cells are strongly stained with the MAb recognising TNEX59 (Fig. 2C), which reduces the gradient.

7th day of regeneration

TNEX59 expression in the nuclei of pharyngeal cells fades. The A-P gradient is definitively re-established.

From these complex dynamics of expression, several characteristics should be highlighted:

(1) The A/P gradient is transiently re-established as early as day 1 of regeneration. This suggests that this factor is an early activator of the re-establishment of the planarian body pattern and supports the hypothesis that the gradient is independent of the cell type and depends on the position of mesenchymal cells along the A/P body axis.

(2) At 1-2 days of regeneration, the nuclei of epithelial cells transiently express TNEX59, perhaps reflecting a general territorial reorganisation, as shown by use of other molecular markers in the same species (TCEN49 [6]; GtPOU-1 [7]).

(3) The strong TNEX59 staining of the nuclei of pharynx primordium cells at 3-5 days of regeneration parallels the strong staining of the apical regenerative blastema, suggesting a similar origin for both structures. In a broad sense, the pharynx primordium can be considered an inner blastema, as both structures are formed by accumulation of undifferentiated but probably committed neoblasts. When the new pharynx starts its maturation, TNEX59 expression fades. The strong TNEX59 staining in the nuclei of both blastema and pharyngeal primordium cells may also suggest that TNEX59 is a regulative factor overexpressed in differentiating cells.

In summary, TNEX59 is a nuclear factor that may be involved in the A/P patterning of mesenchymal cells.

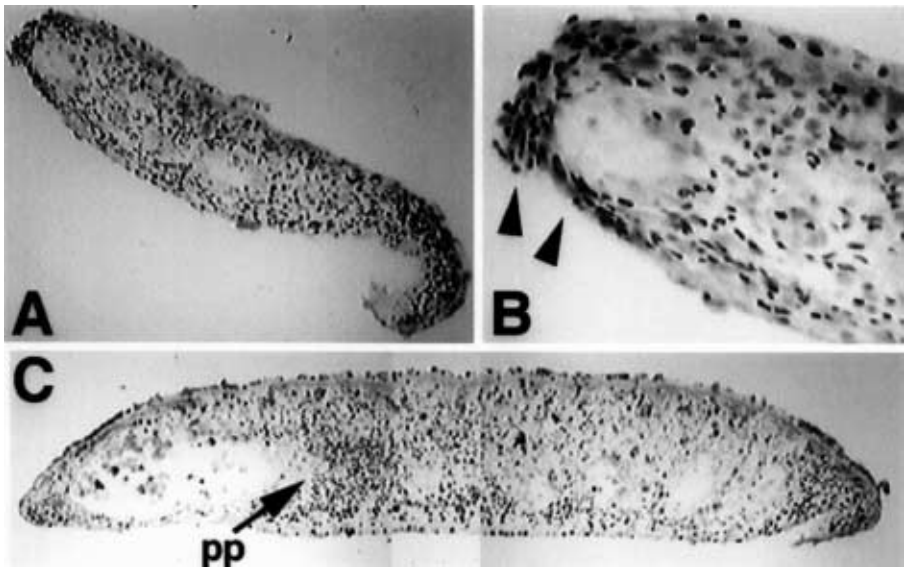

Fig. 2. – Regenerating planarians cut at postpharyngeal level immunostained with TNEX59. Sagittal sections. Anterior is to the left, and dorsal to the top. A) 1st day of regeneration. B) Magnification of the anterior part of A). Note that darker nuclei affect both the blastema and the postblastema (arrowheads). Also note that most nuclei from the epidermis are reactive to TNEX59. C) 5th day of regeneration. Note that nuclei from pharynx blastema cells are strongly stained (arrow). Abbreviations: pp, pharynx primordium.

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Effects of FMRFamide-related peptides and Neuropeptide F on planarian regeneration (Platyhelminthes, Tricladida)

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Due to their high regenerative ability, planarians are model organisms for studying mechanisms of morphological and functional restoration. Attempts to isolate natural endogenous substance(s) that regulate planarian regeneration have been unsuccessful.

Recently, four native FMRFamide-related peptides (FaRPs: GNFFRFa, GYIRFa, YIRFa, RYIRFa) and neuropeptide F (NPF) have been identified within the taxon Platyhelminthes (1). Although the function of these peptides is unknown, they have been found to have concentration-dependent excitatory effects on muscle-strip preparations and on isolated muscle fibres in several flatworm species (1). The presence of GYIRFamide- and NPF-immunoreactivity has been reported in the central and pharyngeal nervous system of the freshwater planarian, *Girardia tigrina* (Girard 1850) (2,3).

The aim of this investigation was to study the effects of exogenously applied neuropeptides (NPF, GYIRFamide and FMRFamide) on the development of nervous system elements and on the restoration of pharyngeal function in the course of regeneration of *G. tigrina*. Two aspects of regeneration were examined: (i) the regeneration of cerebral ganglia in decapitated planarians, and (ii) the restoration of pharyngeal function in cephalic and caudal body fragments. An immunocytochemical study of the influence of NPF (1 μM) on regeneration was performed on wholemount preparations. Five regenerating planarians (at 20° C) from experimental and control groups were flat fixed on Days 1, 1.5, 2, 2.5, 3, 4 and 7 after decapitation and examined for NPF immunoreactivity (IR; for details see (4)). The primary antiserum used was raised in rabbits against the C-terminal decapeptide of NPF (FAIIGRPRF). Demonstration of the muscle system was achieved using TRITC-conjugated phalloidin. Preparations were examined with a Leica TCS NT confocal scanning laser microscope.

Abundant NPF-IR was found in the cerebral ganglia, main nerve cords, pharyngeal nervous system and in the peripheral nerve plexuses of intact planarians. In both experimental and control animals, fine NPF-IR fibres from the subepithelial nerve net appear between days 1-1.5 in the blastema. Day 2 specimens displayed accumulation of NPF-IR in the cut ends of the "old" nerve cords and in the subepithelial nerve net. At day 2-2.5, nerve fibres start to grow into the blastema from the main cords and by day 3 they start to form a delicate arch (the new ganglion) and muscles appear in the centre of the blastema. At day 4 the blastema elongates and becomes triangular as the new ganglion develops. During cephalic regeneration very rapid development of NPF-immunopositive nerves occurs between days 1 and 3. Comparison of the control and experimental animals revealed that NPF (1 μM) stimulates formation of the head blastema. Differences were observed in early stages of the regeneration (1 to 2.5 days post-decapitation).

Analysis of the functional role of neuropeptides was carried out using the restoration of pharyngeal function in cephalic and caudal body fragments. Observations on pharynx regeneration were based on the appearance of the food response (for details see (5)). NPF, GYIRFamide and FMRFamide were added to the media of experimental groups (50 and more animals) and at days 5-12 after pharynx removal the planarians were offered food. The results were analysed using the χ^2 test for alternative distributions. The cumulative number of functioning pharynxes by each day of regeneration was compared in experimental and control groups.

Pharyngeal regeneration in planarian fragments occurs between days 6 and 11. In most animals the pharynx was restored by day 8. In caudal fragments exposed to NPF (1 μM) pharyngeal regeneration was more rapid than that of the control group. FMRFamide (1 μM) stimulated pharyngeal regeneration in cephalic (data not shown) and caudal fragments. GYIRFamide (1 μM) had no significant effect on the process. The positive control experiment with the mixture of amino acids (corresponding to FMRFamide, 1 μM) had no effect on regeneration.

The results indicate that NPF and FaRPs influence the regeneration of nerve elements and the restoration of pharyngeal function. The results are preliminary. Further investigation of the relationships between peptides and the regeneration process is subject of future research and publication.

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Modulation of regeneration of planarians *Dugesia tigrina* (Platyhelminthes, Tricladida) by weak combined magnetic field

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Regenerating planarians were used as a test-system to study the biological effect of weak combined magnetic fields (CMF) (1,2) tuned to parametric resonance for calcium (Ca^{2+}-CMF) or potassium (K^{+}-CMF) ions. To understand the mechanism(s) underlying the effects of CMF, we studied the joint action of CMF and exogenous serotonin, a well-known activator of regeneration.

The experiments were performed with an asexual laboratory race of the planarian *Dugesia tigrina*. The regeneration was initiated by amputation of a head body part containing the cephalic ganglion. Experimental groups were exposed to the Ca^{2+}-CMF or K^{+}-CMF for 72 hours at room temperature in the absence or in the presence of 10^{-6} M serotonin added immediately after sectioning. The control group and the planarians regenerated in the presence of exogenous serotonin alone were placed in the local geomagnetic field (B_{DC}=40.5 μT) at room temperature.

Two different types of CMFs consisting of collinear static (B_{DC}) and alternating ($B_{AC} x \cos 2\pi f$) components, where B_{DC} and B_{AC} - magnetic flux densities, were used: a) CMF tuned to parametric resonance for Ca^{2+}[1,2]: B_{DC}=40.5±0.1μT, B_{AC}=74.5±3.0μT, f_{AC}=31.0±0.1 Hz., and b) CMF tuned to parametric resonance for K^{+}[1,2]: B_{DC}=40.5±0.1μT, B_{AC}=74.5±3.0μT, f_{AC}=47.7±1.0 Hz (the 3d harmonic of the basic frequency).

The mitotic index of cells obtained from the post-blastema region 72 hours after sectioning was determined by counting the metaphases labelled with Hoechst-33342. The metaphases were arrested by adding 0.05% colchicine just after sectioning.

The quantitative estimation of blastema growth by the third day of regeneration was performed using vital computer morphometry based on *in vivo* visualisation of a border between old (pigmented) and new (transparent) body parts. The system of on-line computer image analysis with special software was used to calculate the average ratio (n=30) of blastema area to the whole body area (G=s/S) as a quantitative parameter of growth during regeneration.

A comparative study of mitotic activity of neoblasts and blastema growth in exposed and control animals revealed that regeneration could be either stimulated or inhibited by Ca^{2+}-CMF or K^{+}-CMF respectively. Exposure to Ca^{2+}-CMF accelerated the regeneration of planarians as followed from an increase in mitoses number by 35±5%, and growth of the blastema by 30±5%. In contrast, K^{+}-CMF suppressed blastema growth and mitotic activity by 25±5 %.

Addition of 10^{-6} M serotonin accelerated blastema growth by 30±6% estimated on the third day of regeneration. Similar increase by 30±5% have been obtained after 3 days of exposure of regenerating planarians to Ca^{2+}-CMF. Both factors applied together stimulated the growth of blastemas by 45±5% (Fig. 1). At the same time, serotonin applied together with K^{+}-CMF prevented K^{+}-CMF-induced suppression of regeneration (Fig. 2). The non-additive character of joint action of CMF and serotonin points to similar steps in the pathways by which these two factors affect the regeneration. It is known that serotonin stimulates DNA synthesis through activation of adenylate

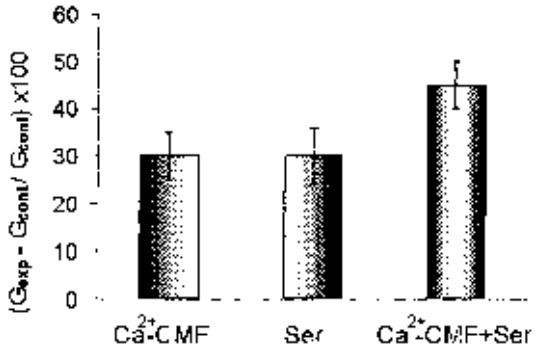

Fig. 1. – The co-operative effect of Ca^{2+}-tuned combined magnetic field (Ca^{2+}-CMF) and serotonin (Ser, 10^{-6} M) on blastema growth by the third day of regeneration. The values represent mean ± S.E.M. of five experiments as % to control ($P< 0.005$). $G_{exp,}$ G_{cont} - relative area (s/S) of blastema in experimental and control planarians.

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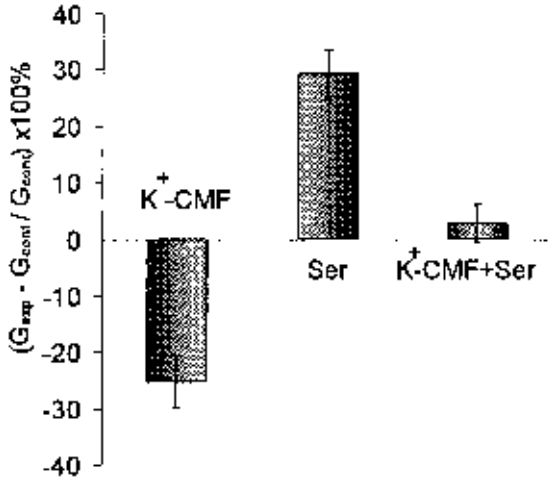

Fig. 2. – The co-operative effect of K^+-tuned combined magnetic field (K^+-CMF) and serotonin (Ser,10^{-6} M) on blastema growth by the third day of regeneration. The values represent mean ± S.E.M. of four experiments as % to control ($P< 0.005$). $G_{exp,}$ G_{cont} - relative area (s/S) of blastema in experimental and control planarians.

cyclase signalling pathway (3) via G-protein-linked receptors (4,5). Moreover, Moraczewski et al. (6) have shown activation of both Ca^{2+}- and cyclic AMP-dependent pathways during regeneration; activation of protein kinase C, a key enzyme of phosphoinositide pathway, preceded activation of adenylate cyclase. LEDNEV (1,2) proposed that Ca^{2+}-dependent kinases are possible targets for weak magnetic fields, and demonstrated this directly using the reaction of myosin phosphorylation in solution as a test system (7). CMF may, probably, affect regeneration by modulating protein kinase C activity. The revealed character of the joint action of serotonin and CMF is indirect evidence in confirmation of this suggestion.

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MORPHOLOGY AND ULTRASTRUCTURE

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Degenerating epidermal cells in *Xenoturbella bocki* (phylum uncertain), Nemertodermatida and Acoela (Platyhelminthes)

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ABSTRACT. Species of the Nemertodermatida and Acoela have a mode of withdrawing and resorbing worn ciliated epidermal cells, through the epidermis and into the gastrodermal tissue. The ultrastructure of these degenerating epidermal cells has been described from very few species, especially concerning the Nemertodermatida. New data are presented here from *Nemertoderma bathycola*. Studies of the body wall of the enigmatic *Xenoturbella bocki* revealed darkened, shrunken cells with epidermal-type cilia enclosed in a vacuole. These cells were found in basal parts of the epidermis and within gastrodermal cells. The cells, or remains of them, were more dissolved in structure the further into the body they were observed. The process of resorbing ciliated epidermal cells in *X. bocki* is essentially similar to that found in the Acoela and Nemertodermatida, thus supporting the hypothesis of a close relationship.

KEY WORDS: Ultrastructure, Metazoan phylogeny, *Xenoturbella*, Platyhelminthes, Acoela, Nemertodermatida, epidermis.

INTRODUCTION

A characteristic feature of members of the platyhelminth taxa Nemertodermatida and Acoela is the mode of internally withdrawing and digesting worn or damaged ciliated epidermal cells. Already in the late 19th century, researchers studying acoels noted peculiar rounded cells with cilia enclosed in a vacuole (e.g. GEDDES, 1879). The nature of these cells has been a matter of debate for a long time. It took over a century, and the advent of electron microscopical methods, until the issue was settled (TYLER et al., 1989; EHLERS, 1992). Putative degenerating epidermal cells have been reported from light microscopical studies of many acoel species (see references in EHLERS, 1992), and from one nemertodermatid, i.e. *Meara stichopi* Westblad, 1949 (see Westblad´s description of the species, plate I, fig. 4). From electron microscopical studies (TEM), degenerating epidermal cells have been reported from three acoel species; *Symsagittifera roscoffensis* (Graff, 1891) by DOREY (1965), *Convoluta pulchra* Smith and Bush, 1991 by TYLER et al. (1989) and *Anaperus tvaerminnensis* (Luther, 1912) by EHLERS (1992). SMITH et al. (1986) mentioned an observation of degenerating epidermal cells in a nemertodermatid specimen similar to the species *Sterreria psammophila* (STERRER, 1970). However, the only TEM micrographs of degenerating epidermal cells in a nemertodermatid published to date are from *Meara stichopi* (LUNDIN & HENDELBERG, 1996).

The degenerating epidermal cells in acoels and nemertodermatids are withdrawn straight through the body wall into the digestive tissue. The actual mechanism responsible for this movement is yet unknown. Autolysis of the cell body often begins already at the level of the epidermal surface, when the cells shrink or are being compressed. The basal portion of the cell with the nucleus becomes compressed at an early stage and is thus hard to recognize. The epidermal cilia and the rigid interconnected ciliary rootlet system withstand degeneration for a longer time (for nemertodermatids this also includes the thick terminal web) and are discernible even within the digestive tissue. When the degenerating cell sinks into the epidermis, the cilia become enclosed in a vacuole. Inside the vacuole the cilia retain some ability to move, at least in the acoels, hence the earlier designation “pulsatile bodies” of degenerating epidermal cells. The sunken degenerating cells most often attain a tilted position, more or less perpendicular to the vertical orientation of the ordinary ciliated cells in the epidermis. The degenerating cells

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always lie in between other cells, without anchoring structures to the membranes of the surrounding cells. When the degenerating cells reach the digestive tissue, the vacuole around the cilia eventually becomes fragmented and disappears, followed by an accelerated autolysis of the cilia.

Degenerating epidermal cells have been suggested as one of the few possible synapomorphic characters for the Nemertodermatida and Acoela (LUNDIN, 1997), supporting the hypothesis that the two groups are sistertaxa forming the Acoelomorpha (sensu EHLERS, 1985). Some of the other possible synapomorphies concern characters of the epidermal ciliary apparatus. However, several of the characters of the ciliary apparatus are also present in *Xenoturbella bocki* Westblad, 1949, an enigmatic vermiform animal whose systematic affinities are disputed (see LUNDIN, 1998, ISRAELSSON, 1999, and references therein). The present study describes degenerating epidermal cells in *Xenoturbella bocki*. Also presented here are the results of a search for degenerating epidermal cells in two species of the genus *Nemertoderma*.

MATERIAL AND METHODS

Specimens of *Nemertoderma bathycola* Steinböck, 1931 and *Nemertoderma westbladi* Steinböck, 1938 were collected in 1997 and 1998 near Kristineberg Marine Research Station on the Swedish west coast. The specimens were fixed with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, followed by postfixation in 1% osmium tetroxide and further preparation with standard methods for transmission electron microscopy. Embedded material of *Xenoturbella bocki* Westblad, 1949, collected in 1976 near Kristineberg Marine Research Station, was kindly provided by Dr. Jan Hendelberg. For the examinations a Zeiss CEM 902 was used, located at the EM Department of the Anatomical Institution, Göteborg University.

ABBREVIATIONS

c	cilia
cr	ciliary rootlets
f	fibrillar extracellular matrix
m	mitochondria
n	nucleus
t	terminal web

RESULTS

In the specimens of *Nemertoderma bathycola* sunken, dark ciliated epidermal cells were very scarce in the epidermis and gastrodermis (Fig. 1 A). These cells correspond well to degenerating epidermal cells reported from other species of the Nemertodermatida and Acoela. No degenerating epidermal cells were found in the studied specimens of *Nemertoderma westbladi.* The degenerating cells of *N. bathycola* were tilted and situated in between other cells, without any visible attachment structures to the lateral cell membranes of the surrounding cells. The basal parts (with the nucleus) of the degenerating cells were greatly reduced in size. The ciliary rootlets of these cells were embedded in a compressed, electron-dense terminal web closely associated with an aggregation of mitochondria beneath it (Fig. 1 A). In the epidermis, the cilia of the degenerating cells were enclosed in a large vacuole. In degenerating cells observed within the gastrodermis, the vacuole around the cilia appeared fragmented (Fig. 1 A, arrowheads), and the cilia showed indications of autolysis.

In *Xenoturbella bocki,* a few sunken, tilted ciliated epidermal cells were observed, occurring in the basal part of the epidermis and of the gastrodermis (Fig. 1 B-D). These cells had cilia and ciliary rootlets of the same type as those found on the epidermal surface. The cilia were enclosed in a large vacuole. In such degenerating cells located basiepidermally (Fig. 1 B, C) there was a central electron-dense region, with what appeared like a compressed cytoplasm and a nucleus. Surrounding the central region was a peripheral area, with less dense cytoplasm and scattered mitochondria. The degenerating cells were situated in between other epidermal cells, without visible cell junctions to the surrounding cells. The intercellular space between the cells was filled with the fibrillar components of the extracellular matrix. In the gastrodermis, remnants of the degenerating cells were found only within large gastrodermal cells (Fig. 1 D). The engulfed degenerating cells showed a high degree of autolysis, and the former vacuole around the ciliary axonemes had vanished. The axonemes were electron-dense, and numbers and patterns of the ciliary axonemes varied greatly as a result of the breakdown of ciliary structure. In the most advanced stages of degeneration observed, the bulk of the degenerating cells began to take on a granulated appearance (as partly visible in Fig. 1 D, arrowheads).

DISCUSSION

The structure of degenerating epidermal cells in specimens of *Nemertoderma bathycola* is similar to that of the nemertodermatid *Meara stichopi* (cf. LUNDIN & HENDELBERG, 1996), with a thick terminal web underlined by an aggregation of mitochondria. Note that in *M. stichopi* the cilia are often, but not always, cast off at the epidermal surface before the cell begins to be withdrawn (LUNDIN & HENDELBERG, 1996). Where the cilia are retained, the degenerating epidermal cells of *M. stichopi* appear almost identical to those of *N. bathycola*. Concerning the apparent absence of degenerating epidermal cells in *N. westbladi*, the process of replacing epidermal cells is not a permanent one, and when present the degenerating cells may nevertheless be scarce. They are often absent from many individuals of acoel and nemertodermatid species from which degenerating epidermal cells have been reported (cf. EHLERS, 1992; own observations of *M. stichopi*). Degenerating cells would probably have

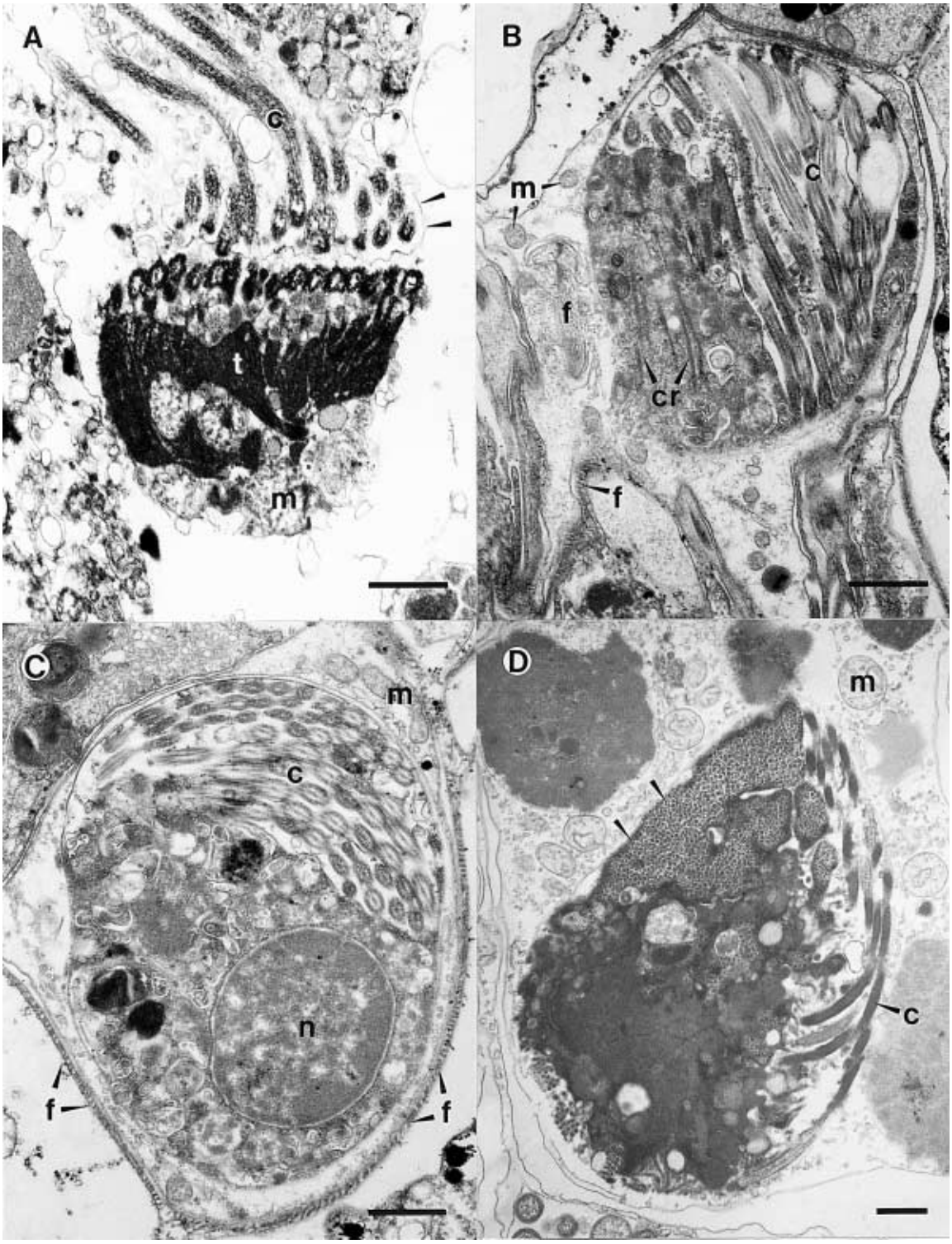

Fig. 1. – Degenerating ciliated epidermal cells of *Nemertoderma bathycola* (A) and *Xenoturbella bocki* (B-D). – A. *N. bathycola*. Degenerating epidermal cell located in the gastrodermal tissue, at 100 µm from the epidermal surface. The cell lies between the gastrodermal cells. The vacuole enclosing the cilia has been fragmented (arrowheads) and the cilia are in a late stage of autolysis. The terminal web (*t*) is underlain by an aggregation of mitochondria (*m*). – B. *X. bocki*. Degenerating epidermal cell located in the basal part of the epidermis, about 150 µm from the epidermal surface. The cilia are enclosed in a vacuole. Note ciliary rootlets (*cr*). Possibly the degenerating cell is enclosed in another cell, as indicated by surrounding unaltered cytoplasmic space with mitochondria (*m*). Fibrillar components of the basal matrix can be seen at the periphery (*f*). – C. *X. bocki*. Basiepidermally located degenerating epidermal cell. Note nucleus (*n*). Fibrillar components of the basal matrix surround most of the cell. – D. *X. bocki.* Degenerating epidermal cells found within a large gastrodermal cell in the gastrodermal tissue. The vacuole around the cilia has vanished and the cilia are darkened from extensive autolysis. The bulk of the degenerating cell has begun to attain a granular appearance (arrowheads) as a final step of the autolysis. TEM. All scale bars: 1 µm.

been found in *N. westbladi* if a larger number of specimens had been investigated. The presence of degenerating epidermal cells in specimens of *N. westbladi* has been confirmed by Ulrich Ehlers (personal communication).

The electron-lucent peripheral areas of the cytoplasm (Fig. 1 B, C) could perhaps imply that the degenerating cells are engulfed by other cells already in the epidermis, but no other structures indicating this were observed. So far, no explanation can be given for the mechanism behind the inward migration of the degenerating cells, or how they pass through the basal lamina in *X. bocki*. In the nemertodermatids and acoels, the extracellular matrix is strongly reduced or absent. EHLERS (1992) discussed the possibility that the mode of internal withdrawal and digestion of epidermal cells could occur in other flatworm groups (rhabditophorans and catenulids) and metazoans as well, but have not yet been detected because the epidermal cells become too strongly diminished when they pass through the basiepidermal layer of extracellular matrix. The presence of degenerating cells in the gastrodermis of *X. bocki,* despite a well-developed basal lamina, speaks against this notion. Based on available ultrastructural data, the mode of withdrawal and digestion of epidermal cells probably represents a unique, homologous character for species of the Nemertodermatida, Acoela and *X. bocki*.

Apart from the degenerating epidermal cells, acoels, nemertodermatids and *X. bocki* also share several distinct characters of the complex ciliary apparatus, suggesting a close relationship. On the other hand, there are molecular and structural indications that *X. bocki* is a strongly modified protobranch bivalve (NORÉN & JONDELIUS, 1997; ISRAELSSON, 1997, 1999). No specific similarities with the ciliary apparatus of *X. bocki* have, however, been found in the Protobranchia, nor in any other of the major molluscan taxa (LUNDIN & SCHANDER, 1999, in press a, b, unpublished studies). A recent study of Bock´s and Westblad´s old microscope slides revealed no morphological trace that would support a bivalvian nature or the existence of a pericalymma-like larva of *X. bocki* (Gerhard Hazsprunar, personal communication).

The apparent incongruence between different character sets concerning the phylogenetic affinities of *Xenoturbella* could be resolved by the hypothesis given recent support by Hox-gene data (BALAVOINE, 1998), that the flatworms arose by progenesis from an interstitial larva of a coelomate with a biphasic life-cycle (RIEGER, 1994; TYLER, 2000). This ancestral coelomate could possibly have been related to *X. bocki*. A problem with this comparison is that the assumed larva of *X. bocki* is relatively advanced in structure. If *X. bocki* really undergoes a far-reaching structural reduction during its development, the evolutionary events leading to this state are nevertheless enigmatic. Usually such a reduction is caused by small size or parasitic life-style, neither of which applies to *X. bocki*. Maybe there were intermediate interstitial (i.e. flatworm-like) larval stages that now are lost.

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I thank Prof. Ulrich Ehlers for sharing his knowledge of the presence of degenerating epidermal cells in *X. bocki*. Ms. Gunnel Bokhede at the EM Department of the Anatomical Institution in Gothenburg assisted with preparations for the TEM. Financial support was received from the Swedish Natural Science Council, the Royal Society for Arts and Sciences in Göteborg, the Helge Axson Johnsons foundation and the Royal Swedish Academy of Sciences (Lars Hierta).

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Ciliopharyngiella intermedia (Plathelminthes, Neoophora) – enigmatic as ever? Ultrastructural features and phylogenetic implications

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ABSTRACT. Ultrastructural features of the epidermis, gonads and photoreceptors are presented. The following features of *Ciliopharyngiella intermedia* are considered to represent autapomorphies of this species or of the taxon *Ciliopharyngiella*: the special design of egg shell-forming granules in vitellocytes, the pattern of marginal granules in germocytes, an intercentriolar body with strong striated supporting beams, cilia with a pair of rootlets in young spermatids, as well as intracerebral photoreceptors with both ciliary and rhabdomeric light-sensing organelles arising from the same cell. Based on present information it is hypothesized, that *Ciliopharyngiella* represents the sister taxon of the Eulecithophora (= Prolecithophora + Rhabdocoela).

KEY WORDS: Platyhelminthes, *Ciliopharyngiella intermedia*, ultrastructure, phylogenetics.

INTRODUCTION

The taxon *Ciliopharyngiella* comprises the species *C. intermedia* Ax, 1952 and *C. constricta* Martens & Schockaert, 1981. Based on anatomical characteristics, i.e. organisation of female gonads and the brain, *C. intermedia* was first ascribed to the Proseriata (see AX, 1952, p. 303ff), albeit with some hesitancy. Later the species was assigned to the "Typhloplanoida" (Rhabdocoela) because of the organisation of the male system and the pharynx, but considered to represent the most basal taxon of all rhabdocoels (see discussion in EHLERS, 1972, p. 71ff).

Meanwhile there exists a wealth of ultrastructural data giving reason to question whether *Ciliopharyngiella intermedia* belongs to the Rhabdocoela (see EHLERS, 1984, 1985; BRÜGGEMANN, 1986; SOPOTT-EHLERS, 1997a, 1999). This study presents some submicroscopic features that lead to a better understanding of the phylogenetic relationships of this taxon.

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MATERIAL AND METHODS

Material derives from sand samples gathered from the type locality (island of Sylt, North Sea). EM – preparation followed conventional steps (see SOPOTT-EHLERS, 1999). Serial sections of four specimens were examined using a Zeiss EM 10B and a Zeiss EM 900 electron microscope.

RESULTS AND DISCUSSION

Epidermis

The epidermis of *C. intermedia* consists of cuboidal, multiciliary cells with intraepithelial nuclei. Clearly marked microvilli project from the surface membrane. Cilia show a short rod-like main rootlet pointing rostrally and a long and slender vertical rootlet. Ultrarhabdites closely beneath the apical membrane as well as processes of true lamellate rhabdite glands are missing.

The apical portion of epidermal cells is studded with secretory vesicles (epitheliosomes) (Fig. 1 A). This layer is similar to those found in Prolecithophora, some dalyellioid and typhloplanoid species (see BEDINI & PAPI, 1974; TYLER, 1984; EHLERS, 1985; RIEGER et al., 1991). This is to say, in respect to the epidermis, *C. intermedia* shares a feature with representatives of some Eulecithophora (= Rhabdocoela + Prolecithophora), but there are no epider-

mal features typical for representatives of the taxon Proseriata.

Female and male gonads

Two strings of vitellarian follicles are differentiated in the prepharyngeal region. These yolk-producing segments of the female gonad are not enwrapped by a sheath of tunica cells (see SOPOTT-EHLERS, 1997a). Cytoplasmic differentiations such as lipid droplets and yolk deposits do not show any peculiarities. Egg-shell forming granules, however, are of special appearance. Electron-lucent material forming islets of different shape is embedded in the osmiophilic polyphenolic ground substance (Fig. 1 B). This is to say, the substructure of eggshell granules does not show a regular pattern, i.e. a meandering pattern, a design of concentric rings or a mosaic pattern as known for other neoophoran species.

Contrary to the vitellaria, the germaria are enclosed by tunica cells (see SOPOTT-EHLERS, 1997a). Mature germocytes do not basically differ in their inclusions from other neoophoran female generative cells. The most conspicuous inclusions, the marginal (cortical) granules, however, differ strongly from known data. Granules are about 1.5µm in diameter and show electron dense material interrupted by electron-lucent substances deposited in winding profiles appearing like a labyrinth (Fig. 1 C). Marginal granules of a substructure as found in *C. intermedia* are unknown for Seriata, Rhabdocoela and Prolecithophora (see GREMIGNI, 1988; LUCCHESI et al., 1995; GREMIGNI & FALLENI, 1998).

Since neither the design of eggshell-forming granules nor the pattern of marginal granules in *C. intermedia* corresponds to features typical of Seriata or of representatives of the Eulecithophora (= Prolecithophora + Rhabdocoela including the Temnocephalida and Neodermata), these characteristics are hypothesized as autapomorphic features of *C. intermedia* or of the taxon *Ciliopharyngiella* (see SOPOTT-EHLERS, 1997a,b; 1999).

The unpaired testis is not enclosed by a layer of tunica cells. Somatic tissue such as gut tissue lies directly adjacent to male generative cells, not even separated by ECM.

Cytogenesis of male gametes is fairly synchronized. Therefore, only preliminary data on the development could be obtained. Nuclei of the rosette stage show evenly distributed chromatin with a few dense islets. These nuclei develop two tip-like dilatations. Between these tips the intercentriolar body is located. It consists of several discs different in electron density. Well developed cross-filaments with split ends and delicate striation form supporting beams extending between the peripheral and central pair of dark plates (Fig. 1 D,E). Bundles of microtubules encircle the basal bodies inserting on the intercentriolar body (Fig. 1 E). The cytoplasm surrounding this organelle is scattered with dense particles, presumably chromatoid bodies. A pair of short rod-shaped rootlets originates close to each basal body. One rootlet of each pair runs to the tip of the nucleus, the other to the cell membrane (Fig. 1 E,F).

The fine structure of mature male gametes corresponds to the pattern of sperm cells typical of free-living Trepaxonemata: filiform in shape, two free cilia of the 9+"1" axonemal pattern, dense bodies, numerous mitochondria, a sheath of cortical microtubules and a thread-like nucleus.

Near the functional frontal tip of the spermatozoa the cortical microtubules leave their peripheral course and turn to the interior in irregular lines (Fig. 2 A-C). At the rear end microtubules form two semicircles enclosing the hind end of the nucleus (Fig. 2 D). In the most caudal tip of the spermatozoa just a single microtubule is seen. Ciliary basal bodies are weakly expressed and surrounded by just a delicate layer of dark material. Special applications such as dense caps or spur-shaped dark appositions do not exist. The transition zone between basal bodies and axonemata has a small diameter (Fig. 2 A, B, G).

Roundish electron-dense as well as grayish dense bodies (Fig. 2 E, F), both with granular structure, extend over two-thirds of the length of the spermatozoa. The first dense body appears in front of the insertion of the cilia (Fig. 2 C). Neither dense bodies nor mitochondria are arranged in a special pattern. Big elongated mitochondria (Fig. 2 F, I) lying slightly staggered to each other, show almost the same extension as the dense bodies. Therefore, different numbers of mitochondrial profiles are seen in transverse sections. The nucleus passes nearly through the whole length of the spermatozoa except for the frontal tip and the very hind end.

While mature spermatozoa correspond to the basic pattern of Trepaxonemata, some features just present during spermiogenesis do not fit the basic pattern. There is first of all an intercentriolar body equipped with strongly developed supporting beams, a pair of striated rootlets inserting on each basal body, and microtubules surrounding the intercentriolar and basal bodies.

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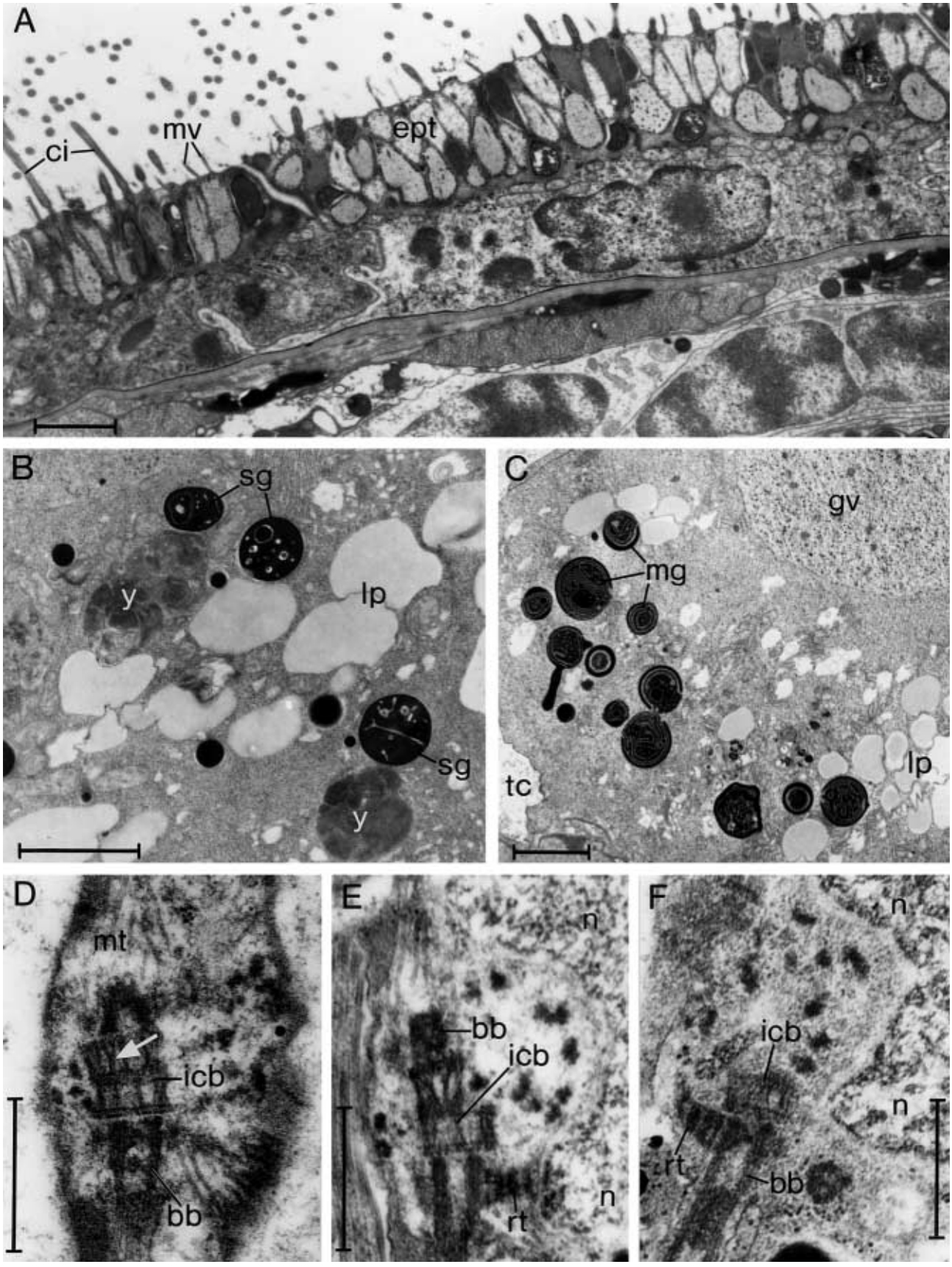
Fig. 1. A. Epidermis with epitheliosomes (ept), microvilli (mv) and cilia (ci). Scale = 2 µm. B. Segment of a vitellocyte. lp – lipid droplet; sg – egg shell-forming granule; y – yolk platelet. Scale = 2µm. C. Segment of a germocyte. gv – germinal vesicle; lp – lipid droplet; mg – marginal granule; tc – tunica cell; . Scale = 2 µm. D-F. Sections of young spermatids. D. Intercentriolar body with split, striated supporting beams (small arrow), basal bodies and microtubular bundles. bb – basal body; icb – intercentriolar body; mt – microtubules. Scale = 0.5 µm. E. Rootlet extending to a tip of the nucleus. bb – basal body; icb – intercentriolar body; n – nucleus; rt – rootlet. Scale = 0.5 µm. F. Rootlet running to the surface membrane of a spermatid. bb – basal body; icb – intercentriolar body; n – nucleus; rt – rootlet. Scale = 0.5 µm.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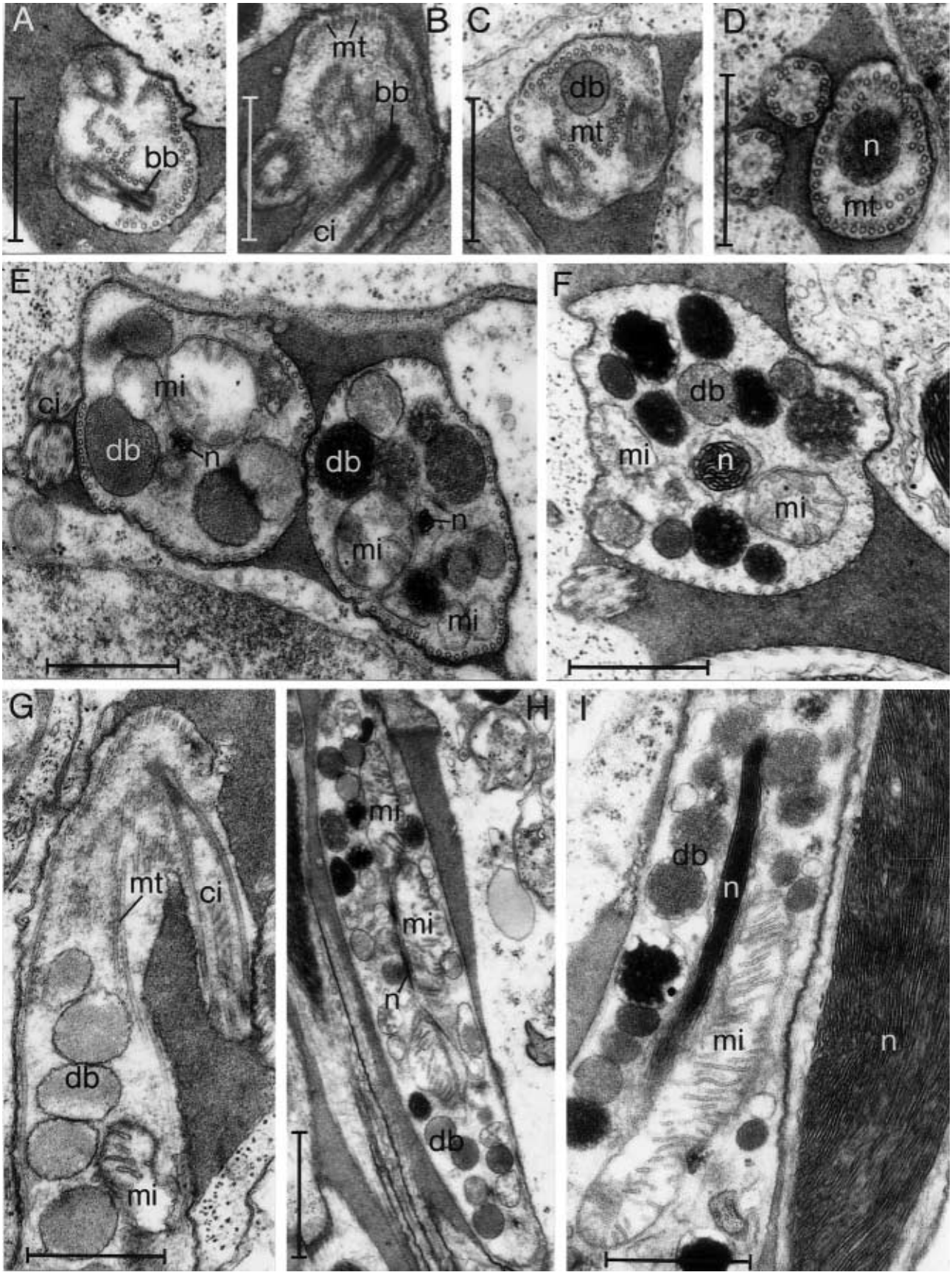
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An intercentriolar body of the type as found in *C. intermedia* has hitherto not been reported for any other species of the Plathelminthes and is hypothesized as an autapomorphic feature of this species or the taxon *Ciliopharyngiella*. Residual rootlets extending towards the apical cell membrane are found in several taxa (see SOPOTT-EHLERS & EHLERS, 1986), and structures similar to an intercentriolar body as found in *Jensenia angulata* (see SOPOTT-EHLERS, 1997b) might also correspond to these residual rootlets. A pair of clearly differentiated rootlets originating from each basal body, however, is unknown for any other taxon of the Plathelminthes. This feature is also considered as an autapomorphy of *C. intermedia*. But, compared to other taxa a pair of rootlets might be a plesiomorphic condition, and residual rootlets a more derived characteristic.

The features, intercentriolar body with strongly developed striated supporting beams and a pair of rod-shaped rootlets originating from each basal body, are hypothesized as autapomorphic features of *Ciliopharyngiella intermedia.*

Bundles of microtubules attached to the basal bodies of young spermatids are not widespread among the Plathelminthes. This feature has been found in the dalyellioid *Jensenia angulata* (see SOPOTT-EHLERS, 1997b) and in a triclad and in a fecampiid species (see i.al. WATSON & ROHDE, 1993; ROHDE & WATSON, 1995). Since data on microtubules inserting on the basal bodies are very sparse, it appears premature to discuss their phylogenetic implication. Furthermore, it cannot be excluded, that these organelles appearing during spermiogenesis, are very short-term transient structures and are therefore rarely reported.

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Fig. 2. A –C. Transverse sections through functional fore ends of spermatozoa . A, B. Insertion of cilia with basal bodies accompanied by dense material. bb – basal body; ci – cilium; mt – microtubules. Scales = 0.5 µm. C. Anterior dense body on the level of the insertion of cilia. db – dense body; mt – microtubules; Scale = 0.5 µm. D. Rear end of a male gamete with posterior segment of the nucleus surrounded spirally by microtubules. mt – microtubules; n – nucleus. Scale = 0.5 µm. E-F. Transverse sections through median segments of spermatozoa. ci – cilia; db – dense bodies; mi – mitochondria; n – nucleus. Scales = 0.5 µm. G-I. Longitudinal sections of spermatozoa. G. Frontal end with a cilium leaving the sperm body, dense bodies and anterior mitochondrion. ci – cilium; db – dense bodies; mi – mitochondrion; mt – microtubules. Scale = 0.5 µm. H. Anterior-median segment with mitochondria arranged staggered to each other, dense bodies and nucleus. db – dense bodies; mi – mitochondria; n – nucleus. Scale = 1 µm. I. Posterior-median segment with long vermiform mitochondrion, dense bodies and nucleus, at the right a rear end containing the nucleus only. db – dense bodies; mi – mitochondrion; n – nucleus. Scale = 0.5 µm.

Photoreceptors

C. intermedia possesses two different kinds of submicroscopic light-sensing organs, five pairs of circumcerebrally located ciliary aggregations and one pair of intracerebral photoreceptors. Ciliary aggregations are presumed light-perceiving organs consisting of a single cell having an intracellular lumen into which axonemata of modified cilia project (Fig. 3 A). Photoreceptors of this type are widespread within representatives of Rhabditophora and do also exist in a representative of the Prolecithophora (see PISKUREK et al., 1998). These differentiations are in all instances combined with fibrous capsules of the brain. Ciliary aggregates are considered either as a plesiomorphic feature or as having evolved more than ones (for ref. see SOPOTT-EHLERS, 1999, 2000).

The intracerebral photoreceptors are built up by an unpigmented mantle cell enveloping a single sensory cell each. Outfoldings of membranes of three modified cilia (see SOPOTT-EHLERS 1999, p.490 fig. 11) and evaginations of the surface membrane of the sensory cell constitute the light-sensing elements (Fig. 3 B). Photoreceptors of this mixed type with both, ciliary and rhabdomeric photoreceptive organelles arising from the apical surface of the same cell, have hitherto not been reported for any other representative of the Plathelminthes. On the contrary, visual cells showing a striking similarity to the ones observed in *C. intermedia* are known for photoreceptors occurring on esthetes of Polyplacophora (for ref. see EERNISSE & REYNOLDS, 1994). This, however, is surely a convergence. The intracerebral photoreceptors of *C. intermedia* are hypothesized as an autapomorphorphic feature of this species or of the taxon *Ciliopharyngiella* (see discussion in SOPOTT-EHLERS, 1999).

CONCLUDING REMARKS

Most of the ultrastructural data presented in this contribution – i.e. egg shell-forming granules of vitellaria, marginal granules in germocytes, submicroscopic anatomy of the intercentriolar body and a pair of rootlets per spermatid cilium, and special intracerebral photoreceptors – are considered as autapomorphic features of the species. These characteristics as well as unpublished findings on some other organs such as protonephridia and adhesive structures indicate that *C. intermedia* is neither a representative of the Seriata nor of the Rhabdocoela.

The feature “secretory vesicles closely beneath the epidermal surface membrane” is shared with some dalyellioid, typhloplanoid and prolecithophoran species and the absence of true lamellated rhabdites with representatives of the Prolecithophora. The fine structure and location of the secretory vesicles, however, might be a convergence. The lack of tunica cells enveloping gonads is also found in some Lecithoepitheliata and Prolecithophora. However, this characteristic is only partly present in *C. intermedia*, since germaria have a tunica sheath.

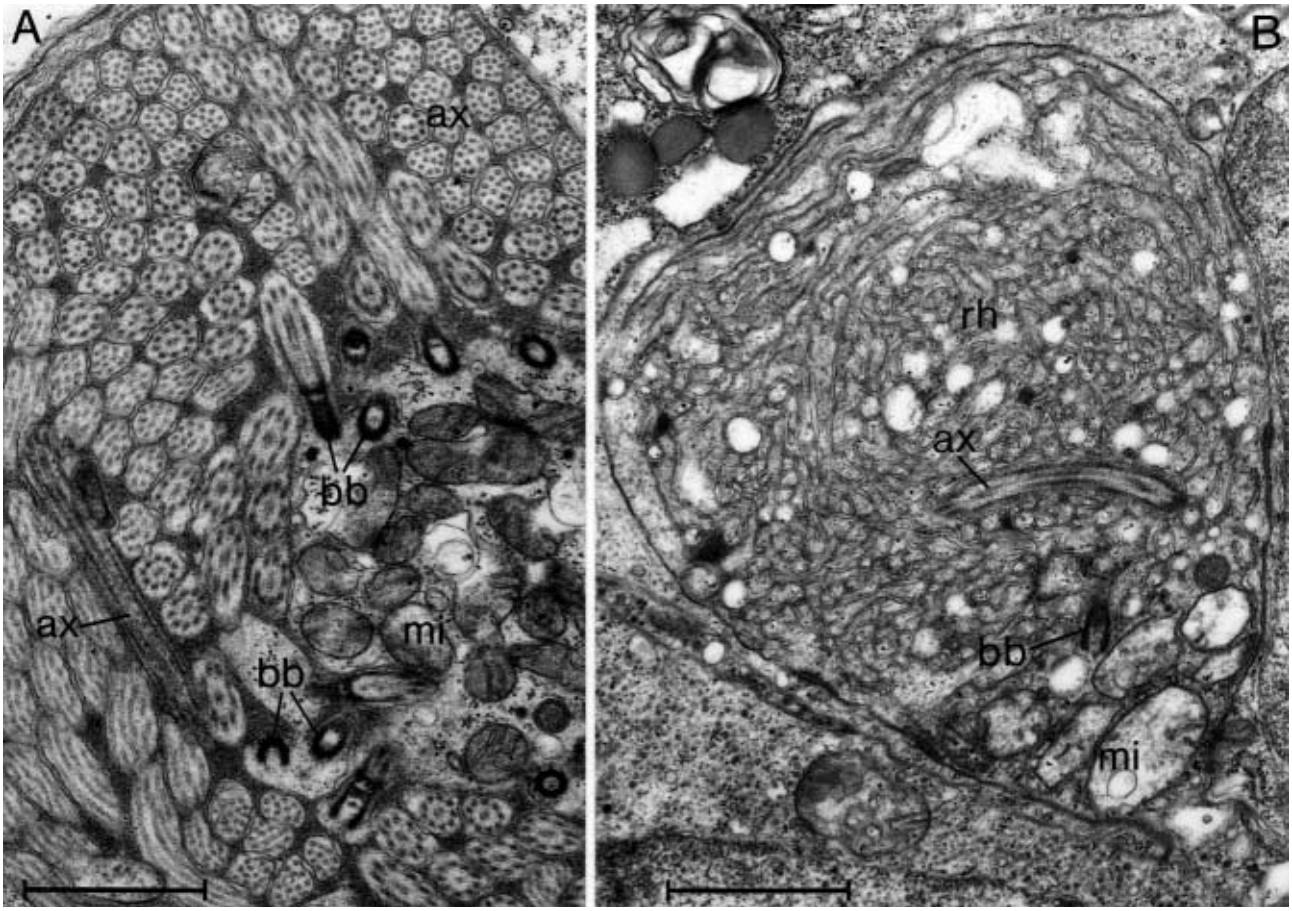

Fig. 3. A. Segment of a ciliary aggregation. ax – axonemata; bb – basal bodies; mi – mitochondria. Scale = 1 µm. B. Dendritic section of a sensory cell of an intracerebral photoreceptor. ax – axoneme; bb – basal body; mi – mitochondrion; rh – rhabdomeres. Scale = 0.1 µm.

Circumcerebral ciliary aggregations are widespread within the Rhabditophora and are either a plesiomorphic feature or have evolved more than once.

So, the data available clearly show that *Ciliopharyngiella intermedia* (or the taxon *Ciliopharyngiella* with both known species) does not belong to any of the taxa Proseriata, Prolecithophora or Rhabdocoela. As hypothesized by Sopott-Ehlers (1997b), Prolecithophora + Rhabdocoela constitute the monophylum Eulecithophora. This hypothesis is based on the special design of female gametes (ultrastructure of eggshell-forming granules in vitellocytes and marginal granules in germocytes). The muscular pharynx in most species of the Eulecithophora is of the 'bulbosus-type' (see Rieger et al., 1991), a more or less barrel-shaped pharynx with an internal isolating muscular septum. Such a pharynx bulbosus is also present in *C. intermedia* and *C. constricta*, but not in any species of the Proseriata (the bulbosus-like pharynx in *Bulbotoplana acephala* lacks a septum). One hypothesis is that the pharynx bulbosus present in quite a number of prolecithophorans (here often named a pharynx variabilis) has evolved from a pharynx plicatus (as known from distinct prolecithophorans), independently of the pharynx bulbosus of all rhabdocoels (here called pharynx rosulatus and pharynx doliiformis). But it is also possible that the different subtypes of the pharynx bulbosus are homologous and that a pharynx bulbosus can be hypothesized for the basic pattern of the Eulecithophora. This would support the view of a sister-group relationship of *Ciliopharyngiella* and Eulecithophora.

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The mosaic of the epidermal syncytia in *Didymorchis* sp. (Didymorchidae, Temnocephalida) from South America

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ABSTRACT. Two species of *Didymorchis* (Turbellaria, Temnocephalida) from Lago Nahuel Huapi, Argentina were studied using a scanning electron microscope and silver nitrate staining to demonstrate the presence of syncytial plates and map the epidermal mosaic. The *Didymorchis* species studied have twenty syncytia, more than are present in the Australian and New Zealand species. Otherwise, the same six morphological groups found in the other species can be recognised. The syncytial plate topography of the South American species of *Didymorchis* has the following distinguishing characteristics: the presence of ventro lateral posterior syncytia, the absence of outer intermediate syncytia, the higher number of plates than present on Australian and New Zealand species, including the replications, and the position of the nephridiopores. The results presented provide data relevant to the origin and relationships of the Didymorchidae within the Temnocephalida, and also their relationships with their hosts.

KEY WORDS: Platyhelminthes, *Didymorchis*, Temnocephalida, South America, syncytial epidermis.

INTRODUCTION

Only four species of *Didymorchis* Haswell, 1900 have been described: one from New Zealand (*D. paranephropis* Haswell, 1900), two from Australia (*D. astacopsidis* Haswell, 1915 and *D. cherapsis* Haswell, 1915), and one from Uruguay, South America (*D. haswelli* Mañe Garzon, 1960). A variety of the last species (*D. haswelli* var. *australis* Dioni, 1972) has been described from Argentina and three undescribed species were reported from Australia (JOFFE et al, 1995a). All these species are commensal on parastacid crayfish and, in South America, they are also found in the branchial chamber of crabs, *Aegla neuquensis* Schmidt, 1942 (Crustacea Anomura). The systematic position and the relationships of these species were uncertain because some authors linked them with the dalyelliids and others with the temnocephalids.

WILLIAMS (1979, 1986) demonstrated the multisyncytial plates of the epidermis of the temnocephalids. Further studies showed that this characteristic is a synapomorphy that distinguishes Temnocephalida from the dalyelliids (JOFFE et al. 1995b). JOFFE et al. (1995a) demonstrated that the epidermis of the Australian species of *Didymorchis* has several syncytial plates and later SEWELL & CANNON (1998) mapped the epidermal mosaic of the type species of the genus, *Didymorchis paranephropis*. *Didymorchis* is now considered an early derived group of the Temnocephalida.

The South American species have been little studied: the presence or the topography of the epidermal plates remains unknown. The aim of this work is to demonstrate the syncytial plates of South American species of *Didymorchis*, to map their topography and compare this with the epidermis of the other species of the genus.

MATERIAL AND METHODS

Two species of *Didymorchis* (*Didymorchis* sp. A and *Didymorchis* sp. B) were found in the branchial chamber of *Aegla neuquensis* Schmitt, 1942 (Crustacea Anomura Aeglidae) from Lago Nahuel Huapi, 2 km west to San Carlos de Bariloche city, Río Negro Province, Argentina (41º07'54.3 S - 71º19'51.5 W), collected by Martín García Asorey (Universidad Nacional del Comahue) and

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transported alive to the laboratory. The worms could be easily differentiated by the structure of the male copulatory organ, but a full description of these species will be presented only after a more detailed morphological and histological study.

Worms were removed from the host with the aid of a stereo-microscope and observed alive under a microscope and then fixed.

The method of JOFFE & CANNON (1998) was used to study the syncytial plates. First, worms were killed with 5% hot (ca. 60º C) silver nitrate, then exposed to cold light for about 5 minutes, and finally washed in distilled water, dehydrated and mounted in Eukitt.

For Scanning Electron Microscope (SEM) observation, the worms were fixed in hot 10% phosphate buffered formalin. They were then dehydrated in ethanol, subjected to critical point drying, mounted and examined with a Jeol SEM.

The terminology used for the syncytial plates and the functional groups follows JOFFE et al. (1995a) and SEWELL & CANNON (1998).

Figures and Photographs were scanned and edited using Adobe Photoshop.

RESULTS

Both, silver nitrate staining and SEM revealed the epidermal mosaic of both *Didymorchis* spp. Twenty syncytial plates can be recognised (Fig.1) with clear borders separating the plates. The mouth and gonopore are midventral in the anterior and posterior quarters of the body respectively, and the nephridiopores are lateral, somewhat dorsal in the middle of the body.

The ventral surface is covered by (from anterior to posterior) several ciliated plates: the ventral frontal syncytium (VF) (Fig. 2a-b), an anterior ventral intermediate syncytium (AVI) (Fig. 2c), two ventro-lateral frontal syncytia (VLF), a preoral syncytium (PrO), four (two side by side pairs) post oral syncytia (PtO), six (three longitudinal pairs) ventral trunk syncytia (VT), and a single posterior intermediate (PI) syncytium. Posteriorly is the unciliated adhesive field syncytium (AD) (Fig. 2d).

The dorsal trunk syncytium (DT) (Fig. 2a) and two ventro-lateral syncytia (VLP) cover the dorsal and lateral surfaces of the animal (Fig. 3a and e). The VLP are elongated plates that contact with the ventral trunk ones lying behind the level of the nephridiopores (Fig. 3c).

Both DT and VLP are similar, unciliated plates. Numerous ciliated receptors can be observed in this surface. On the VLP plates a row a receptors can be recognised (Fig. 3c).

The borders between both the DT and the VLP syncytia with those of the VT syncytia are very clear (Fig. 3d). The ventral region has a covering of dense locomotory cilia. The ventral region is composed of six VT syncytia, all alike. The borders between these syncytia are evident with nitrate staining, but very difficult to see under SEM, because of the numerous, long cilia.

There is a single posterior intermediate syncytium (PI), which is ciliated and looks similar to the VT syncytia. It bears the gonopore. In two specimens it was split longitudinally in two. Behind this lies the AD syncytium. In general, it is a single plate with a horse-shoe shape, but in

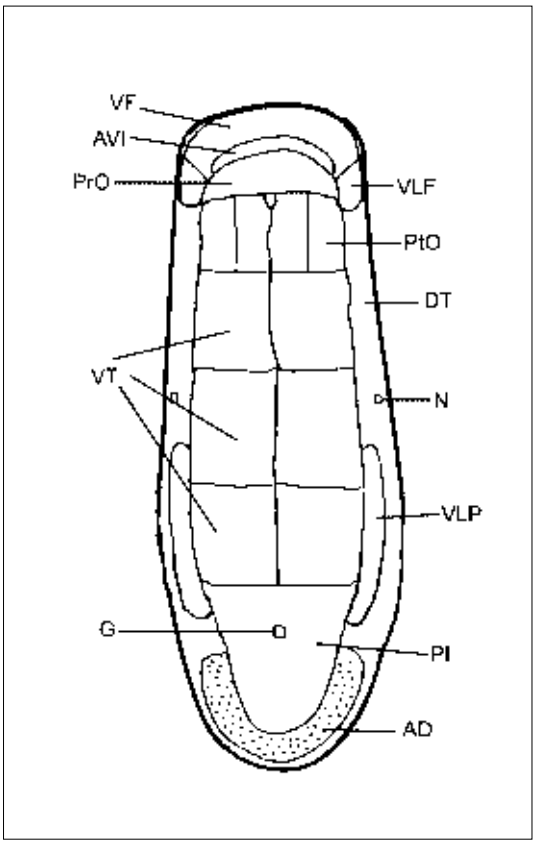

Fig. 1. – Mosaic of epidermal syncitia for *Didymorchis* sp. B. Syncytia: AD, adhesive field; AVI, anterior ventral intermediate; DT, dorsal trunk; PI, posterior intermediate; PrO, preoral; PtO, postoral; VLF, ventral lateral frontal; VLP, ventral lateral posterior; VF, ventral frontal; VT, ventral trunk. G: gonopore; N, nephridiopore (terminology after JOFFE et al., 1995a).

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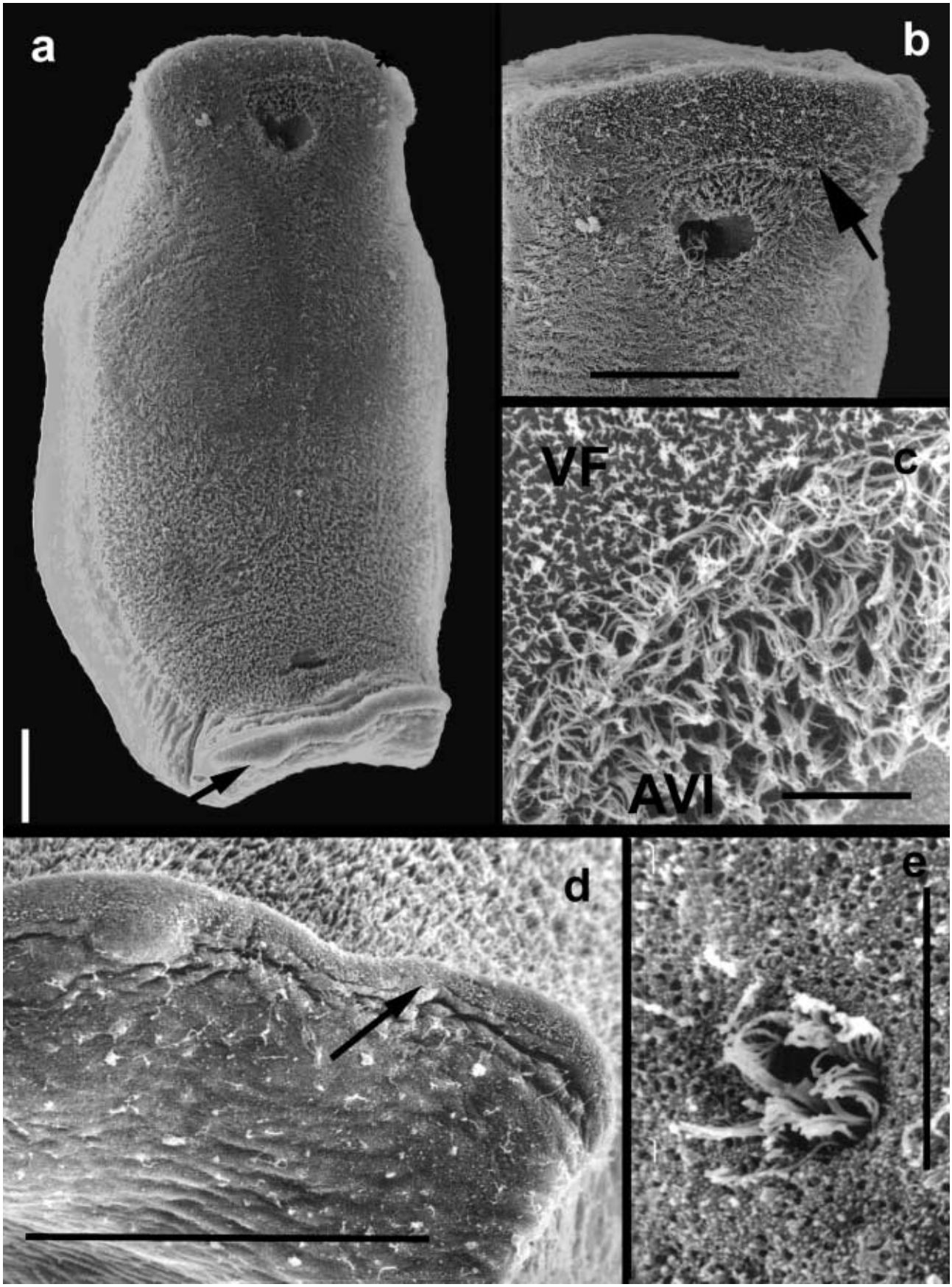
Fig. 2. – SEM of *Didymorchis* sp. B.

a. ventral view of whole specimen showing the ventral cilia and dorsal unciliated surface. Arrow indicates border between AD and DT. Scale: 100 µm.
b. VF syncytium. Arrow indicates the border between VF and AVI. Scale 100 µm.
c. Detail of the VF and AVI. Scale: 10 µm.
d. Posterior end. The border between the AD and DT is indicated by the arrow. Scale 100 µm.
e. Nephridiopore. Scale: 10 µm.


a
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VF
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some specimens it is split into two small, ovoid plates. The AD abuts anteriorly with the PI and posteriorly and laterally with the DT (Fig. 2d).

In the anterior ventral region, several ciliated syncytia can be recognised. The ventral frontal syncytium (VF) has few cilia (Fig. 2c). The AVI is a narrow structure with longer cilia than the more anterior VF (Fig. 2c) and behind is the PrO syncytium just before the mouth. Immediately behind the mouth lie four syncytia side by side (PTO), which bear dense cilia, similar to those of the VT.

Two other syncytia, the VLF, are also evident. They are anterior and lateral, joined to the DT and to the VF. The cilia are short and few in number.

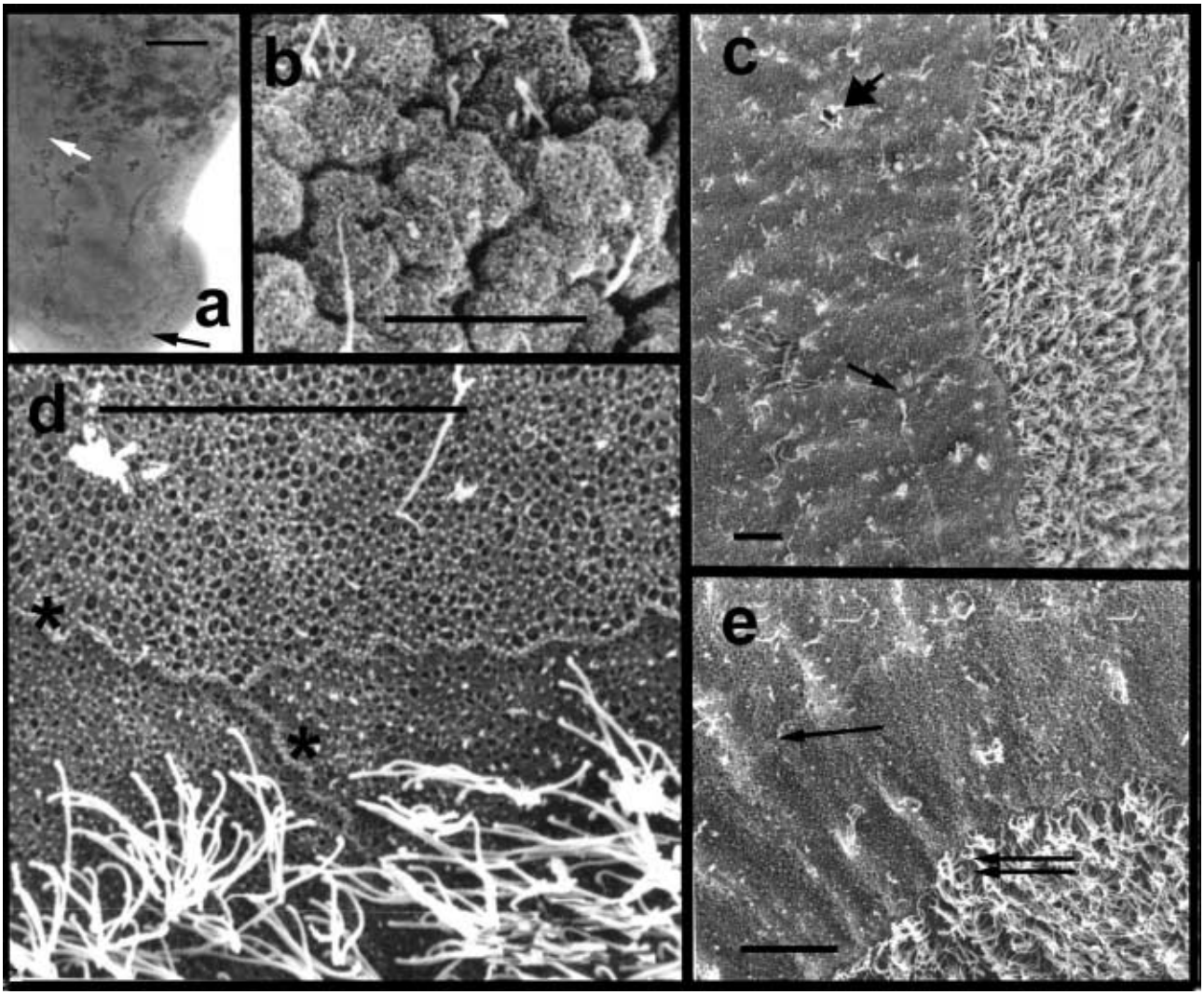

Fig. 3. – a. Lateral view of *Didymorchis* sp. B stained with silver nitrate. White arrow: the border between the VLP and PI; black arrow: the border between the AD and PI. Scale: 50 µm.
b-e. SEM of *Didymorchis* sp.
b. Dorsal surface. Scale: 10 µm.
c. Lateral view showing the ciliated VT, DT and VLP. Small arrow the border between DT and VLP; big arrow the nephridiopore. Scale: 100 µm.
d. Detail of the border between DT and VT and between two VT, *show the borders. Scale: 10 µm.
e. Detail of the VLP, border between DT and VLP single arrow; border between VT and VLP double arrow. Scale: 10 µm.

DISCUSSION

The species of *Didymorchis* are now placed in the Temnocephalida, due to the observed mosaic syncytial plate pattern. The South American species studied here share this characteristic, so they are clearly Temnocephalida.

The *Didymorchis* species studied from Argentina have numerous syncytia, more than the Australian and New Zealand species. Otherwise, the same six morphological groups found in the other species can be recognised. These groups were described for Australian species by Joffe et al. (1995a) and observed again in the New Zealand species (Sewell & Cannon, 1998).

The ventro-lateral posterior plates found in the South American worms, however, have not been found in any other species of the genus. They have the same morphology as the dorsal trunk plate and could be considered as a division of the same functional group.

The morphology of some plates shows variability. Variability, as already noted by JOFFE & CANNON (1998), seems characteristic of the Didymorchidae and although some minor differences are observed in plate topography within the other Temnocephalida it is much less obvious. The most striking variation was found in the adhesive field. Some specimens have this plate split into two. This has been observed by JOFFE et al. (1995a) for the Australian species. Another notable, but less frequent split, was observed in the posterior internal (PI) syncytium.

Both New Zealand and Australian species have the nephridiopores opening on the ventral trunk syncytia. In the South American specimens they open on the dorsal trunk syncytium where they are more anterior and somewhat dorsal.

The position of the gonopore is on the posterior intermediate syncytium, as is the case also with the New Zealand species. In contrast, the Australian specimens have the gonopore on the single ventral trunk syncytium, and the inner posterior intermediate plate is very small. Since the South American species lack the outer intermediate syncytium of the Australian species we call this single plate the posterior intermediate syncytium.

The ventro-lateral frontal syncytia are present both in Australian and South American species, but are absent in the New Zealand one.

If the topography of the syncytial plates is considered alone, we can affirm that the South American species are close to the New Zealand ones, because of the high number of syncytia, the split of the ventral trunk syncytia, the dense cilia of the posterior intermediate plate and the position of the gonopore in the posterior intermediate plate. The South American species are similar to the Australian ones, however, they differ in the presence of the ventro-lateral frontal syncytia and in the form and variation of the adhesive field. Furthermore, the plate topography of the South American species of *Didymorchis* is unique in having the following characteristics: (1) the presence of the ventro-lateral posterior syncytia, (2) the absence of the outer intermediate syncytia, present in all other species studied until now, (3) the higher number of plates, including the replications (laterally of the PRO and longitudinally with the VT) and (4) the position of the nephridiopores. Such differences may prove to be worthy of generic redesignation (see DAMBORENEA & CANNON, 2001).

The genus has a Gondwanan distribution and shares crustacean hosts (Astacidea and Anomura). The results presented here provide valuable data relevant to the origin and relationships of the Didymorchidae within the Temnocephalida, and also their relationships with their hosts.

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The sensory border of the land planarian *Bipalium kewense* (Tricladida, Terricola)

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ABSTRACT. The lunular headplate of *Bipalium kewense* is limited all around its margin by a distinct sensory organ, consisting of a row of papillae, intercalated with ciliated pits. We have investigated the detailed morphology of this sensory region by means of scanning and transmission electron microscopy, as well as, immunohistochemistry. Epithelial cells of the papillae have insunk nuclei and a microvillar border. The dense cytoplasm contains a system of smooth, elongate, vesicles. Monociliated ends of multipolar dendrites come to the surface of the headplate through the epithelial cells. Pear-shaped ciliated pits insert among the papillae. These pits are also provided with sensory dendrites having longer cilia, that are interpreted as chemo-receptors. The distal ends of receptors at both locations are provided with belt-like septate junctions, especially evident following lanthanum impregnation. Immunohistochemical experiments by indirect immunofluorescence have localized substance P in the ciliated receptors, thereby confirming their sensory nature.

KEY WORDS: Platyhelminthes, sensory border, ultrastructure, immunohistochemistry, substance P.

INTRODUCTION

Most sensory elements of flatworms have a rather simple construction (monociliary receptors), although some more elaborate forms exist (Bedini et al., 1975; Ehlers, 1985; Rieger et al., 1991; Wright, 1992). Epithelial ciliated receptors are free endings of neurons, whose perikarya are located in the parenchyma, making contact with the brain (Welsch & Storch, 1976). The functions ascribed to these various receptors remain for the greater part speculative, other than the eyespots and ocelli.

The land planarian *Bipalium kewense* bears a flattened headplate bordered by numerous papillae, supposedly having sensory functions. To our knowledge, ultrastructural aspects of these papillae have received little attention (Storch & Abraham, 1972; Curtis et al., 1983). Given the unique character of this headplate and its presumed involvement in the detection of prey (Hyman, 1951; Bullock & Horridge, 1965), a detailed study of these receptors, including immunohistochemistry, seemed desirable. Recently, terrestrial triclads have become a subject of great concern in certain locations as predators of earthworms, and also due to a high proliferative rate (e.g. Ogren, 1995).

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MATERIAL AND METHODS

Specimens of *Bipalium kewense* Moseley, 1878, most of them over 10 cm in length, were collected locally at the University campus and in Bariri, state of São Paulo, under boards, leaves and flagstones. They were reared within closed pots in the laboratory, fed every two weeks with live earthworms. Only the cut off headplates were used for the present experiments, the remaining bodies being left to regenerate.

Light microscopy (LM)

The heads were fixed in 4% paraformaldehyde (PF) in 0.1M phosphate-buffered saline (PBS), pH 7.4 at 4ºC, for 6h, embedded in Historesin and cut at 3 μm; sections were stained with haematoxylin / eosin.

Immunohistochemistry

The indirect immunofluorescence technique of COONS et al. (1955) was used. Heads fixed as above for 4 h, were cryoprotected in phosphate buffered sucrose, pH 7.4, then embedded in Tissue Tek, sectioned at 8-10 μm at -20ºC, transferred to gelatin-coated glass slides, allowed to dry and frozen at -70ºC. The sections were thawed and immersed in PBS (phosphate buffered saline) with 1% bovine serum albumin (BSA) and 0.2% Triton X-100, at room temperature, for 2 h, then incubated with the primary antibody (anti-rabbit substance P, Sigma, 1:200), for 48 h at 4ºC. Sections were rinsed in PBS and further incubated for 2 h with the secondary anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (1:50) (FITC-Jackson Immunoresearch Lab.). Sections were rinsed again in PBS, mounted in 80% glycerol plus 2.4% antifade (Dabco - Sigma), in 0.1M PBS and stored in the dark, until examination under a Zeiss-510 confocal scanning laser microscope.

Transmission electron microscopy (TEM) and Tracing experiments

Fixatives used were: a) 2.5% glutaraldehyde:1.5% paraformaldehyde (GTA:PF) followed by 1% OsO_4 or b) 3% GTA containing 0.1% $CaCl_2$, followed by 2% OsO_4; c) fixation in cacodylate buffered 3.5% GTA; post-fixation in 2% OsO_4 containing 3% lanthanum nitrate in s-collidine buffer, pH 7.6; dehydration, and Epon embedding (REVEL & KARNOVSKY, 1967); d) fixation in 3% GTA + 0.5% cetylpyridinium chloride for 2h, followed by 1% OsO_4 + 1% lanthanum nitrate in s-collidine buffer, pH 8.0, 2h. (SHEA, 1971). Embedding was in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The electron microscopes used were: Siemens Elmiskop 101 operated at 100 kV, Philips CM-200 (at 200 kV) and Jeol 100 CX-II (at 80 kV).

Scanning electron microscopy (SEM)

Fixation at 85ºC in 2.5% GTA, pH 7.2-7.4 (SEWELL & CANNON, 1995). After cooling to room temperature, the pieces were dehydrated, critical point-dried, and coated with gold. A JSM 840-A microscope was used.

RESULTS

The sensory margin of the headplate of *B. kewense*, consists of a regular row of flattened papillae (Figs 1, 2). In contrast to the overall ciliated surface of the head, this sensory margin is covered by only microvilli and secretory droplets (Figs 2,3). The individual papilla measures about 15 x 25 μm (Fig. 2). Tufts of cilia (Fig. 3) occur at the entrance to a number of alternating pits, which vary between 20-40 μm in depth, depending on the plane of sectioning and/or contraction state of the head (Figs 4,5). Many uniciliated receptors protrude at the surface of the papillae, and can be recognized among the secretory droplets as stiff rods, about 1-2 μm high (not shown). Immunostaining experiments for the neuropeptide substance P demonstrated reactive sites all along the sensory border of the headplate (Fig. 5)

Epithelial cells covering the papillae have an expanded distal cytoplasm and the cell body insunk well beyond the basal membrane and muscular layer. Nuclei are found below the pits (Fig. 4); this arrangement is also evident in Fig. 6. The papillae are covered by microvilli and traversed by many multipolar neurons (Figs 6,7). The microvilli exhibit a glycocalyx that stains selectively with cetylpyridinium-lanthanum (Fig.10). The cytoplasm of epithelial cells is always dense, in any fixation method (compare Figs 6, 11). This high density was reinforced when accidental penetration of lanthanum occurred, revealing a characteristic population of clear vesicles (Figs 6, 7,11), scattered at random throughout the cytoplasm, except for a thin marginal layer (Figs 7, 10). Most vesicles are elongate or tubular, with dilated ends, about 200 x 45 nm, and are usually empty. On face view some vesicles seem disk-shaped. At higher magnification, a definite limiting membrane is resolved in them (Fig. 12). It has not been possible to further characterize these elements.

Sensory receptors located at the surface of the papillae are uniciliated ends of multipolar dendrites, circular in cross section, containing small mitochondria and microtubules (Figs 6, 7). Many dendrites traverse a single epithelial cell, each one establishing with it, at the distal end, a belt-like, septate junction of the pleated type (Figs 8, 9). Similar junctions occur around receptors concentrated at the bottom of the pits. Receptors found at the surface of the papilla have short "9+2" cilia, apparently stiff, showing a system of thin unstriated fibrils running from the basal body (Fig. 8). A similar construction is found for receptors of the pits, except that in this case the ciliary shaft is longer. In both locations, the junctions were clearly enhanced with lanthanum (Figs 7, 8, 9).

Legends to the figures (see opposite page)

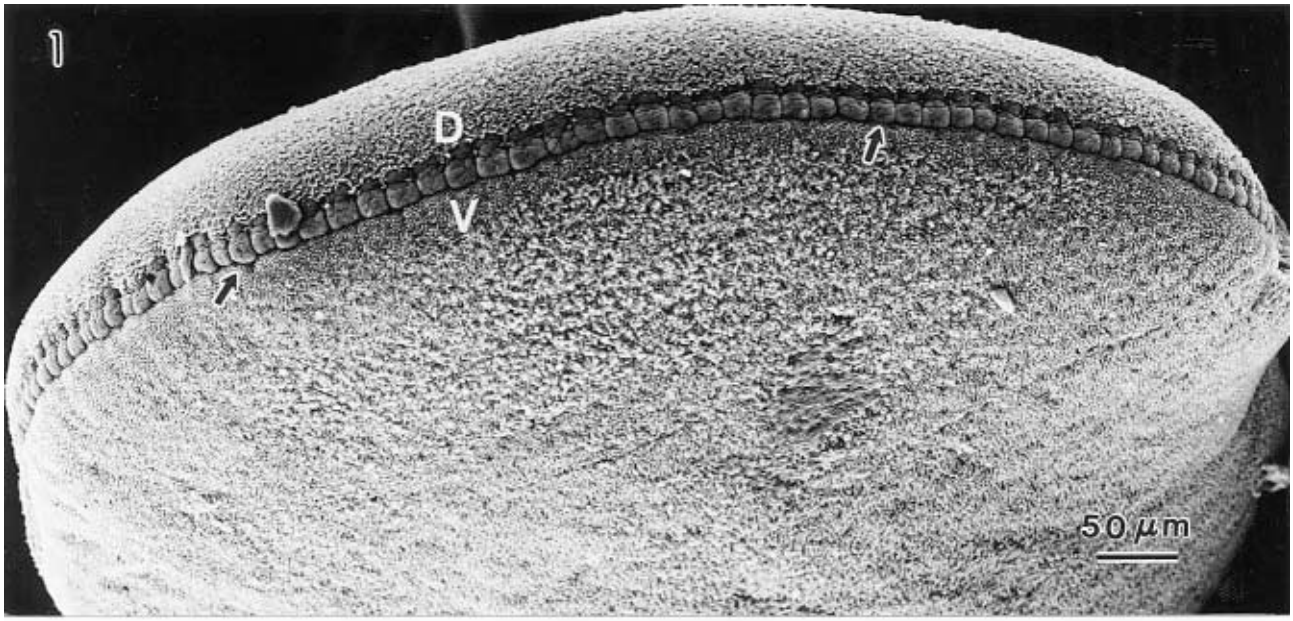
Fig. 1. – Whole view of the sensory border (arrows) delimiting dorsal (D) and ventral (V) faces of the headplate. SEM.

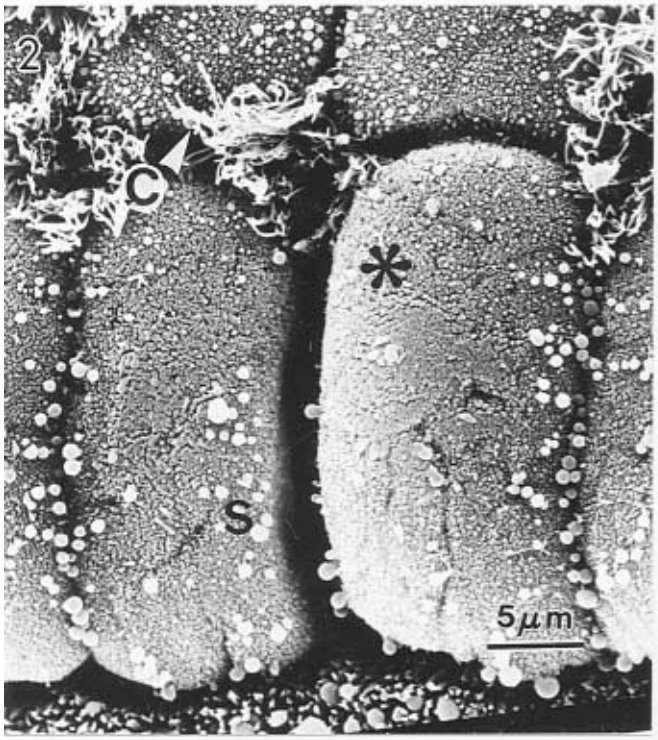
Fig. 2. – Surface view of papillae. Note the pebbled appearance of microvillar covering (*) and many secretory droplets (S); tufts of cilia (C, arrowhead) at the entrance of pits. SEM.

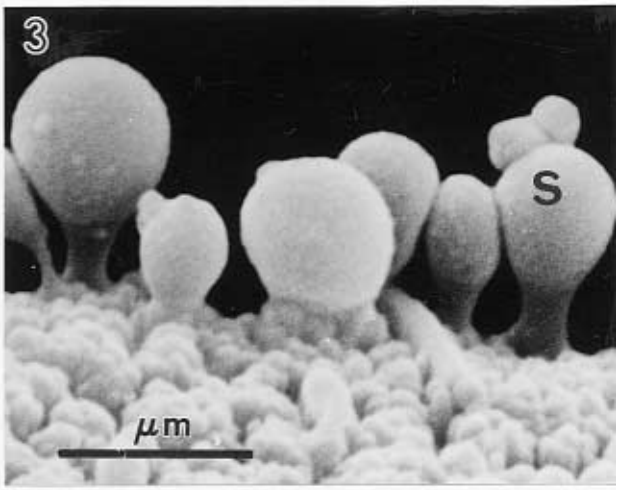
Fig. 3. – Enlarged view of secretory droplets (S), presenting a short stalk. SEM.

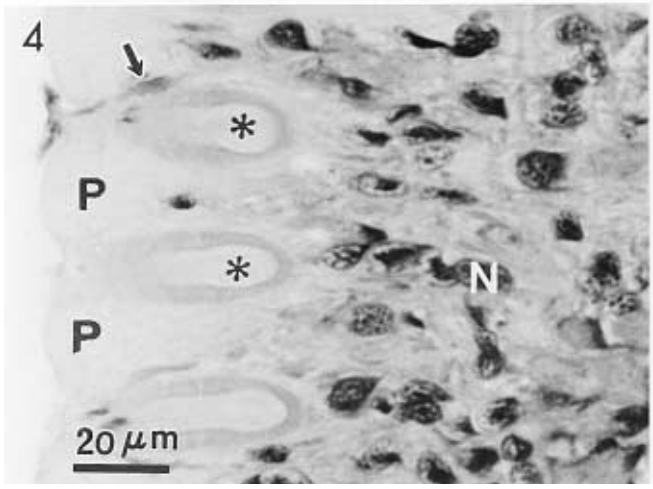
Fig. 4. – Section through the sensory margin, showing alternate arrangement of papillae (P) and ciliated pits (*). Parenchymal nuclei (N). Arrow points to secretion trail. LM section, stained with HE.

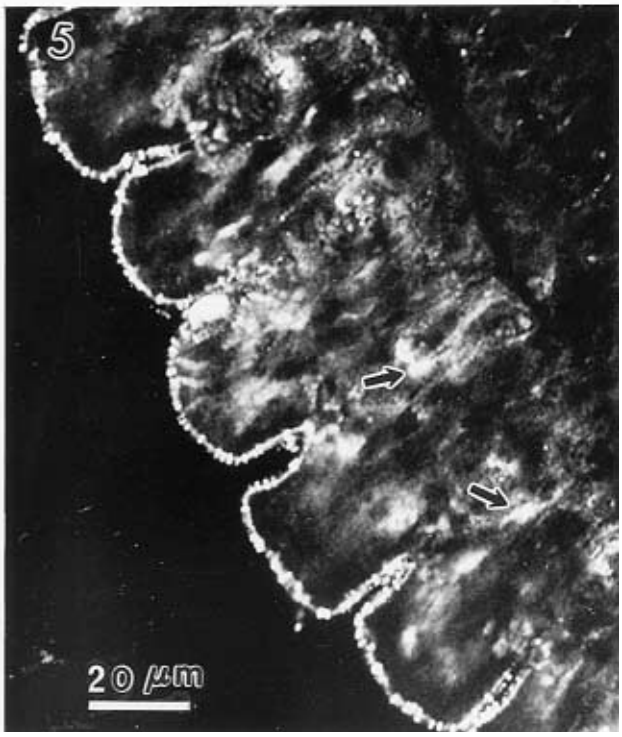
Fig. 5. – Section equivalent to Fig.4, to illustrate reactive sites for substance P along the surface of the papillae, as well as on sensory neurons (arrows). Confocal laser scanning.

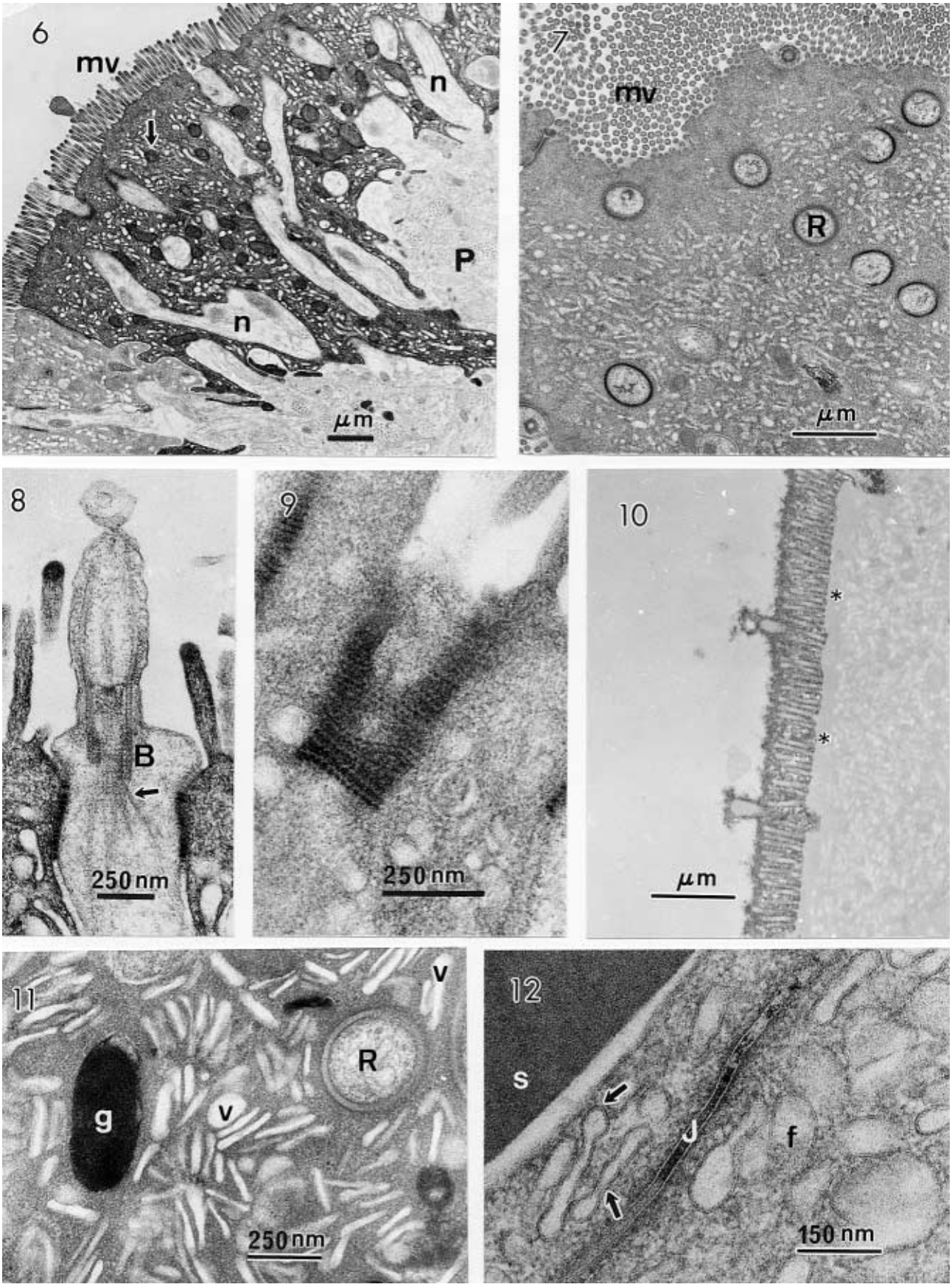

1
D
V
50 μm


2
C
*
S
5 μm


3
S
μm


4
*
P
*
N
P
20 μm


5
20 μm


6
mv
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v
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s
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150 nm

DISCUSSION

Electron microscopic studies on terrestrial triclads are not numerous (STORCH & ABRAHAM, 1972; BAUTZ 1977; STORCH & WELSCH 1977; CURTIS et al., 1983; MINMIN et al., 1992; MCGEE et al.,1997), especially considering the wide distribution of such worms (WINSOR, 1983). The present study confirms the sensory function of, and adds ultrastructural details to the receptors originally described by STORCH & ABRAHAM (1972). Lanthanum impregnation showed the path of sensory neurons across the epithelium, also evidencing the septate junctions engaged in cellular (mechanical or electrical) coupling (see GREEN, 1984). The occurrence of the peptide substance P all along the border of the head, and along the neurons, undoubtedly means that the papillae integrate a sensory organ, connected to the nerve plate. The reactive sites correspond to the surface receptors and to the processes of neurons extending toward the parenchyma of the headplate. It is interesting that sensory receptors of the catenulid *Stenostomum leucops* also contain substance P (WIKGREN & REUTER, 1985); the neuropeptide is also present around the eyes of *B. kewense* (FERNANDES, 2000). In particular, as seen in our experiments, the immunoreactivity demarcates the profile of all ciliated receptors.

The presence of a brush-border of microvilli on the outer surface of a land dwelling worm is somewhat surprising. As in other systems, microvilli provide a significant increase on the surface area of the epidermis (ALBERTS et al, 1994). These cytoplasmic extensions are made rather rigid due to a filamentous cytoskeleton. Its glycocalyx coat would conceivably provide the chemical component involved in signal reception, necessary for the impulse transduction (THURM, 1983; HUFNAGEL, 1992). This glycocalyx contains acidic muco-substances, likely complexed to a protein (SHEA, 1971).

Dense epithelial cells containing vesicular elements were not observed in other regions of *Bipalium kewense*, but they occur in the auricular epithelium of *Dugesia tigrina* (personal observations; see also MACRAE, 1967). The vesicles could correspond to a sort of smooth reticulum, maybe retaining Ca^{2+} ions or some elusive material not preserved by our preparative methods; they could even represent just a storage site for smooth membranes. The contents of the vesicles might contribute to the bright autofluorescence seen under the confocal microscope, in whole heads. Similar vesicles, though having a dense core, are present in the rostellar tegument of *Hymenolepis nana* (KUMAZAWA & YAGYU, 1988). Epithelial cells of *Geoplana pasipha*, another land planarian studied by us, also contain smooth vesicles that incorporate ruthenium red (unpublished observations).

The headplate, a characteristic feature for the genus *Bipalium sp.*, is a muscular organ, richly innervated and involved in the search for food (HYMAN, 1951; FERNANDES, 2000). Specialized devices for sensing chemical and mechanical stimuli are therefore expected to occur in the headplate. It is likely that the uniciliated receptors found at the surface of the papillae have tactile function, whereas those located in the recessed pits would be chemical receptors. Unfortunately, the ultrastructural approach to such identification is neither sufficient nor trivial, even in Protozoa (HUFNAGEL, 1992); it would depend on the identification of transduction molecules, ion channels, etc. It seems that each component of a receptor, however simple in construction it may be, is responsible for eliciting one step of the sensorial/excitatory function (THURM, 1983). Septate junctional complexes like those of *Bipalium kewense* are widespread among invertebrates, providing the pathway for mechanical or electric coupling between sensory dendrites and epithelial cells (GILULA et al., 1970; THURM et al., 1983).

Legends to the figures (see opposite page)

Fig. 6. – Section through a papilla. Dense epithelial cells are penetrated by nerve endings (n). Parenchyma (P); mitochondria (arrow); microvilli (mv). Lanthanum-impregnation. TEM.

Fig. 7. – Grazing section through the epithelial border. Septate junction around each receptor (R) was permeated by La^{3+}. Microvilli in cross sections (mv). TEM.

Fig. 8. – Longitudinal section of a ciliated receptor. The "9+2" cilium is short, attached to a basal body (B) and associated, unstriated fibrils (arrow). The ciliary shaft is slightly swollen, forming a balloon at the tip. TEM.

Fig. 9. – Tangential section through a junctional belt around a receptor, following Lanthanum impregnation. Note the wavy arrangement of the septa. TEM.

Fig. 10. – Brush border-like microvilli from epithelial cell of a papilla. Glycocalyx selectively stained according to Shea (1971). Homogeneous apical cytoplasm (*) contains no vesicles. TEM.

Fig. 11. – Epidermal cell fixed in GTA/OsO_4. Many elongate vesicles (v) stand out on dense ground cytoplasm. Secretion granules (g); receptor (R). TEM.

Fig. 12. – Cytoplasmic vesicles in profile and on face view (f) exhibit a neat unit membrane (arrows). Septate junction (J); secretion granule (s). TEM.

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The resorptive vesicle of *Temnocephala jheringi* (Temnocephalida)

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ABSTRACT. The wall of the resorptive vesicle is a merocrine secretor and a phagocytic syncytium, and its free plasma membrane shows deep and intertwined invaginations. Redundant foreign spermatozoa are engulfed by these invaginations, digested and incorporated into the individual's metabolism. The observations presented in this paper lead us to conclude that, during copulation, the resorptive vesicle acts as a temporary receptive organ for the foreign male gametes (bursa copulatrix role).

KEY WORDS: Platyhelminthes, *Temnocephala*, resorptive vesicle, reproductive biology, ultrastructure, epithelia

INTRODUCTION

To date there is no ultrastructural information available on bursal organs of *Temnocephala* (RIEGER et al. 1991). The resorptive vesicle was originally called receptaculum seminis by HASWELL (1888) and WEBER (1889) who assigned it a function as a reservoir for spermatozoa. Later, HASWELL (1924) changed his mind and called it vesicula resorbiens following MERTON's (1914) identification of the true receptaculum seminis, and in view of the fact that it contained yolk, and no spermatozoa. According to FYFE (1942), the vesicula resorbiens "is a reservoir of superfluous reproductive material which can be absorbed at intervals into the gut".

Recently, WILLIAMS (1994) using light microscopy (with haematoxylin/eosin and Mallory's trichrome staining) claimed that it functions in the digestion of excess spermatozoa.

In the present work we postulate, on the basis of our studies, and in accordance with HYMAN´s physiologic concept (HYMAN, 1951), that the resorptive vesicle of *Temnocephala jheringi* acts as a bursa copulatrix. We also postulate that phagocytic activity takes place in the epithelium of the resorptive vesicle, and remnant foreign sexual products are digested.

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MATERIAL AND METHODS

Specimens of *Temnocephala jheringi* (Haswell, 1893) were extracted from the paleal cavity of *Pomacea canaliculata* (Mollusca) from the marshy coastal waters of the River Plate, just in front of the University of Buenos Aires Campus (Ciudad Universitaria). Sexually active specimens were fixed in Bouin-Hollande fixative and embedded in paraffin wax.

Longitudinal oblique and transversal serial sections of 6-8 μm thickness were obtained. The Mann-Dominici´s method, Masson´s trichromic and PAS reaction (GABE, 1968) were employed for staining.

For transmission electron microscopy (TEM) specimens were fixed in 3% glutaraldehyde in sodium cacodylate at pH 7.3, post-fixed for a one hour in 1% osmium tetroxide in 0.25 M cacodylate with 0.12 M sucrose, and embedded in Spurr´s resin. Following staining with uranyl acetate and lead citrate, thin serial sections were scanned by means of a JEOL-JEM-100C apparatus. Light microscopy was performed to study 1 μm sections after staining with Loeffler´s alkaline methylene blue (GRAY, 1975).

RESULTS

Temnocephala jheringi individuals are hermaphroditic. The female system includes an ovovitelline duct, paired

seminal receptacles, and a resorptive vesicle. The first leads to the common genital atrium, and in the post-copulation stage its anterior end acts as a sphincter. The other end narrows and opens into the resorptive vesicle. The resorptive vesicle has the shape of a hollow hemisphere of diameter approximately 300 µm. The connection to the ovovitelline duct lies centrally in its base. The seminal receptacles are connected to the ovovitelline duct through openings that are contiguous with the entrance to the resorptive vesicle; each is approximately 80 µm long with muscle fibers at the entrance. In sections, they are seen as notches over the wall of the duct.

No connection of any type (permanent or transitory) was observed between the resorptive vesicle and the neighboring intestine. No female gametes, no vitelline cells, and no characteristic vitellus were seen amongst the contents of the vesicle.

The epithelium of the resorptive vesicle shows a basal or proximal part and an apical or distal part (Fig. 1). The basal part is pierced by dorso-ventral and askew oriented muscular fibers.

The few nuclei are in the basal part; they are irregular and their main axis lies parallel to the cellular limit. They measure 20µm x 24µm, have a 7µm nucleolus, and, in some cases, have an extra one that is smaller in size. The nuclear membrane shows small internal indentations that limit the nuclear pores and constitute a prickly surface. In this zone there are abundant mitochondria and dictyosomes. The latter produce a secretion that is discharged through the plasma membrane at the distal zone (merocrine extrusion). The distal or apical region is constituted by a tubular system and cisterns with vacuolated cytoplasm.

The parietal cytoplasm appears differently colored depending on the staining used; with the Mann-Dominici´s method, the basal part stains light violet (basophilic) and the apical one that limits the vesicular lumen stains pinkish (acidophilic). With Masson´s trichromic, the apical part stains lilac, and the basal one red. With PAS technique, the apical is positive. No cellular junction structures were detected at the vesicular wall level.

After copulation, the resorptive vesicle holds foreign spermatozoa, which are free, together with prostatic secretion ejaculated by the sexual partner. Bundles of sperm cells lie between the seminal receptacles and the lumen of the resorptive vesicle (Fig. 2). A viscous fluid accompanies the moving sperm cells bundles. The fluid fills the lumen of each receptacle and, in some cases, protrudes into the ovovitelline duct cavity.

In the post-copulation stage, the apical zone of the resorptive vesicle shows a system of tubules and cisterns formed by deep invaginations of the distal plasma membrane of the vesicular syncytium (apical labyrinth). The apical labyrinth is connected to the vesicular lumen. The cytoplasm of the apical zone contains vacuoles and mitochondria. Circumscribed areas from the apical labyrinth may protrude into the vesicular lumen. The remnant gametes that lie in juxtaposition to the parietal epithelium free edge become engulfed within vesicles through a phagocytic process (Fig. 3). When few spermatozoa remain into the resorptive vesicle, there are lytic products from the digestion of remnant spermatozoa and remains of their cilia near the nuclei (Fig. 4).

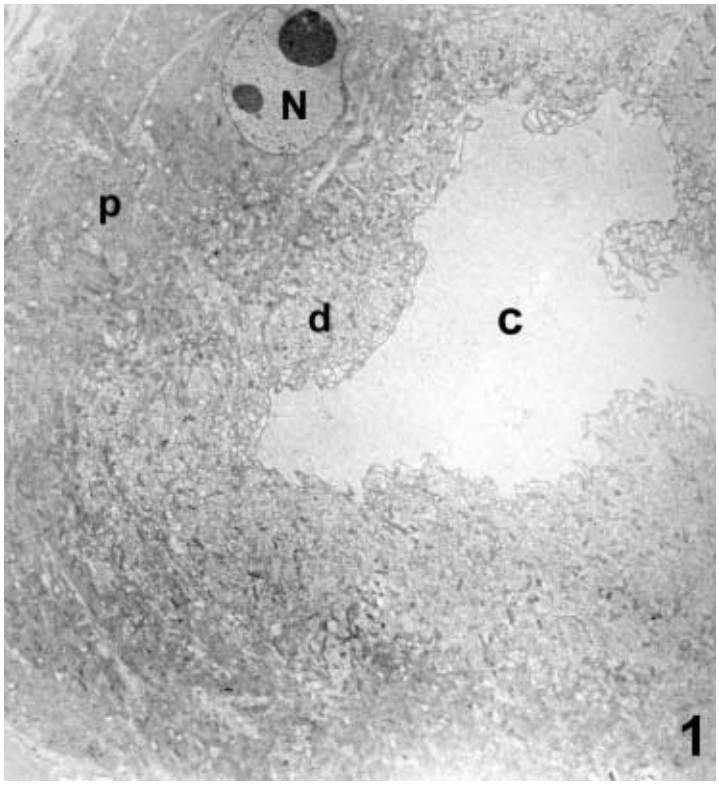


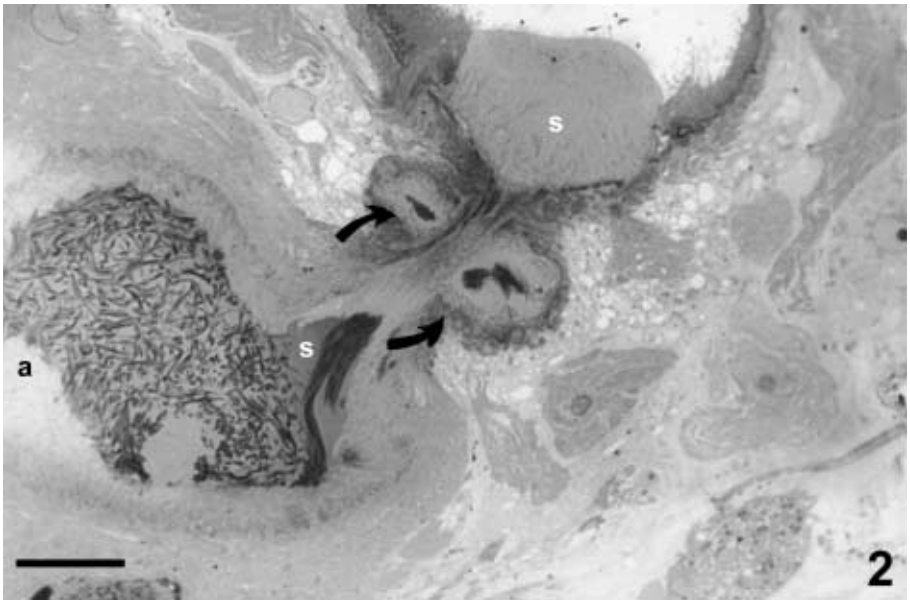
Fig. 1. – Oblique section of resorptive vesicle. The two regions of its epithelium. p = proximal (or basal) region with nucleus (N); d = distal (or apical) region; c = vesicular cavity. TEM. (X 2000)

Legends to the figures (see opposite page)

Fig. 2. – Post-copulation stage. Bundles of spermatozoa going into the seminal receptacles (arrows). Note the secretion (s) accompanying the spermatozoa and filling the seminal receptacle region. The apical labyrinth (a) of the resorptive vesicle engulfs spermatozoa. Longitudinal section (1µm). Loeffler´s methylene blue. Scale bar: 20µm.

Fig. 3. – Some spermatozoa engulfed by the apical labyrinth. (arrow) TEM, 20000 X.

Fig. 4. – Vesicles containing lytic products from the digestion of spermatozoa in the proximal (basal) epithelial zone of the resorptive vesicle. TEM, 8000 X.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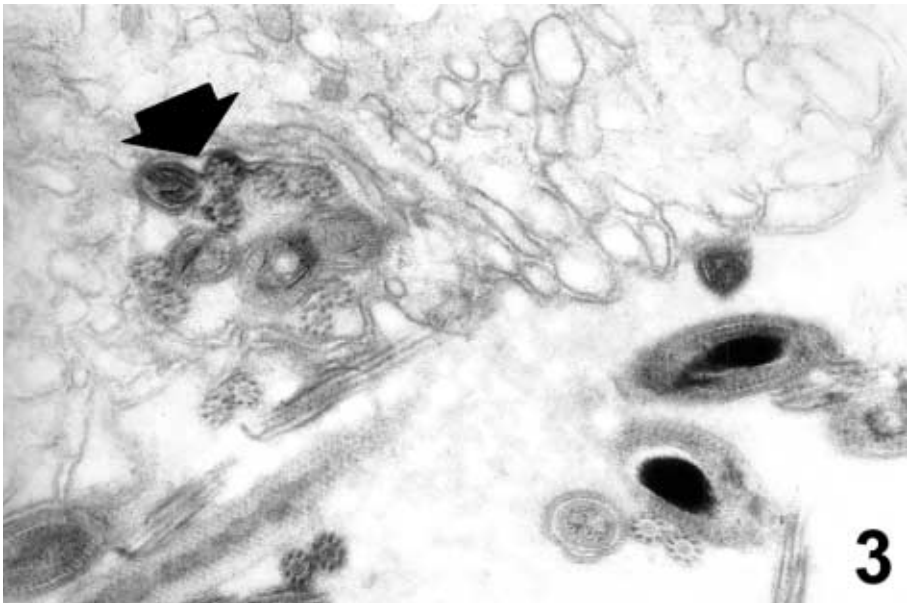
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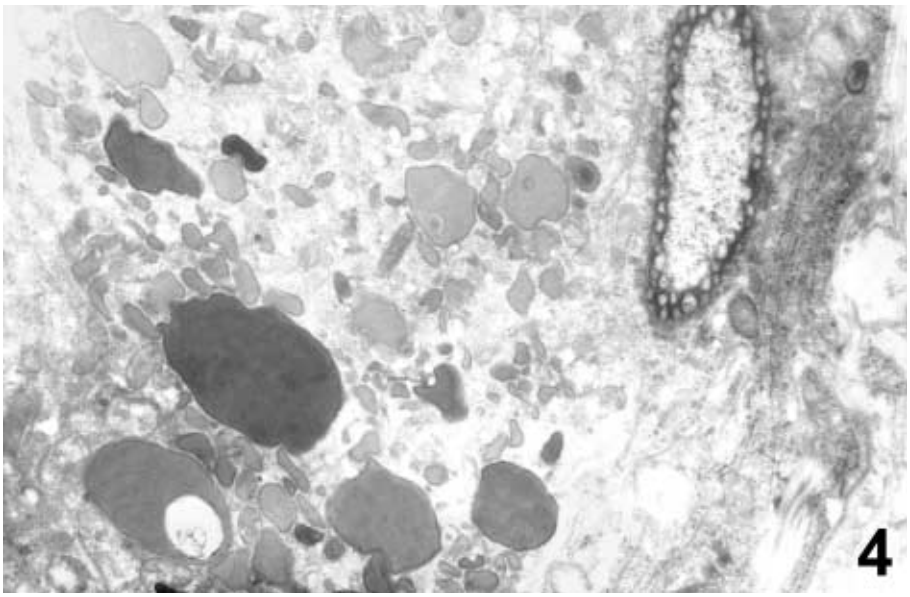
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DISCUSSION AND CONCLUSION

According to WILLIAMS (1994), insemination in *Temnocephala genoma* would occur through the gonopore into a ventral expansion of the ovovitelline duct (a particular characteristic of that species), which would act as bursa copulatrix. The resorptive vesicle´s only function in that species would be digestion of the excess foreign sperm cells. As degenerated spermatozoa were observed intraepithelially, she concluded that at least some part of the digestive process occurs within the epithelium.

According to Hyman´s criterion (HYMAN, 1951), however, the resorptive vesicle of *Temnocephala jheringi* may be regarded as a bursa copulatrix, because it is a temporary holder of the foreign male gametes during copulation. In the post-copulation stage the foreign sperm cells would be selected on the motility of the bundles in which they are grouped and for their capacity to escape from the bursa to the seminal receptacles. The fluid that accompanies the sperm cells and fills the region of the seminal receptacles may be protective, and may originate in the epithelium of this region. This extrusion is stained with Loeffler's alkaline methylene blue.

Remnant and redundant bundles would be destroyed within the bursa, their spermatozoa lysed, and the resulting products incorporated into the individual´s metabolism. These findings are consistent with what we have previously observed in other species of South American Turbellaria (MORETTO, 1996). After copulation, the bursa retains the foreign male sexual products for a short time. We therefore suggest that the term bursa seminalis proposed by FISCHLSCHWEIGER & CLAUSNITZER (1984) is not suitable in Turbellaria.

In *Temnocephala,* the remnant spermatozoa are phagocytized by a pseudopodial activation at the distal zone of the bursal epithelium.

Under a static vision, the apical or distal labyrinth observed in the bursal epithelium of *T. jheringi*, in the post-copulation stage, would correspond to the smooth endoplasmic pseudo-reticulum that can be found in fish gills cells. This constitutes a tubule system that connects directly to the cellular surface. This surface is formed by deep and wide invaginations of the plasma membrane (WELSCH & STORCH, 1976).

In conclusion, during copulation the resorptive vesicle of *Temnocephala jheringi* receives the foreign sexual products. In the post-copulation stage, the bursal epithelium becomes a merocrine glandular syncytium and lyses all the spermatozoal bundles that do not escape in time from within the bursa, and the resulting products are incorporated into the metabolism of the individual.

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Critical reading of the manuscript by Dr. S. Tyler was most helpful. Technical advice and help by Mrs. Isabel Farías (Buenos Aires) and Lic. Viviana Sorrivas de Lozano (CRIBBAB-Bahía Blanca) made the research effort possible. Mr. Ángel Fusaro provided expert help with photography. Dr. Silvina Heisecke and Mr. Héctor Chiocchio helped with technical support.

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SHORT NOTES

Spermiogenesis and ultrastructure of the spermatozoon in the dioecious marine planarian *Sabussowia dioica* (Platyhelminthes, Tricladida)

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The phylogeny of the Platyhelminthes is a subject of controversy. Recently a number of attempts to reconstruct a valid phylogenetic scheme of Turbellaria-Platyhelminthes have been based on ultrastructural data of several organs (1, 2). Spermiogenesis and spermatozoon ultrastructure are often used for understanding both phylogeny and reproductive biology in the phylum (3, 4, 5). In this respect, a small number of triclads have been studied. The marine planarian *Sabussowia dioica* (Claparède, 1863) is the only known gonochoristic triclad and the rarest dioecious organism in Platyhelminthes. Males of this species transfer sperm by depositing sclerotic spermatophores onto the body wall of the female, and it is only from these capsules that sperm are discharged into the parenchyma of the partner. This sperm transfer method is unique in two respects: (a) external deposition of spermatophores is unique within the Tricladida and (b) formation of spermatophores is unique within the Maricola. Fertilization of oocytes takes place within the ovary, contrasting with the usual situation of fertilization in the proximal section of the oviductus (6). *S. dioica* also shows unusual features of the developing female germ cells and their associated cells (7).

In this study by transmission electron microscopy, we report the first data on spermiogenesis and spermatozoon ultrastructure of male specimens of *S. dioica* collected in the Gulf of Tunis. Three mitoses and the meiosis give rise to clustered structures of 32 biflagellated spermatids. Young spermatids are round shaped (Fig. 1). The spermiogenesis consists mainly of nuclear condensation and elongation (Figs 1, 2), and fusion of the numerous mitochondria of the spermatid resulting in a single giant one (Figs 4, 6).

The spermatozoon is thread-like (50µm in length). The elongated nucleus is made up of two distinct components one being filamentous, electron-dense chromatin with a helical structure (Fig. 6) and the other of uniform electron-light appearance, suggested to be residual protein (8). The nucleus and the single elongated mitochondrion are coiled around each other in a screw-like fashion. Two free flagella are coupled and lie parallel to one another (Figs 5, 7). Their axonemal microtubules are arranged in a "9+1" structure (Fig. 5). There is a single layer of peripheral microtubules running longitudinally beneath the plasma membrane (Fig. 4). By reconstruction from transverse and longitudinal sections taken through the testis and seminal vesicle, a diagrammatic drawing of the structure of the mature spermatozoon of *S. dioica* is given in Fig. 8.

This research shows that spermiogenesis and spermatozoon ultrastructure of *S. dioica* are mainly in agreement with the literature data regarding Platyhelminthes and Turbellaria Tricladida: (a) Two free flagella in the spermatids and spermatozoa are widespread in free living flatworms and should be considered the plesiomorphic structure for the Platyhelminthes (3, 4, 5). (b) Axonemal microtubules with "9+1" structure have been reported for Turbellaria and parasitic groups (4, 5, 9). (c) As in other triclads (10, 11, 12) and other Platyhelminthes (4, 5, 12), the spermatozoon of *S. dioica* lacks an acrosomal structure.

Some aspects that appear to be confined to species of Tricladida are also noted in the spermatozoon of *S. dioica*: **(a)** the occurrence of a complex nuclear structure with two distinct components coiled around each other in a screw fashion (8,13,5, the present study). **(b)** The nucleus and the single elongate mitochondrion also coil around each other (4, 5, this study). **(c)** Mature sperm lack the numerous dense bodies characteristic of many other turbellarians (4, 13, 5, this study).

Meanwhile, a particular feature of the spermatozoon of *S. dioica* is that the two free flagella are coupled and parallel to one another.

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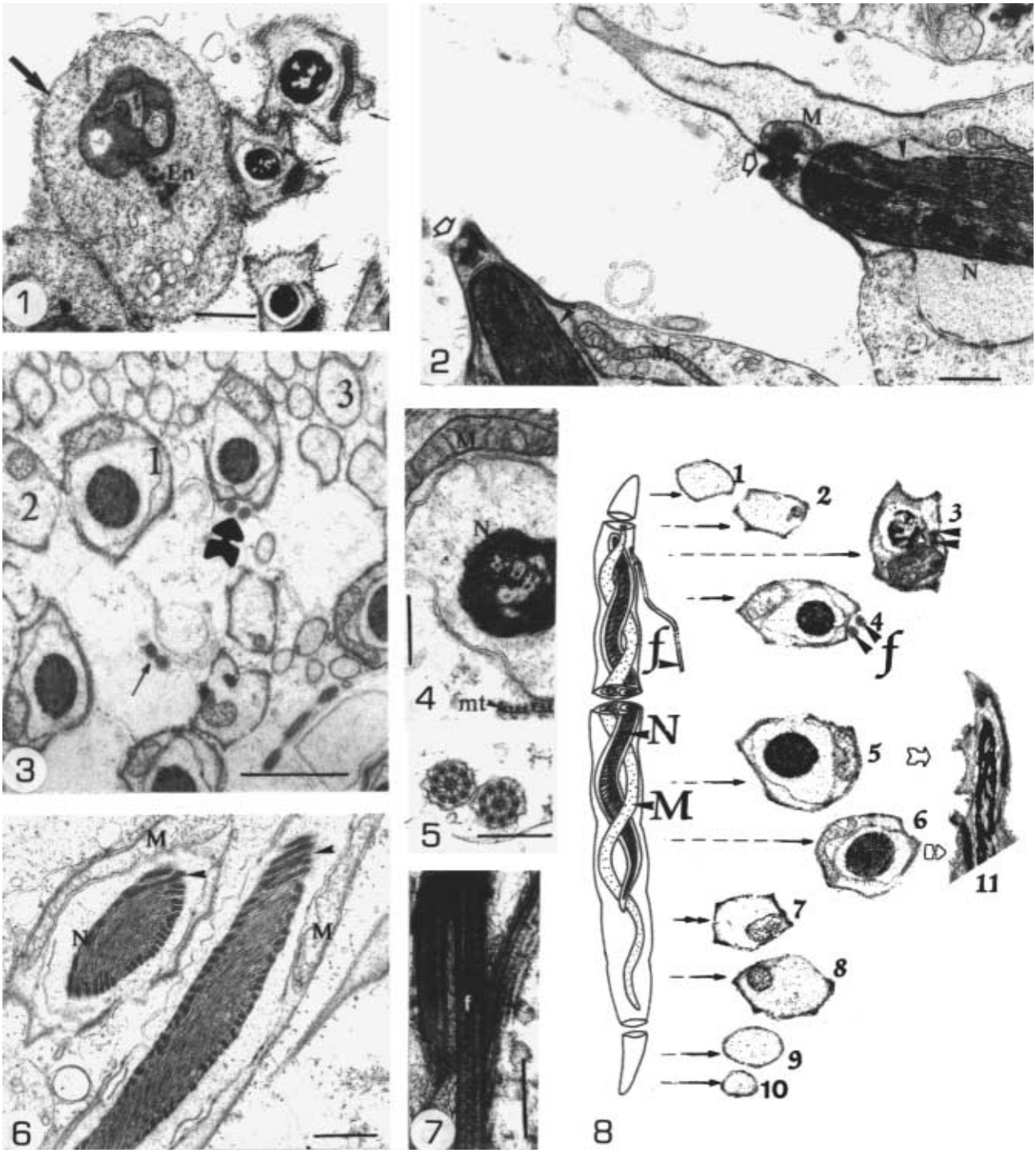

Fig. 1. – A young spermatid (strong arrow) showing nuclear emissions (En) and three sections of spermatozoa (small arrows) in the testis; scale bar = 1 µm.

Fig. 2. – Spermiogenesis of two spermatids showing elongation of the condensed nuclear material (heads of arrows) and departure of the two flagella (light arrows); M: mitochondria; N: nucleus; scale bar = 1 µm.

Fig. 3. – Transverse sections of spermatozoa in the seminal vesicle; three section levels (1: nucleus & mitochondrion; 2: mitochondrion; 3: 0); arrows: two flagella; scale bar = 2 µm.

Fig. 4. – Transverse sections of a spermatozoon showing the complex nuclear structure (N) and peripheral microtubules (mt); scale bar = 0.5 µm.

Fig. 5. – Transverse section of the two coupled flagella with "9+1" structure; scale bar = see 0,5 µm.

Fig. 6. – A transverse (on left) and a longitudinal (on right) section of spermatozoa showing the nucleus (N) with spiral-shaped condensed chromatin (heads of arrows) and the giant mitochondrion (M); scale bar = 1 µm.

Fig. 7. – Longitudinal section of the two coupled and parallel flagella (f); scale bar = 0.5 µm.

Fig. 8. – Reconstruction of *S. dioica* spermatozoon structure (on left) showing the nucleus (N), the mitochondrion (M) and the two free flagella (f); transverse (1-10) and longitudinal sections (11). Heads of arrows at profile 3 indicate two dense structures where the flagellar axonemes originate in the shaft of the spermatozoon.

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The ultrastructure of oocyte and vitellocyte inclusions in a scutariellid (Platyhelminthes, Rhabdocoela, Temnocephalida) with phylogenetic implications

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Ultrastructural investigations of the female gonad have provided useful data on platyhelminth phylogeny (1). In particular, the structure and composition of eggshell-forming globules in vitellocytes, as well as those of peripheral egg granules, and the presence or lack of nutritive materials in the oocytes, have been considered suitable features for understanding the phylogenetic relationships (2, 3, 4).

Members of Rhabdocoela (Dalyellioida, Kalyptorhynchia, Typhloplanoida) studied so far, display peripheral egg granules with a granular content in the oocytes, and eggshell-forming globules with a multigranular/mosaic-like pattern in the vitellocytes. These characteristics of the female gonad are considered apomorphic features shared with the taxon Prolecithophora (synapomorphies of the two taxa) and have contributed to the assignation of the name Eulecithophora to the monophylum Prolecithophora+Rhabdocoela (5).

To date, ultrastructural data on the female gonad of Temnocephalida are still scarce. The only detailed ultrastructural investigation examined *Temnocephala dendyi* and *T. minor* belonging to the family Temnocephalidae (6). In the present study we have examined the germarium and the vitellarium of a temnocephalid belonging to the family Scutariellidae, *Troglocaridicola* sp., in order to obtain additional information on the structure and composition of oocyte and vitellocyte inclusions. These ultrastructural findings are compared with those from other platyhelminths.

Specimens of *Troglocaridicola* sp. were removed from the gill cavity of the shrimps *Troglocaris* sp. collected in the cave of Comarie, locality Doberdò del Lago, near Gorizia (Italy). Specimens were processed for transmission electron microscopy, and cytochemical tests were performed according to the procedure described in Raikova et al. (7)

The female gonad of *Troglocaridicola* sp. consists of a single germarium located posterior to the pharynx, and of several paired, dorsal vitelline follicles. The germarium is delimited from the surrounding somatic tissues by a sheath of flattened accessory cells and a thin extracellular lamina. Packed free-ribosomes, mitochondria, chromatoid bodies, annulate lamellae, RER profiles and Golgi complexes are the main organelles of the developing oocytes (Fig. 1). RER and Golgi complexes appear to be involved in the production of two types of vesicle, some smaller containing an electron-dense material, others larger with a translucent material (Fig. 2). Repeated fusions of the electron-dense vesicles give rise to a few acorn-shaped granules (1.3-1.5 μm in diameter), which remain scattered throughout the cytoplasm during oocyte maturation (Fig. 1). They have a protein content that is partially extracted by protease (Fig. 3), and do not contain polyphenols. These egg granules differ from those observed in most rhabdocoels where they have a cortical localization and a granular content with polyphenols. The larger, translucent Golgi-derived vesicles undergo only a minimal coalescence process and, as soon as they are formed, migrate to the cortical ooplasm (Fig. 4). Some of them are seen in the process of fusing with the plasma membrane (Fig. 5). They have never been observed in other rhabdocoels.

Vitellocytes are similar to secretory cells with well-developed RER and Golgi complexes involved in the production of two types of membrane-bound inclusions. The first type to appear has an electron-dense content, which, in early stages of maturation, may show a mixed pattern consisting of both multigranular and concentric material, and in later stages a pattern with alternating dark and clear rings (Fig. 6). These inclusions measure 1.8-2 μm in diameter, contain polyphenols (Fig. 7) and have been interpreted as eggshell globules. The peculiar design of their content does not correspond either to the multigranular pattern prevailing in representatives of the Rhabdocoela and Prolecithophora or to the homogeneous/convoluted pattern of the Lecithoepitheliata; in fact, it somehow resembles that of eggshell globules in Proseriata and Tricladida. The second type of membrane-bound inclusion in the vitellocytes has a homogeneous, glycoprotein content of medium electron-density, is devoid of polyphenols, and represents yolk (Fig. 8). In addition, mature vitellocytes contain glycogen and lipid droplets.

In conclusion the female gonad of *Troglocaridicola* sp. exhibits some autapomorphic features (the presence of ooplasmic peripheral translucent vesicles, the substruc-

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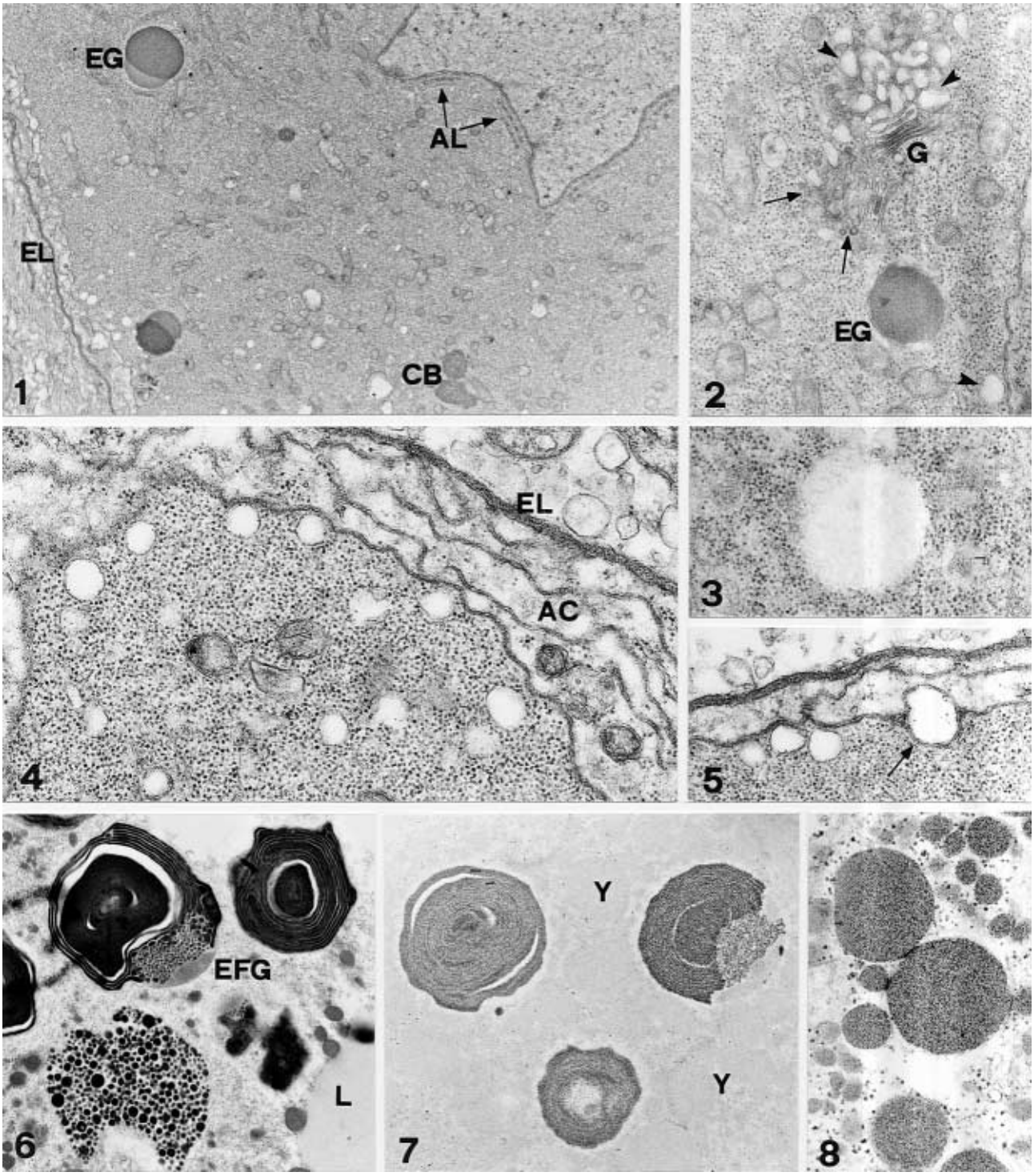

Fig. 1. – Growing oocyte. AL, annulate lamellae; CB, chromatoid body; EG, egg granule, EL, extracellular lamina. x17 500

Fig. 2. – Growing oocyte. A Golgi area (G) and a nascent egg granule (EG). Arrows point to small vesicles with a content of medium electron-density, arrowheads indicate larger vesicles with a translucent material . x24 000

Fig. 3. – Growing oocyte. Enzymatic extraction, Pronase incubation. The egg granule content is partially digested. x28 500

Fig. 4. – Nearly mature oocyte. Some translucent vesicles under the oolemma. EL, extracellular lamina, AC, accessory cells. x30 000

Fig. 5. – A translucent vesicle (arrow) in the process of fusing with the plasma membrane and releasing the content into the extracellular space. x25 000

Fig. 6. – Developing vitellocyte. Some eggshell-forming globules (EFG) at different stages of maturation. L, lipid. x12 800

Fig. 7. – Mature vitellocyte. Locke and Krishnan test for polyphenols, unstained section. A silver precipitate is exclusively on the eggshell-forming globules. Y, yolk. x12 000

Fig. 8. – Mature vitellocyte. Thiéry test for polysaccharides and glycoproteins, unstained section. A fine silver precipitate is visible on the yolk globules and on glycogen particles. x23 500

ture of eggshell-forming globules) rather than characteristics typical of the taxon Rhabdocoela + Prolecithophora. These data support the observation that the spermiogenesis of *Troglocaridicola* sp. differs from that of other temnocephalids and shows features that resemble those of Proseriata (8). Taken together, these ultrastructural findings on the male and female gonads give rise to some doubts about the place of scutariellids in the monophylum Eulecithophora and strongly suggest reconsideration of the supposed close phylogenetic relationships between Scutariellida and Temnocephalida.

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TAXONOMY AND ECOLOGY

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Taxonomic redescription of *Phagocata sibirica* and comparison with *Phagocata vivida* (Tricladida, Paludicola)

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ABSTRACT. Insufficient knowledge on the morphology and anatomy of the planarian flatworm *Phagocata sibirica* (Zabusov, 1903) has hampered adequate comparisons between species of *Phagocata* from the Far East and the assessment of biodiversity in this group of species. On the basis of an old sample of specimens, collected near the type locality in the vicinity of Lake Baikal by Russian workers who earlier had made important contributions to our knowledge on this group of flatworms, a redescription of the species is provided. The study of these specimens, together with information published by Russian workers, contributes to a much more detailed and comprehensive knowledge on the anatomy of *Ph. sibirica*. Furthermore, this new evaluation points to the fact that there is only one character in which the species *Phagocata fontinalis* (Zabusov, 1903) differs from *Ph. sibirica*. Previously, the species *Phagocata vivida* was sometimes considered to be conspecific with *Ph. sibirica.* On the basis of the examination of new material of *Ph. vivida* (Ijima & Kaburaki, 1916), collected from a new Siberian locality, together with information from the literature, it is concluded that *Ph. sibirica* and *Ph. vivida* cannot be considered as conspecific.

KEY WORDS: Platyhelminthes, Tricladida, Paludicola, *Phagocata sibirica*, *Phagocata vivida*, redescription, taxonomy, morphology

INTRODUCTION

Systematic biological revisions frequently are hampered not only by the complexity of nature itself but also by inadequate species accounts, according to modern standards, and by taxonomic descriptions in a language that prevents easy consultation by the world community of scientists. The Holarctic genus *Phagocata* Leidy, 1847 (Fig. 1) illustrates all of these complexities.

Recently, SLUYS et al. (1995) explored the phylogenetic systematics of the genus, as currently defined. Although it was clear that the current genus *Phagocata* subsumes a heterogeneous group of species, they found only few apomorphic characters suggesting some presumably monophyletic groups within *Phagocata* s.l.

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In a first preliminary, Russian and German account ZABUSOV (1903a) provided a superficial diagnosis of *Phagocata sibirica* (Zabusov, 1903) (olim *Planaria sibirica*) in that it lacks relevant anatomical and histological information. In a second preliminary report ZABUSOV (1903b) gives an equally short and non-informative description of a new variety, *Planaria sibirica* var. *fontinalis*, which is now usually considered to be a separate species, viz. *Phagocata fontinalis* (Zabusov, 1903). That *Ph. fontinalis* represents a separate species was for the first time suggested by LIVANOV & ZABUSOVA (1940), who redescribed the species and placed it into their new genus *Penecurva* Livanov & Zabusova, 1940; later, *Penecurva* was synonymized with *Phagocata* by KENK (1974). The same workers published a redescription (also in Russian) of *Ph. sibirica* under the generic name *Penecurva* (LIVANOV & ZABUSOVA, 1940). The only other more or less detailed, Russian accounts on *Ph. fontinalis* and *Ph. sibirica* are those of DYGANOVA & PORFIRJEVA (1990).

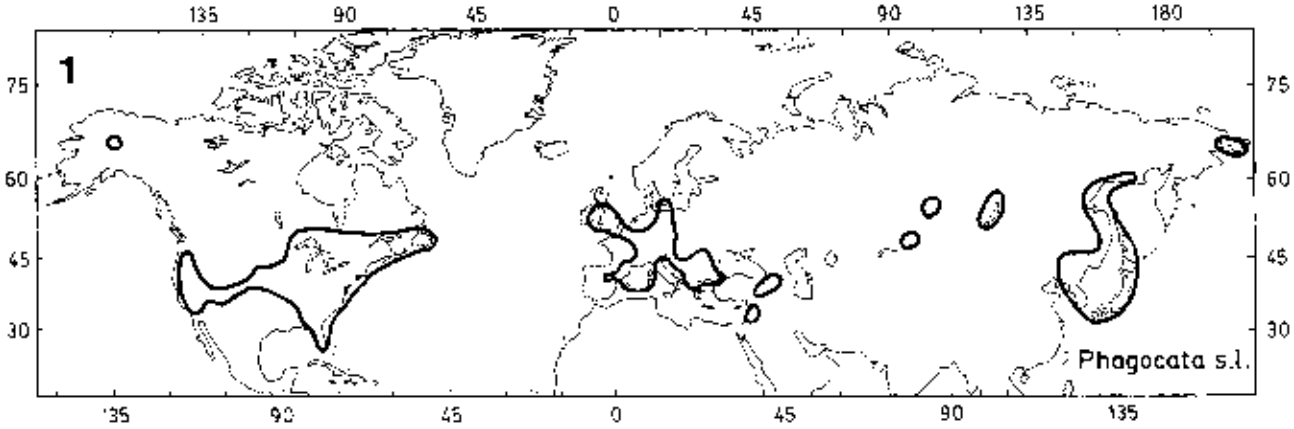


Fig. 1. – Generalized distribution map of the genus *Phagocata* sensu lato.

Present insufficient knowledge on *Ph. sibirica* has already hampered adequate comparisons between species of *Phagocata* from the Far East (KAWAKATSU et al., 1994, 1995), although this has not stopped several workers from assigning their laboratory organisms to the species *Ph. sibirica* (VYKHERESTYUK & KLOCHKOVA, 1984; CHELOMINA & PASHKOVA, 1991; BURENINA, 1993). Therefore, it is fortunate that the collections of the Museum für Naturkunde in Berlin (ZMB) house specimens presented by Livanov and identified as *Penecurva sibirica*. The present paper describes these specimens, thus contributing to a better understanding of the specific characteristics of *Phagocata sibirica*. Furthermore, the morphology of the species is compared with that of an Asian congener, *Phagocata vivida* (Ijima & Kaburaki, 1916), for which we describe specimens from a new Siberian locality that are now housed in the Zoological Museum Amsterdam (ZMA).

SYSTEMATIC SECTION

Order Tricladida Lang, 1884
Suborder Paludicola Hallez, 1892
Family Planariidae Stimpson, 1857
Genus *Phagocata* Leidy, 1847

Phagocata sibirica (Zabusov, 1903)

Material examined

ZMB 8074, Siberia, Baikal mountains, River Tscheremschan (= Cheremshanaya), preserved specimens. Two of the animals were sectioned at intervals of 6 µm and stained in Mallory-Heidenhain (Mallory-Cason): ZMB 8074-1, sagittal sections on 4 slides; ZMB 8074-2, sagittal sections on 6 slides.

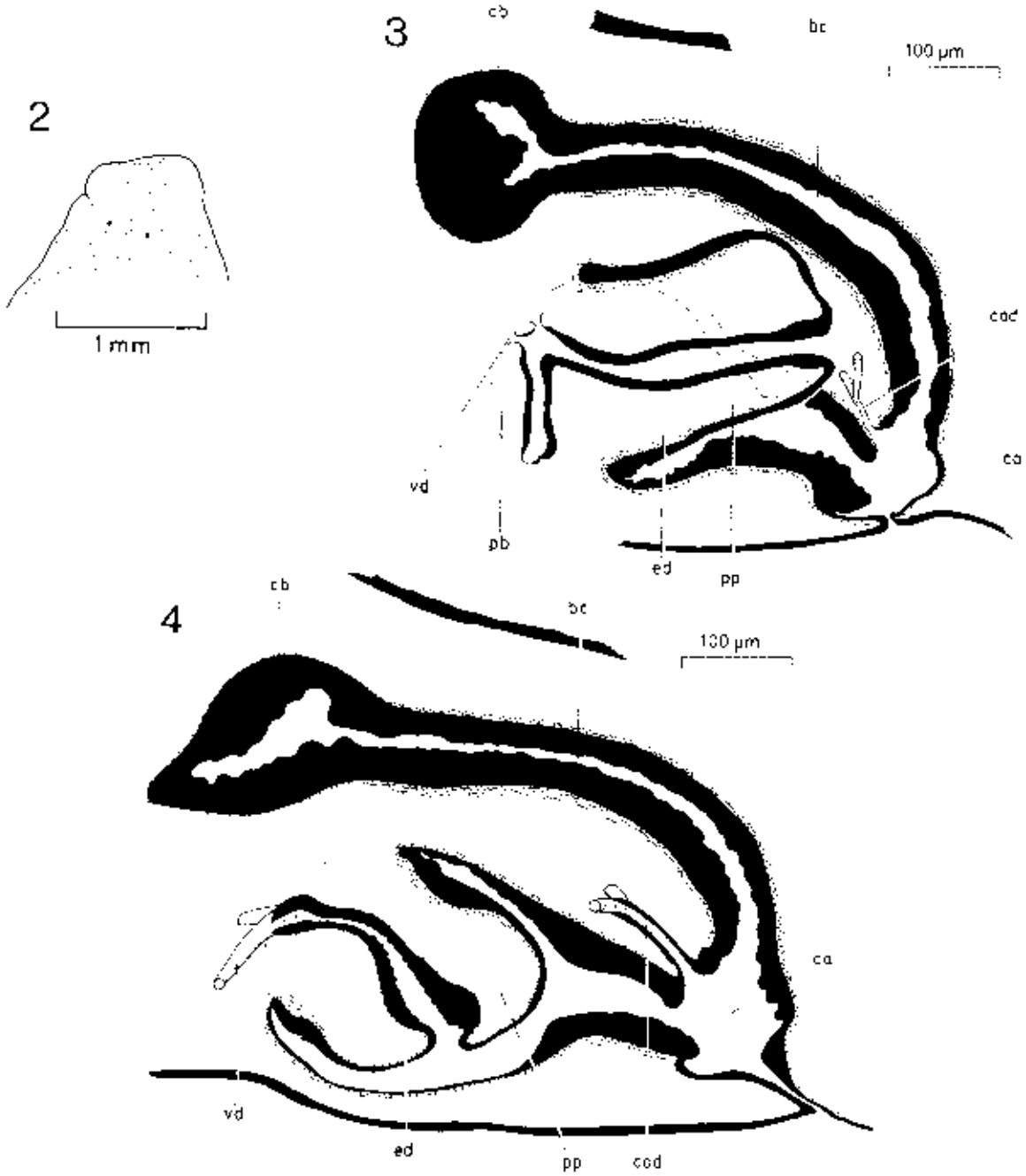
With respect to this sample the entry in the catalogue of the museum reads as follows: "Penecurva sibirica Sab., Sibirien, Baikalgebirge, Fluss Grosser Tscheremschan, 3. VI. 1931, N. Livanov Kasan ded., Frau Sa..... det., Eing. 1934". According to the late Dr. Hartwich (in litt.) of the museum, the name of the person who identified the material is hardly legible, but according to the length of the word and the number of letters it may be the name "Sabussov". According to Dr. Harwich the situation may be interpreted such that Livanov's co-author Zabusova (cf. LIVANOV & ZABUSOVA, 1940) identified this material (and probably also collected the sample) and that the specimens were presented as a gift to the museum by Livanov in 1934.

Redescription

The largest preserved specimen is about 11 mm long and 1.5 mm wide. The dorsal surface is brownish, the ventral surface is pale. The anterior end shows two unpigmented auricles (Fig. 2). In the preserved specimens, the pharynx measures about one-third of the body length. The mouth opening is situated at the posterior end of the pharyngeal cavity.

The numerous small testes are situated ventrally and extend from the level of the ovaries into the posterior end of the body. The paired ovaries are situated directly posterior to the brain; vitellarian follicles, located mainly in the ventral body region, occur throughout the body length.

The penis papilla is a blunt, stubby structure lined with a nucleate epithelium, which is underlain with circular and longitudinal muscles, respectively; the penis bulb is moderately muscular (Figs. 3, 4). The animals are characterized by a slightly asymmetrical penial papilla, due to a ventrally displaced ejaculatory duct that opens at the tip of the papilla. Immediately after having penetrated the musculature of the penis bulb, the vasa deferentia open into the slightly expanded proximal section of the ejaculatory duct. From hereon, the ejaculatory duct narrows slightly before expanding again and opening at the tip of the penial papilla; the duct is lined with tall, nucleated cells. In specimen ZMB 8074-1 the proximal section of the ejaculatory duct gives rise to a, probably atypical, ventrally directed duct that seems to extend somewhat beyond the perimeter of the penial bulb.



Figs. 2-4. – *Phagocata sibirica*. (2) ZMB 8074, anterior end of preserved specimen; (3) ZMB 8074-1, sagittal reconstruction of the copulatory apparatus; (4) ZMB 8074-2, sagittal reconstruction of the copulatory apparatus. Abbreviations: bc, bursal canal; ca, common atrium; cb, copulatory bursa; cod, common oviduct; ed, ejaculatory duct; pb, penis bulb; pp, penial papilla; vd, vas deferens.

The male atrium that houses the stubby penial papilla narrows considerably before communicating with the common atrium; the female atrium is very small.

The common oviduct opens at the junction between male and common atrium. Shell glands could not be observed unequivocally, maybe due to the relatively weak staining of the preparations.

A small copulatory bursa is situated antero-dorsally to the penial bulb; there appeared to be no difference between the lining of the bursa and that of the bursal canal. The bursa communicates with a bursal canal that opens into the dorsal portion of the common atrium; the canal is lined with a tall, nucleated epithelium that is overlain with a well developed layer of intermingled longitudinal and circular muscles.

Phagocata vivida (Ijima & Kaburaki, 1916)

Material examined

ZMA: V.Pl. 914.1, Khor River, near Khabarovsk, Siberia, Russia, 4 September 1997, sagittal sections on 8 slides; V.Pl. 914.2, ibid., sagittal sections on 7 slides; V.Pl. 914.3, ibid., sagittal sections on 13 slides. Sections were made at intervals of 8 µm and were stained in Mallory-Heidenhain (=Mallory-Cason).

Description

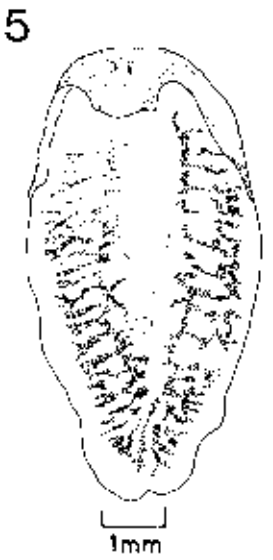
The largest preserved specimen measured about 10 x 3.5 mm. The dorsal surface is brown, the ventral body surface pale; the anterior end is provided with two unpigmented auricles (Fig. 5). The pharynx is located in the middle of the body and measures between one-fourth and one-fifth of the body length, in preserved specimens. The mouth opening is located at the posterior end of the pharyngeal cavity.

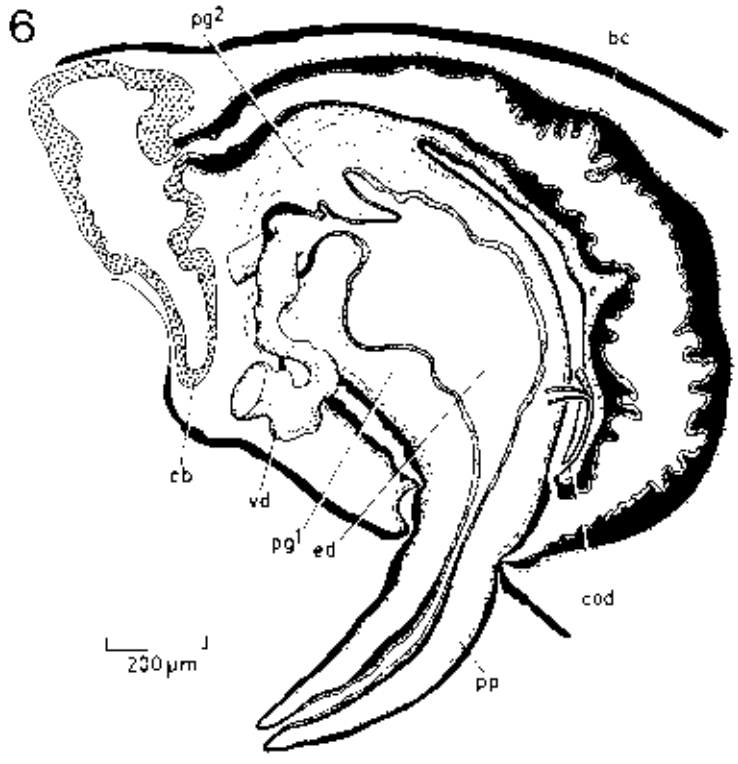
The testis follicles occupy most of the dorso-ventral space and are distributed throughout the body length. The ovaries are located at a short distance (approx. 300 µm) behind the brain. Vitellarian follicles are well developed, occupying the entire dorso-ventral space and extending throughout the body.

In all three of the specimens examined the penial papilla extends through the gonopore (cf. Fig. 6), most likely as a result of the fixation in 70% ethanol. In these specimens the penial papilla is an elongated cone, covered with a nucleated epithelium, which is underlain with a well developed layer of circular muscle, followed by a layer of longitudinal muscle.

At the level of the copulatory apparatus the vasa deferentia expand to form large spermiducal vesicles. Subsequently, the ducts narrow and recurve before penetrating the penis bulb. Once within the bulb, the vasa deferentia expand again before separately opening into the intrabulbar seminal vesicle; the intrabulbar, expanded section of the vasa deferentia is surrounded by a coat of mostly circular muscle fibres. The seminal vesicle, which is lined with a nucleated epithelium, communicates with the highly expanded proximal section of the ejaculatory duct. It may well be that in animals with a more contracted penis papilla this expanded proximal portion of the ejaculatory duct is located within the penial bulb and then represents a sort of bulbar cavity. In the present specimens this presumed bulbar cavity gradually narrows to form the ejaculatory duct, which traverses the penis to open at the tip of the penial papilla; the ejaculatory duct is lined with a cuboidal to tall, nucleated epithelium.

The presumed bulbar cavity and the interconnecting duct between the cavity and the seminal vesicle receive the secretion of abundant eosinophilic glands. Another type of penis gland is also highly developed and discharges a more granular erythrophilic secretion into the





Figs 5-6. – *Phagocata vivida* (5) ZMA: V.Pl. 914, ventral view of preserved specimen; (6) ZMA: V.Pl. 914.1, sagittal reconstruction of the copulatory apparatus. Abbreviations: bc, bursal canal; cb, copulatory bursa; cod, common oviduct; ed, ejaculatory duct; od, oviduct; pg^1, first type of penial gland; pg^2, second type of penial gland; pp, penial papilla; sg, shell gland; spe, spermatophore; sv, seminal vesicle; vd, vas deferens.

ejaculatory duct; most of the glands open into the proximal section of the duct, whereas the distal part of the ejaculatory duct receives much less secretion.

Before uniting to form the common oviduct, the distal sections of the separate oviducts receive the secretion of erythrophilic shell glands; the glands do not open into the common oviduct. Oviducts and common oviduct are lined with a cuboidal, nucleated epithelium. The common oviduct opens through the roof of the most distal (posterior) section of the male atrium, i.e. at the junction of the male and common atrium (a separate female atrium practically being absent); the male atrium is lined with a cuboidal, nucleated epithelium.

A relatively large copulatory bursa is located directly anterior to the hemispherical penis bulb; the bursa of specimen V.Pl. 914.2 contains the distinct remains of a spermatophore. A broad bursal canal connects the bursa with the common atrium. The bursal canal is lined with a nucleated, glandular epithelium, the secretion accumulating in the apical sections of the cells; the canal is covered with a subepithelial layer of circular muscle, followed by a layer of longitudinal muscle.

COMPARATIVE DISCUSSION

Phagocata sibirica

When the anatomy of the specimens from the River Cheremshanaya is compared with the descriptions of *Ph. sibirica* provided by Zabusov (1903a) and Livanov & Zabusova (1940), it is important to note that the gross morphology of the reproductive apparatus of the specimens examined suggests that they are not fully mature. Notably their small copulatory bursae, densely packed with small cells, and the apparent absence of shell glands seem to indicate that the animals are not in a state of full maturity. However, the same appears to have been the case with some of the animals studied by Zabusov (1903a) and Livanov & Zabusova (1940). The latter mentioned that the majority of their specimens, collected in 1931, and some collected by Zabusov (1903a, b) have evidently no mature genital organs. Furthermore, Livanov & Zabusova (1940) provide a description of specimens that are not completely mature but also give an account of the anatomy of animals that are in a state of full maturity. It is to be expected that the animals from the River Cheremshanaya will resemble the not fully mature specimens described by Livanov & Zabusova (1940) since they probably stem from the same sample taken in 1931.

According to Zabusov (1903a), *Ph. sibirica* is characterized by a male atrium that via a kind of channel communicates with the common genital atrium. Although Livanov & Zabusova (1940) interpreted this feature to be an artefact, it does hold true also for the Cheremshanaya specimens, in which the male atrium narrows considerably before communicating with the common genital atrium.

Zabusov (1903a) and Livanov & Zabusova (1940) described for *Ph. sibirica* an acentral, ventrally displaced ejaculatory duct running through a short and blunt penial papilla, representing features that are present also in the Cheremshanaya animals. Livanov & Zabusova also mentioned that in fully mature animals the ejaculatory duct receives the secretion of shell glands; another type of secretion would be discharged into the small seminal vesicle at the proximal end of the ejaculatory duct.

As is the case with the material examined, Livanov & Zabusova (1940) were also unable to discern shell glands opening into the oviducts and/or common oviduct of their not completely mature specimens; Zabusov does not mention the shell glands, but his drawing (Zabusov, 1903a, fig. 14) suggests that they open into the common oviduct. Livanov & Zabusova observed that in their fully mature animals shell glands open into the distal sections of the oviducts and into the proximal part of the common oviduct.

Zabusov (1903a) did not say anything about the testes, but Livanov & Zabusova (1940) mentioned that the follicles extend to the dorsal body surface when they are fully developed. This implies, in our opinion, that the testes are ventral in less mature specimens, such as the Cheremshanaya animals.

According to Livanov & Zabusova (1940) the copulatory bursa occupies only the dorsal half of the body in less mature specimens, as we observed in the Cheremshanaya animals, but that the bursa occupies the entire dorso-ventral space in fully mature specimens. Livanov & Zabusova described the musculature of the bursal canal as consisting of intermingled layers of longitudinal and circular muscle fibres, both in fully mature and less mature specimens; an intermingled muscle coat was observed also in the Cheremshanaya animals.

One last, and less important, feature in which the material examined agrees with the accounts of Livanov & Zabusova (1940) and Zabusova-Zhdanova (1962) on *Ph. sibirica* is that the pharynx measures about one-third of the body length.

On the basis of the comparisons discussed above, we have concluded that the Cheremshanaya specimens indeed concern representatives of the species *Ph. sibirica*, albeit in a not fully mature state.

When the characteristics of *Ph. sibirica* are compared with those of *Ph. fontinalis*, as described by Zabusov (1903b) and Livanov & Zabusova (1940), there is actually very little that might be considered supportive of the presumed specific status of the last-mentioned species. Initially, the species was merely recognized as a variety of the species *Ph. sibirica* on the basis of a slightly different configuration of the intestinal branches: *Ph. sibirica* var. *fontinalis*. However, according to Livanov & Zabusova

(1940) *Ph. fontinalis* also differs from *Ph. sibirica* in details of its copulatory apparatus. Although LIVANOV & ZABUSOVA (1940) gave a comprehensive description of *Ph. fontinalis* there is actually only one character that seems to be really different between the two species, i.e. the presence of a non-intermingled coat of circular and longitudinal muscle on the bursal canal of *Ph. fontinalis*, thus contrasting with the coat of intermingled muscle in *Ph. sibirica* .

Phagocata vivida

Our conclusion that the Khabarovsk specimens belong to the species *Ph. vivida* has been reached after detailed comparison of their features with other, congeneric species. Notably, for only a few species of *Phagocata* sensu stricto (cf. SLUYS et al., 1995) have testes been reported that occupy most of the dorso-ventral space and extend throughout most of the body: *Ph. kawakatsui* Okugawa, 1956 (from Shikoku, Chûgoku, Kinki, and Chûbu Regions in Honshû, central Japan), *Ph. suginoi* Kawakatsu, 1974 (from Hokuriku Region in Honshû, central Japan), *Ph. tenella* Ichikawa & Kawakatsu, 1963 (from Hokkaidô, northern Japan), *Ph. teshirogii* Ichikawa & Kawakatsu, 1962 (from Tôhoku Region in Honshû, northern Japan), *Ph. vivida* (from Kyûshû, Shikoku, Honshû, and Hokkaidô, Japan; also distributed in the Korean Peninsula and northeastern China). However, the Khabarovsk specimens do not exhibit a sphincter on the bursal canal (as in *Ph. kawakatsui*), an expanded common oviduct (as in *Ph. suginoi*), an expanded and tall epithelium at the distal, vaginal end of the bursal canal (as in *Ph. tenella*), or the enlarged, intrabulbar parts of the vasa deferentia opening into the equally sized bursal cavity (as in *Ph. teshirogii*). The copulatory apparatus of the Khabarovsk animals, however, conforms to the situation described for *Ph. vivida*. Particularly noteworthy in *Ph. vivida* are the expanded intrabulbar parts of the vasa deferentia, opening into a small seminal vesicle that, via a constriction, communicates with the bulbar lumen, the latter tapering to form the ejaculatory duct (cf. KAWAKATSU et al., 1982, 1994); these are all features that also occur in the Khabarovsk specimens. Furthermore, *Ph. vivida* has been described with well developed penial glands opening into the bulbar lumen and the ejaculatory duct, and with a highly glandular epithelium of the bursal canal (KAWAKATSU et al., 1982, 1994), being features that are present also in the specimens from Khabarovsk. The only difference between previous accounts of *Ph. vivida* and the present material concerns the presence of a much more developed muscle coat on the distal section of the bursal canal, consisting of a thin inner layer of longitudinal muscle, a thick layer of circular muscle, followed by a well-developed layer of longitudinal muscle. This thick coat of muscle contrasts with the thin and simple musculature covering the bursal canal of the Khabarovsk animals.

Confusion and distribution

In the past there has been some confusion about the identity of *Ph. sibirica* and *Ph. vivida*, which were sometimes considered as conspecific (cf. TU, 1939). However, on the basis of the descriptions provided above it can be concluded that they are separate species, each characterized by several distinct features.

Ph. vivida occurs both in Japan and on the mainland in the Far East (cf. KAWAKATSU et al., 1995, fig. 2). *Ph. sibirica* was first described from near Lake Baikal and since then has been found at several localities in the vicinity of the lake. Furthermore, the last-mentioned species has been reported also from near Lake Belove and from localities in Primorskiy Kray and Khabarovsk Kray (cf. KAWAKATSU et al., 1995, fig. 2); in addition, findings have been reported also for the Zabaykalsk (DYGANOVA & PORFIRJEVA, 1990) and Krasnoyarskiy territories (ZABUSOVA-ZHDANOVA,1962). In view of the better understanding that we now have of the anatomy of *Ph. sibirica* it is desirable that records far from the type locality and the environs of Lake Baikal are substantiated by new findings and by voucher material.

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The genus *Humbertium* gen. nov., a new taxon of the land planarian family Bipaliidae (Tricladida, Terricola)

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ABSTRACT. A new generic name is proposed for a group of 23 bipaliid species that share a unique feature of the female copulatory organ: the ovovitelline ducts turn dorsally before reaching the gonopore, and enter the female organ from an antero-dorsal aspect. They are distributed in Madagascar, India, Sri Lanka, China, Sarawak, and West Malaysia.

KEY WORDS: Platyhelminthes, Terricola, land planarian, copulatory apparatus, *Humbertium* n.g., geographic distribution

INTRODUCTION

The land planarian family Bipaliidae Stimpson, 1857 exhibits species with a diverse morphology, which is prominent in their copulatory organs. VON GRAFF (1899) used the diversity of head form as a basis for the new genera *Placocephalus* and *Perocephalus*, but did not use copulatory organs in his taxon definitions. More recently, OGREN & SLUYS (1998) have used copulatory organ morphology to unravel phylogenetic relationships in the family Bipaliidae. By examining a number of characters related to the copulatory organ, and presumed to be of phylogenetic importance, they constructed a phylogenetic tree for five major, *a priori* groups. Their Group A, comprising species with a pseudophallus (an elongate penis sheath), has been recently named as the new genus *Novibipalium* Kawakatsu, Ogren, & Froehlich, 1998. This paper proposes a new generic name for Group B1 + Group B2, which is characterized by the proflex condition of the ovovitelline ducts. In this paper the same basic characters, definitions and symbols are used as in the former paper (OGREN & SLUYS, 1998).

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RESULTS

The 23 species falling within the groups B1 and B2 recognized by OGREN & SLUYS (1998), clearly belong to the family Bipaliidae in that they possess its defining characters: semilunar head, narrow creeping sole, continuous sensory groove, collared pharynx, and penis papilla. Moreover, the 23 species that will constitute the new genus are part of a selected group in Bipaliidae because they share the vertical position of the female organ (FCA-1), with genera *Bipalium* and *Novibipalium* (OGREN & SLUYS, 1998: fig. 7). These species of the new genus are considered to be a natural group or monophylum because they possess the same derived character, viz., the antero-dorsal entrance of the ovovitelline ducts into the female organ (character OVD-1). This suggests common ancestry. Therefore, we do here propose to recognize this group of proflex bipaliid species as a separate taxon, for which we provide the following new generic name and diagnosis:

Humbertium gen. nov.

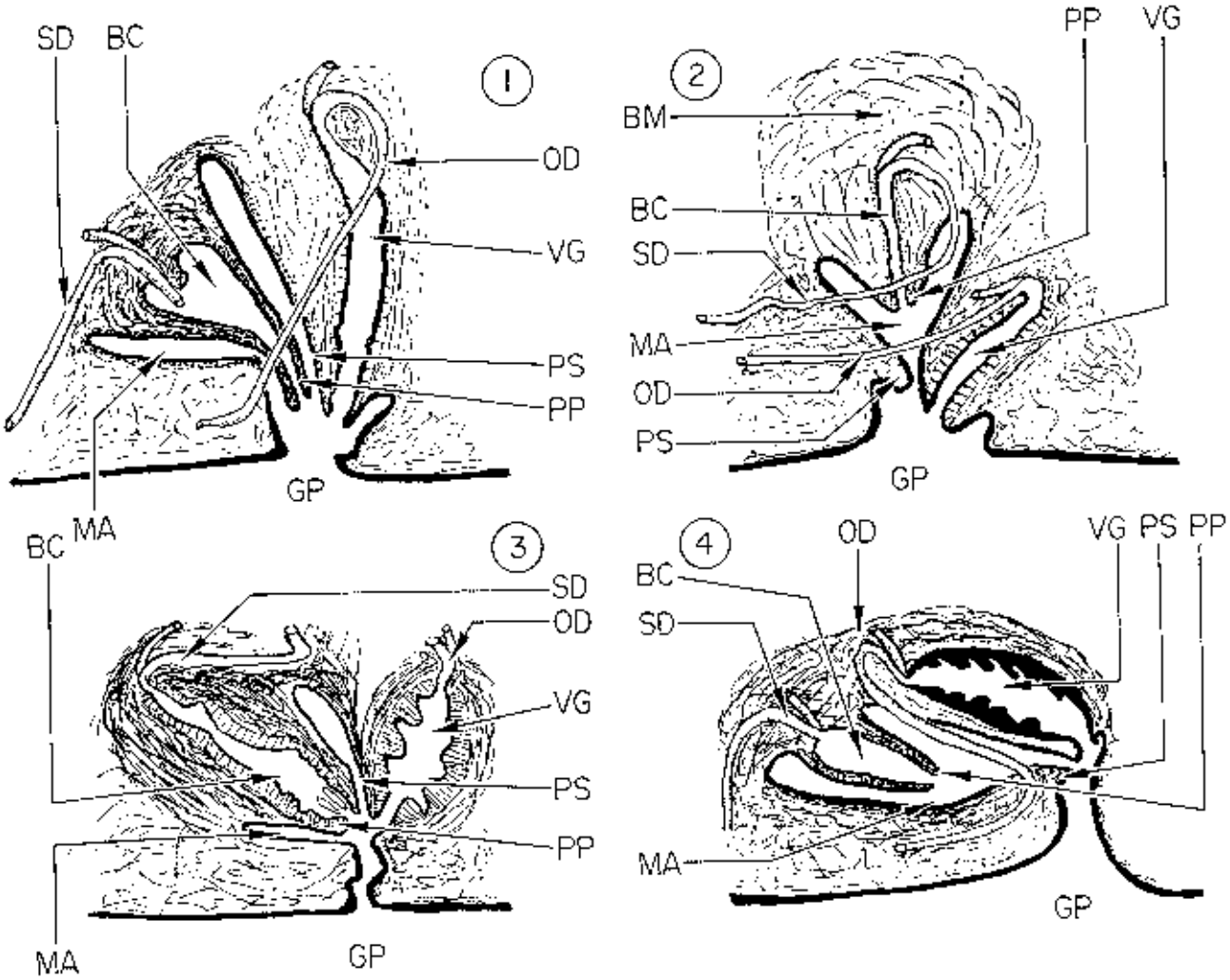
Diagnosis: Bipaliidae with ovovitelline ducts turning dorsally before reaching the genital pore and having an antero-dorsal entrance to the female organ. Type species: *Perocephalus ravenalae* von Graff, 1899. [Copulatory apparatus described by MELL (1903)].

Etymology: the genus is named for Aloïs Humbert whose early paper (Humbert & Claparède, 1862), describes several bipaliid species from Sri Lanka, now included in the new genus.

In our previous paper (Ogren & Sluys, 1998) schematic profiles of the copulatory apparatus were provided for the *Humbertium* species *H. ravenalae* (von Graff, 1899) and *H. proserpina* (Humbert & Claparède, 1862). Repetition of these diagrams is not considered necessary to the present paper. Nevertheless, for the purpose of the current discussion, additional examples of copulatory apparatus are provided for *H. kelleri* (von Graff, 1899), *H. umbrinum* (Geba, 1909), *H. dodabettae* (De Beauchamp, 1930), and *H. woodworthi* (von Graff, 1899) (Figs 1-4). In the new genus *Humbertium* the male copulatory organ has essentially those features found in the genus *Bipalium*: large rounded muscular bulbus, cone-shaped penis papilla and well-developed penis sheath. The female organ is vertical with the vagina opening into the common antrum. However, in *Humbertium* the ovovitelline ducts approach from the anterior, turn dorsally near the gonopore, and enter the female organ from an anterior-dorsal aspect (Figs 1-2).

The species that we consider to belong to the new genus *Humbertium* are listed in Table 1. For bibliographic information on the species we refer to Ogren & Kawakatsu (1987, 1988).

As pointed out previously, within *Humbertium* there is a group (Group B2 of Ogren & Sluys, 1998) consisting of three species with a prolapsed condition of the female organ (character FCA-2, organ tilted anteriorly); viz., *H. ceres, H. proserpina, & H. woodworthi*. They appear to



Figs 1-2. – Copulatory apparatus profiles showing location of ovovitelline ducts. Fig. 1. *Humbertium kelleri* (von Graff, 1899) (olim *Bipalium*) profile after Mell (1903, pl. 32, fig. 6); Fig. 2. *H. umbrinus* (Geba, 1909) (olim *Placocephalus*), profile after Geba (1909, pl. 20, fig. 5). Figures represent schematic sagittal views, modified and simplified; anterior is to the left, ventral is bottom; male organ on the left. BC, bulbus cavity; BM, bulbus muscle; GP, genital pore (=gonopore); MA, male genital antrum; OD, ovovitelline duct; PP, penis papilla; PS, penis sheath; SD, sperm duct; VG, vagina (= female canal).

Figs 3-4. – Copulatory apparatus profiles showing location of ovovitelline ducts. Fig. 3. *Humbertium dodabettae* (de Beauchamp, 1930) (olim *Bipalium*), profile after de Beauchamp (1930: 687, fig. 8); Fig. 4. *Humbertium woodworthi* (von Graff, 1899) (olim *Bipalium*), profile after Mell (1903, pl. 31, fig. 4). Abbreviations as in Figs 1-2.

constitute a distinctive phylogenetic sister group to the other members of *Humbertium*, represented in Group B1 (cf. OGREN & SLUYS, 1998) of which the monophyletic status remains to be supported by apomorphic characters.

Table 1 shows that the greatest external variation occurs in Madagascar. External dorsal appearance of species shows no clear pattern and varies between species from one stripe, to two, three, four and four-five stripes. There is also reported intraspecific variation in number of stripes. There are three species with transverse banding known from Sarawak and West Malaysia. Two species with prolapsed female organ (*H. ceres* and *H. proserpina*) occur on Sri Lanka; Another prolapsed species (*H. woodworthi*) is found only on Madagascar.

DISCUSSION

Taxonomy

The general characters of the male organ in *Humbertium* are shared with species of the genus *Bipalium* as shown in the character matrix of OGREN & SLUYS (1998). *Humbertium* species are different from the genus *Novibipalium* because in *Humbertium* species the male antrum wall (character MAW) does not generally form a pseudophallus. However, one species, *Humbertium pseudophallicum* (de Beauchamp, 1925) has an elongated, moderately muscular pseudophallus (without an inner, thick layer of circular fibers) formed by the male antrum wall (MAW). This is considered an unusual feature for a species of this genus. The several characters employed in this study were not considered the result of distortion from fixation or preservation.

The present generic definition is based on the single apomorphic condition (OVD-1) that can be learned only after histological sections have been made and examined. External body characters are readily observed, but do not correlate with the presence of the proflex (OVD-1) condition. For example, head form is regularly placoid, with a few species having a head with prominent recurved auricles. Great variability exists for dorsal body patterns. Some species are plain dorsally; or have longitudinal stripes; or show transverse banding. Because these features are not unique to *Humbertium*, and do not correlate with the proflex condition, they are not useful as reliable features to supplement the generic definition.

Biogeography

In the map published by OGREN et al. (1992: p. 99, Plate I) the distribution of Bipaliidae is shown (see also KAWAKATSU & OGREN, 1998: 8, fig. 11); their distributional records are also registered in a biogeographic database of all nominal species of terrestrial planarian (SLUYS, 1998, 1999). Species of *Humbertium* occur primarily in Madagascar, India, Sri Lanka and Sarawak. How can this disjunct distribution of *Humbertium* species be

TABLE 1

Species of *Humbertium*, with an indication of their external appearance and geographic distribution.

Ψ: This symbol indicates the species has the prolapsed condition (FCA-2) of female organ.
*: The asterisk indicates assignment is probable but not certain because the OVD-1 character is not clearly shown in diagram or text. Although these were reported as part of 23 species in Group B1 + B2 (OGREN & SLUYS, 1998), it is clear that we must await new knowledge of their copulatory organs before their present temporary inclusion within *Humbertium* can be supported. (Since in Madagascar all Bipaliidae species, where the copulatory organ is known, have the OVD-1 character, it is expected that this feature will be confirmed in the three marked species).

Madagascar
Humbertium ferrugineoideum (Sabussowa, 1925) comb. nov. (plain black)*
Humbertium ferrugineum (von Graff, 1899) comb. nov. (3 stripes)
Humbertium girardi (von Graff, 1899) comb. nov. (5 stripes)
Humbertium kelleri (von Graff, 1899) comb. nov. (3 stripes)
Humbertium ravenalae (von Graff, 1899) comb. nov. (2-4 stripes)
Humbertium sikorai (von Graff, 1899) comb. nov. (1-3 stripes)*
Humbertium umbrinum (Geba, 1909) comb. nov. (plain brown, paler medially)
Humbertium voigti (von Graff, 1899) comb. nov. (2 medial stripes)
Humbertium woodworthi (von Graff, 1899) comb. nov. (4 stripes) Ψ
India, Sri Lanka, China
Humbertium core (de Beauchamp, 1930) comb. nov. (3 stripes, India)
Humbertium depressum (Ritter-Záhony, 1905) comb. nov. (3 stripes, India & Sri Lanka)
Humbertium dodabettae (de Beauchamp, 1930) comb. nov. (plain, India)
Humbertium negritorum palnisium (de Beauchamp, 1930) comb. nov. (India)*
Humbertium ceres (Moseley, 1875) comb. nov. (2 medial stripes, Sri Lanka) Ψ
Humbertium diana (Humbert, 1862) comb. nov. (2 medial stripes, Sri Lanka)
Humbertium longicanale (Sabussowa, 1925) comb. nov. (1 stripe, China)
Humbertium phoebe (Humbert, 1862) comb. nov. (2 marginal stripes, Sri Lanka)
Humbertium proserpina (Humbert, 1862) comb. nov. (4 stripes, Sri Lanka) Ψ
Humbertium univittatum subboreale (Sabussowa, 1925) comb. nov. (plain, China)
Sarawak and West Malaysia
Humbertium penrissenense (de Beauchamp, 1925) comb. nov. (transverse band, Sarawak; cf. KAWAKATSU et al., 1998)
Humbertium pseudophallicum (de Beauchamp, 1925) comb. nov. (transverse band, Sarawak)
Humbertium penangense (Kawakatsu, 1986) comb. nov. (transverse band, W. Malaysia)

explained? It is possible that human migrations accidentally introduced species, for example from Madagascar to India. However, Continental Drift may provide an alternative explanation for the distribution pattern. Before major continental drifting these regions (Madagascar, India, Sri Lanka) were nearly adjacent land masses, being parts of Gondwanaland in the early Jurassic Period (HALLAM, 1994). Bipaliidae presumably had evolved before the time of the Cretaceous Period and populated part of Gondwanaland. By the late Cretaceous the landmasses had become separated, thus enabling speciation in the various land planarian populations. According to this viewpoint, *Humbertium* species of Madagascar would represent the result of speciation. Under this scenario, *Humbertium* species may have dispersed into West-central China, West Malaysia, and Sarawak after India had collided with the Asian land mass between 10 and 20 million years ago. This scenario therefore implies that Asian *Humbertium* species are more closely related to Indian species than to species from Madagascar. Future and more in-depth phylogenetic analyses of the genus may be able to test this prediction.

Humbertium species are the primary Bipaliidae on Madagascar. There are 23 bipaliids known from Madagascar (See the list of species for Madagascar in OGREN et al., 1997; those marked + have known copulatory apparatus). There are 11 species with copulatory apparatus known and all except one belong to *Humbertium*. The one exception is *Bipalium kewense* Moseley, 1878, which does not have the proflex (OVD-1) character. This species is known to be a cosmopolitan migrant with human activities (WINSOR, 1983), and is not considered part of the indigenous fauna of Madagascar. *Humbertium* species on Madagascar display the widest variety of longitudinal stripe patterns for the genus.

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Redescription of Japanese *Bdellocephala annandalei* from Lake Biwa-ko with comparative redescription of the Far Eastern and Kamchatkan *Bdellocephala* species (Tricladida, Paludicola)

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ABSTRACT. *Bdellocephala annandalei* Ijima et Kaburaki, 1916, is an endemic planarian of Lake Biwa-ko in Central Japan. This lake-dwelling species inhabits the muddy bottom of the northern basin of the lake (20-103 m in depth). The species is characterized by its comparatively large size, uniformly light brown coloration, two small eyes, and a subterminal, ventral adhesive organ on the frontal end. A detailed taxonomic redescription of *B. annandalei* is given here and compared with the other three Far Eastern and Kamchatkan *Bdellocephala* species inhabiting epigean waters.

KEY WORDS: Platyhelminthes, Dendrocoelidae, *Bdellocephala*, Lake Biwa-ko, Japan, taxonomy.

INTRODUCTION

Lake Biwa-ko, located in the central part of Honshû, is the largest and oldest lake in Japan. Topographically, the lake consists of two parts, the northern basin (a wide and deep part; deepest point, 103.6 m) and the southern basin (a narrow and shallow part). The stratigraphic records of sediment indicate that the former has existed continuously for 0.43 m. yr. (MEYERS et al., 1993).

The occurrence of a large, flat, leech-like animal in the profundal zone of the lake has been recognized by fishermen of Lake Biwa-ko. Scientific samples of this large planarian were first collected from the profundal area of the lake by T. N. Annandale (1876-1924) and T. Kawamura (1883-1964) in the autumn of 1915. Then, the species was described as *Bdellocephala annandalei* by IJIMA & KABURAKI (1916). Although this original description was very simple, it contains woodcuts of a general view of the body, an adhesive organ and a sagittal view of the copulatory apparatus. Later, KABURAKI (1922) gave a rather old-fashioned redescription of this species with three figures (an animal in color, and newly prepared figures of the adhesive organ and the copulatory apparatus).

The limnobiological survey made after the 1950s confirmed the presence of *B. annandalei* from various areas of the northern basin (KAWAKATSU, 1969; 80-81, pl. VIII, fig. 16; KAWAKATSU & NISHINO, 1993: 100, pl. III; 1994: 97-100; NISHINO & WATANABE, 2000). The latest distribution map of *B. annandalei* was shown in a paper by OKI et al. (1998: 316, fig. 1; see also KAWAKATSU & NISHINO, 1993: 100, pl. III; KAWAKATSU et al., 1996, 8: fig. 7). A complete list of literature for this lake-dwelling species will be found in KAWAKATSU & NISHINO (1993, 1994).

The basic purpose of this paper is to give a detailed redescription of this species from a modern taxonomic point of view. Chromosome numbers and the karyotype of *B. annandalei* have already been reported (OKI et al., 1998). The genus *Bdellocephala* de Man, 1875, is poorly defined (SLUYS et al., 1998). Based on the new data on the compar-

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ative anatomy of the copulatory apparatus of the known *Bdellocephala* species, some taxonomic remarks are made.

MATERIAL AND METHODS

Kawakatsu has numerous preserved specimens of *B. annandalei* from various stations of the lake. For the present taxonomic study, the following samples in his collection were used; Specimen Lot Number registered in Kawakatsu's fixing notebook (KSL No.; simply shown as MK in the following sections) is given for each vial of samples.

KSL No. 381: Off Ômi-Hachiman City (depth, 40 m); fixed with 3% formalin solution (Oct. 23, 1962; coll. Dr. M. Nagoshi). / KSL Nos. 392 and 393: Off Oki-no-shima Island (depth, 30-45 m); fixed with Bouin's fluid (Sept.-Oct., 1963; coll. Mr. R. Chaya). / KSL No. 394: Off Onoe (depth, 40-60 m); fixed with Bouin's fluid (Sept.-Nov., 1963; coll. Mr. S. Matsuoka). / KSL No. 428: the same area as KSL Nos. 392 and 393; fixed with Bouin's fluid (Sept.-Oct., 1964; coll. Mr. R. Chaya). / KSL No. 1666 (2 sexual and 2 asexual specimens of *Bdellocephala* sp. according to Dr. Teshirogi's team): Off the Tsuzurao-zaki Cape (depth, 40-60 m); fixed with Bouin's fluid (Sept. 29, 1981; coll. Mr. M. Yagihashi).

Three large sexual specimens from the KSL No. 392 stained with borax-carmine were prepared as whole mounts. Many sets of serial sections (7-8 µm) of the sexual specimens from the KSL Nos. 381, 392-394, 428, and 1666 were prepared. These sagittal, transverse and horizontal sections were stained with Delafield's hematoxylin and erythrosin or Mallory's triple stain.

Taxonomic comparison of lake-dwelling *B. annandalei* has been made with another epigean water species, *Bdellocephala brunnea* Ijima & Kaburaki, 1916. The following samples of the latter collected from the Lake Biwa water system were employed.

KSL Nos. 2287 (1 sexual and 1 asexual specimens) and 2288 (3 sexual specimens): Downstream of the Amagase Dam, Uji-gawa River (approximately 18 km downstream from the outlet of the lake), Uji City, in Kyôto Pref.; fixed with 70% ethanol (Oct. 31, 1997 and Jan.10, 1998; coll. Nishino, in cooperation with Dr. H. Abe in the former and Mr. N. Kobayashi in the latter. Serial sections with Mallory-Carson hematoxylin and eosin staining were prepared by Sluys and are now housed in the Zoological Museum, University of Amsterdam: V. Pl. 946.1 (sagittal sections on 2 slides; MK 2287-1); V. Pl. 946.2 (sagittal sections on 8 slides; MK 2287-2); V. Pl. 947.1 (sagittal sections on 8 slides; MK 2288-1); V. Pl. 947.2 (sagittal sections on 14 slides; MK 2288-2).

For comparative purpose, serial sections of *Bdellocephala borealis* Kawakatsu, 1978 (Kawakatsu's collection) and *Bdellocephala grubiiformis* (Zabusova, 1929) (Timoshkin's collection; loc. Dalnee Lake, Kamchatka) were restudied by Kawakatsu and partly by Sluys.

RESULTS

Systematic

Suborder Tricladida Lang, 1884
Infraorder Paludicola Hallez, 1892
Family Dendrocoelidae Hallez, 1892
Genus *Bdellocephala* de Man, 1875

Description of *Bdellocephala annandalei* Ijima et Kaburaki, 1916

Photographs of *B. annandalei* in life are shown in KAWAKATSU & NISHINO (1993: 98, pl. I, fig. A; see also fig. B for the preserved condition) and KAWAKATSU et al. (1996: 7, fig. 6 D) (Fig. 1). Large, sexually mature specimens measure up to 35-40 mm or more in length and 10 to 12 mm in width. The body is of a low rotundate shape with a pair of bluntly protruded, elongated auricles on each side. The colorless sensory organ with elongated oblanceolate shape is visible on the outer side of the auricles. Two small eyes, each surrounded by a lanceolate, pigment-free ocular area, are present at middle level of the head. The space between them is nearly one half the width of the head at the level of eyes. Behind the auricles, the body first narrows slightly, then widens at the level of pharynx and copulatory apparatus. The posterior end of the body is obtuse.

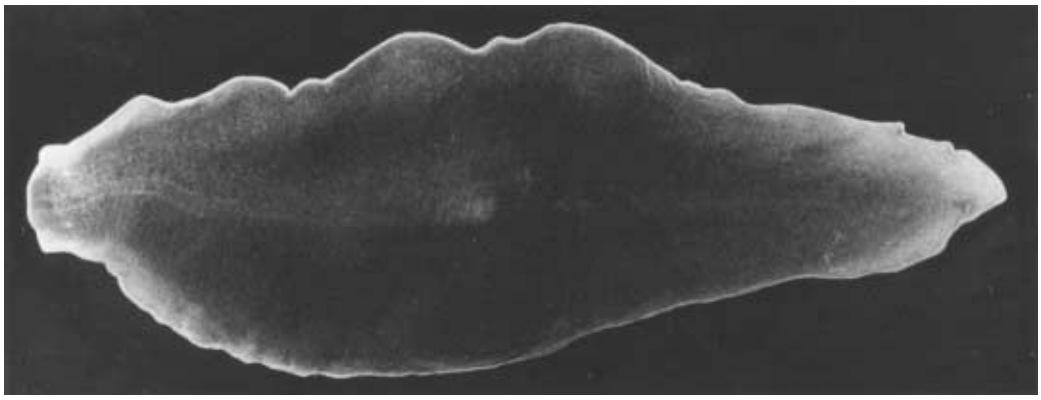

Fig. 1. – *Bdellocephala annandalei*. A dorsal view of a live specimen (ca. 50 mm long).

The ground color of the dorsal surface of the body is uniformly light brown to grayish tint. The body margin and the areas above the pharynx and copulatory apparatus are of a lighter hue. A narrow, indistinct, whitish, mid-dorsal double line can be seen on the prepharyngeal region of the body. The ventral surface is pale compared to that of the dorsal body.

B. annandalei has a frontal, subterminal, adhesive organ located on the ventral side of the body. It is a shallow, reniform organ with longitudinal folds. The surface of the organ is covered with glandular epithelium. Numerous, thickly swollen erythrophilic gland ducts filled with a granular secretion are found here; their cell bodies are scattered through the mesenchyme of this region. The muscular system of the organ is rather weakly developed. The marginal adhesive zone is well developed.

The subepithelial musculature of the dorsal body consists of the following layers: 1) a rather thick layer of circular fibres, with the more inside fibres being more loosely arranged, 2) a layer of diagonal fibres, and 3) a rather thick layer of loose, longitudinal (partly diagonal) fibres. On the ventral body, the subepithelial muscle zones are: 1) a rather thick layer of circular fibres, with the more inside fibres being more loosely arranged, 2) a thick layer of longitudinal muscle, and 3) a layer of diagonal fibres.

The pharynx is located at about the middle of the body and is one-fifth to one-sixth the body length. Its internal muscle zone consists of a very thick layer of intermingled circular and longitudinal fibres. The outer muscle zone of the pharynx consists of three layers: 1) thin, longitudinal fibres, 2) very thick, circular fibres and 3) rather thick, longitudinal fibres.

It was clear from the examination of whole mounts and histological sections of fully sexual specimens that the dorsal testes are small in size and numerous. Testes occur from behind the ovaries to nearly the posterior end of the body. In the prepharyngeal region, they are arranged on either side of the midline in eight to ten longitudinal zones, whereas in the pharyngeal and postpharyngeal regions there may be up to six zones. The spermiducal vesicles located on either side of the pharyngeal and postpharyngeal regions, from the anterior level of the mouth to the middle level of the penis bulb, are well developed in *B. annandalei*.

A pair of small ovaries occurs on the ventral side of the anterior region between the third and fourth intestinal diverticula. The two oviducts run posteriorly and unite to form a rather long common ovovitelline duct slightly anterior to the level of the genital pore. The yolk glands (or vitellaria) are distributed in the surrounding parenchyma.

The sagittal view of the copulatory apparatus of a well-extended specimen is shown in Fig. 2 (MK 392-a: redrawn from a pencil sketch of the organ from the same specimen in KAWAKATSU et al., 1996: 8, fig. 8).

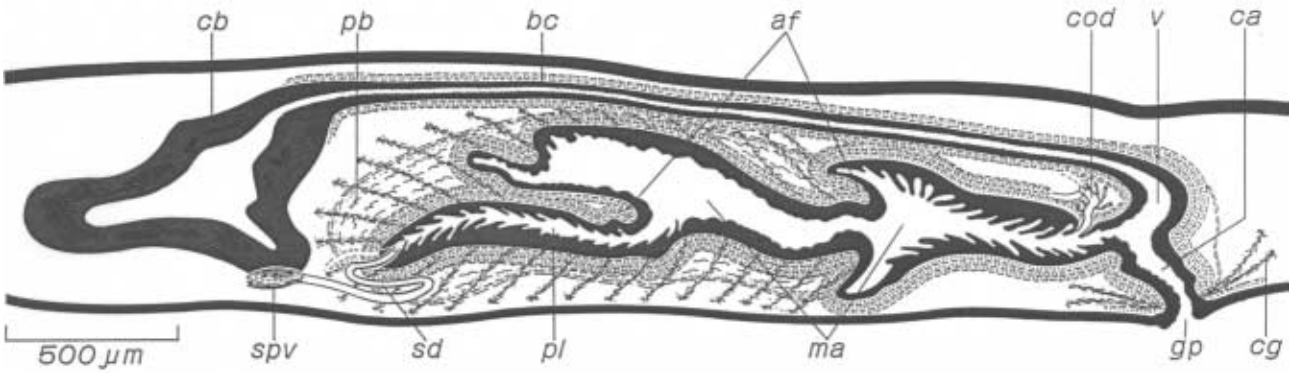

Fig. 2. – *Bdellocephala annandalei*. Sagittal view of the copulatory apparatus (MK 392-a). af, atrial fold; bc, bursal canal; ca, common genital antrum; cb, copulatory bursa; cg, cement gland; cod, common ovovitelline duct; gp, genital pore; ma, male genital antrum; pb, penis bulb; pl, penis lumen; sd, sperm duct (vas deferens); spv, spermiducal vesicle; v, vagina..

The male copulatory apparatus consists of two parts. An ovoid penis, of which the underside and the posterior half of the upperside, is embedded in the parenchyma; a wide, tubular, posterior portion has conspicuous folds. The former represents the penis bulb and is highly muscular. The tubular penis lumen is lined with a thick, highly glandular, nucleate epithelium with many plicae. Its surrounding muscular coat consists of three layers, i.e., a thin longitudinal, a thick circular layer intermingled with a few longitudinal fibres, and a rather thick, longitudinal layer. The two vasa deferentia (sperm ducts) open separately into the penis lumen, which opens posteriorly into the anterior portion of the male genital antrum.

The shape of the male antrum varies between sectioned specimens. It is essentially a wide, tubular cavity that opens into the anterior section of the common genital antrum; sometimes, it shows a uroceolate or a calceolate cavity. The male antrum has several well-developed, dorsal and ventral folds. Sometimes, the antrum is almost separated into two cavities by a pair of well-developed

folds. The entire wall of the male antrum is covered with a very thick, glandular, nucleate epithelium (with many plicae in the posterior narrow portion) below which there are two muscle layers, one thick circular layer intermingled with longitudinal fibres, and the other longitudinal. The penis bulb and the wall of the anterior half of the male antrum are pierced by many erythrophilic gland ducts. The common ovovitelline duct opens dorsally near the posterior terminal portion of the male antrum.

In many slides of *B. annandalei*, a poorly developed, penis papilla-like projection is formed by the atrial folds located at the anterior portion of the male genital antrum. This structure may function as the penis papilla, found commonly in many other species and genera of triclad turbellarians. The penis lumen in this species functions as the seminal vesicle and ejaculatory duct.

The copulatory bursa is a middle- to large-sized organ of an ovoid, or pear shape. The bursal canal is a very long, slender duct that runs dorsally to the penis bulb and male antrum, and opens into the roof of the common antrum. The canal is lined with a tall, glandular, nucleate epithelium. The muscular coat surrounding the anterior and middle sections of the canal consists of an inner, thin layer of longitudinal fibres, a middle, thin layer of circular ones and an outer, thin layer of longitudinal muscle fibres. The posterior terminal section of the canal forms a moderately developed vagina lined by a thick, glandular epithelium and a moderately thick muscle coat (especially the circular muscle fibres). The short, tubular, common genital antrum communicates ventrally into the genital pore. Weakly erythrophilic cement glands open into the terminal part of the common antrum.

The cocoon of *B. annandalei* is spherical in shape (3-4 mm in diameter), having no stalk.

Bdellocephala sp. reported as a new species (YAGIHASHI et al., 1995) is undoubtedly a juvenile form or a small-sized specimen of *B. annandalei* (OKI et al., 1998: 316). The sagittal view of the copulatory apparatus of one of these small specimens is shown in a previous paper (KAWAKATSU et al., 1996: 9, fig. 9, MK1666-a).

Comparative redescription of the Far Eastern and Kamchatkan *Bdellocephala* species

Among the three known *Bdellocephala* species in Japan, *B. brunnea* is a species distributed from Kyôto Prefecture to Aomori Prefecture in Honshû (KAWAKATSU, 1969). The occurrence of this species in Okushiri Island in Southern Hokkaidô was recently reported by NISHITANI et al. (1995; see also NISHITANI, 1998). This species is usually found in rather cool waters, such as shallow springs, spring-fed streams and outlets of lakes.

B. brunnea has a dark to blackish coloration and measures between 10 and 20 mm in length and 3 to 5 mm in width. The head is truncate (or retuse when in an elongated condition) with a pair of blunt auricles; two eyes are conspicuous. Morphologically and histologically, the adhesive organ in this species is more developed than in *B. annandalei*; both erythrophilic and cyanophilic glands can be found.

The animals examined from the Amagase Dam (KSL Nos. 2287-2288) have brownish tint in coloration, which is rare in this species. On the dorsal body, the subepidermal musculature consists of the following layers: 1) a thin, subepidermal layer of circular muscle, 2) a thin layer of longitudinal muscle, 3) a layer of diagonal fibres, and 4) a layer of longitudinal muscle fibres. On the ventral surface, the musculature is somewhat more complex: 1) a thin, subepidermal layer of circular muscle, 2) a thin layer of longitudinal muscle, 3) a layer of diagonal fibres, 4) a thick layer of longitudinal muscle, followed by 5) a thin layer of loosely arranged circular muscle fibres.

The outer pharyngeal musculature consists of three layers: 1) directly underneath the outer pharynx epithelium a thin layer of longitudinal muscle, 2) a thin layer of circular muscle, and 3) a distinct layer of longitudinal muscle. The zone of muscles adjacent to the inner pharynx epithelium consists of a thick layer of intermingled circular and longitudinal muscle fibres.

The sagittal view of the copulatory apparatus of this Amagase specimen is shown in Fig. 3. The anatomy of the copulatory apparatus of *B. brunnea* is very similar to that of *B. annandalei* (except for their dimensions). The subepithelial muscle zone of the genital antra and the muscle coat of the bursal canal (including the vaginal portion) are more weakly developed in *B. brunnea* than those of *B. annandalei*. According to Kawakatsu's study of the copulatory apparatus of *B. brunnea* from many locations in Japan, the degree of development of musculature in the copulatory apparatus varies to some extent in animals from different localities (unpublished data except for fig. 7 C and D in KAWAKATSU et al., 1978, reported erroneously as *B. borealis* from Okushiri Island in Hokkaidô). In the Amagase specimen, the vagina has a thin muscle coat.

Bdellocephala borealis Kawakatsu, 1978, is a middle-sized species known only from its type locality (an outlet of Hime-numa Pond) in Rishiri Island, Northern Hokkaidô (KAWAKATSU et al., 1978). The external appearance of this species is very similar to that of *B. brunnea*. However, the dorsal surface of *B. borealis* is uniformly grayish brown with numerous, small, indistinct, reddish brown pigment spots. The adhesive organ is well-developed in *B. borealis* (cf. KAWAKATSU et al., 1978: 84-85, figs. 3 A-D, 4).

The subepithelial muscle zone of the dorsal body consists of a thin, circular layer underlying the epithelium, next a rather thin layer of diagonal fibres, and the third, thick layer of loosely arranged, longitudinal fibres. The ventral subepithelial muscle zone consists of a thin, circular layer adjoining the ciliated epithelium, next a thin layer of diagonal fibres, the third, rather thick, longitudi-

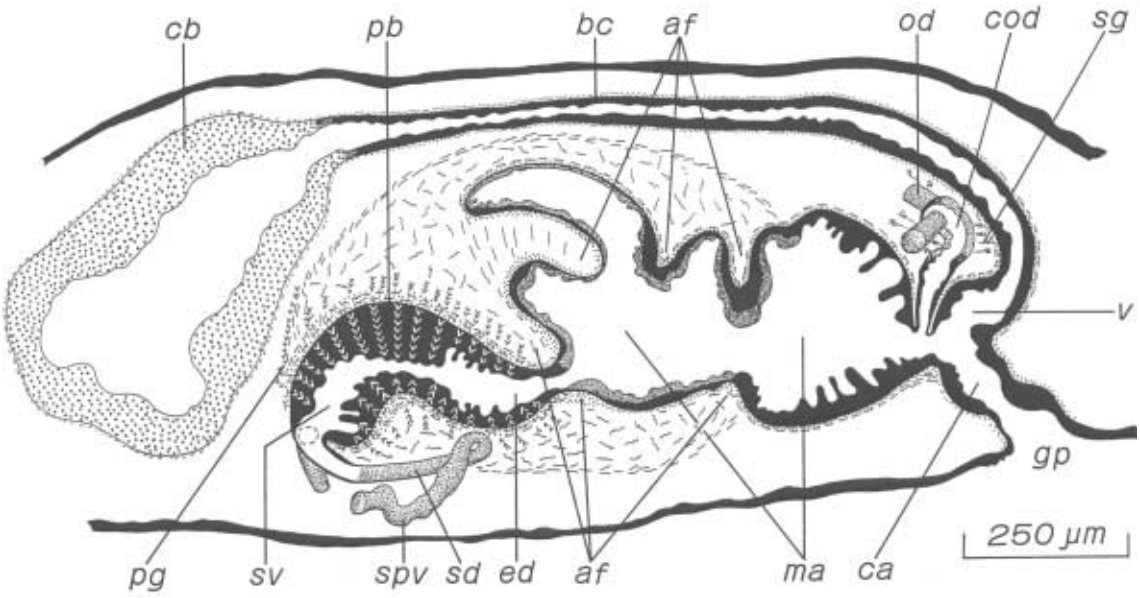

Fig. 3. – *Bdellocephala brunnea*. Sagittal view of the copulatory apparatus of a specimen from the Amgase Dam in the Lake Biwa water system (V. Pl.947.1; MK 2288-a). od, oviduct (ovovitelline duct); sg, shell gland; ed, ejaculatory duct; pg, penis gland; sv, spermiducal vesicle (bulbar cavity). For other abbreviations, see Fig. 2.

nal fibres; inside the third layer, the fourth, thick, diagonal fibres can be seen.

The outer pharyngeal muscle zone consists of five layers: a thin layer of diagonal fibres, next a thin layer of circular fibres, the third, thin layer of longitudinal fibres, the fourth, thick later of diagonal fibres, and the fifth, rather thick, longitudinal fibres.

In the copulatory apparatus of *B. borealis*, the musculature of the penis bulb and genital antra is well developed; the beginning of the penis lumen forms a narrow, napiform seminal vesicle; the muscle coat surrounding the vagina is well developed (cf. KAWAKATSU et al., 1978: 88, fig. 7 A and B, 91, fig. 8 C-E, 93, fig. 9 A-F).

Bdellocephala grubiiformis (Zabusova, 1929), is now considered a separate species, widely distributed only in Kamchatka, Primorskiy and Sakhalin (PORFIRJEVA et al., 1979; DYGANOVA & PORFIRJEVA, 1990: 81, fig. 47). Morphologically and anatomically, this species from the Russian Far East is similar to Japanese bdellocephalid species.

The subepithelial muscle zone of the dorsal body consists of a thin layer of circular fibres, next a thick layer of diagonal fibres, and the third, rather thick layer of loosely arranged, longitudinal fibres. The ventral subepithelial muscle zone consists of a thin, circular layer, next a thin layer of longitudinal fibres, and the third, rather thick layer of diagonal fibres.

The histology of the outer pharyngeal musculature of *B. grubiiformis* is very similar to that of *B. borealis*. The fifth layer of longitudinal fibres is less developed in the former than in the latter.

In the copulatory apparatus of *B. grubiiformis*, a globose penis bulb with a calceolate penis lumen, a rather wide, male genital antrum with well-developed atrial folds, and a poorly developed vagina are conspicuous (Fig. 4).

Bdellocephala sp. from Shumshu (Simusyu) Island, the North Kurile Islands (MIYADI, 1937: 450) seems to be *B. grubiiformis*. The occurrence of *Bdellocephala* sp. in the NE China was reported by LIU (1993: 125, fig. 3, etc.). There is a fair possibility that this unidentified species is *B. grubiiformis* because the Chinese localities are located adjacent to Primorskiy (cf. KAWAKATSU, 1994: 53, fig. 3; 1996: 6, fig. 4).

Karyology

The chromosome numbers and the karyotypes of three Japanese *Bdellocephala* species are as follows:

B. annandalei: 2x = 28 and n = 14, with a karyotype of 2m + 2sm + 2sm + 2sm + 2sm + 2m + 2m + 2m + 2m + 2m + 2m + 2m + 2m + 2m (OKI et al., 1998: 317, fig. 2, bottom A-E; see also YAGIHASHI et al., 1995; NISHITANI, 1998).

B. brunnea: 2x = 28 and n = 14. Karyotype: 22 M + 6SM (DAHM, 1963; NISHITANI et al., 1995; NISHITANI, 1998). In the Okushiri population, animals with 42 chromosomes were also found (NISHITANI et al., 1995); NISHITANI (1998) considered it to be a triploidic form (i.e., 3x = 42 according to our karyological formula).

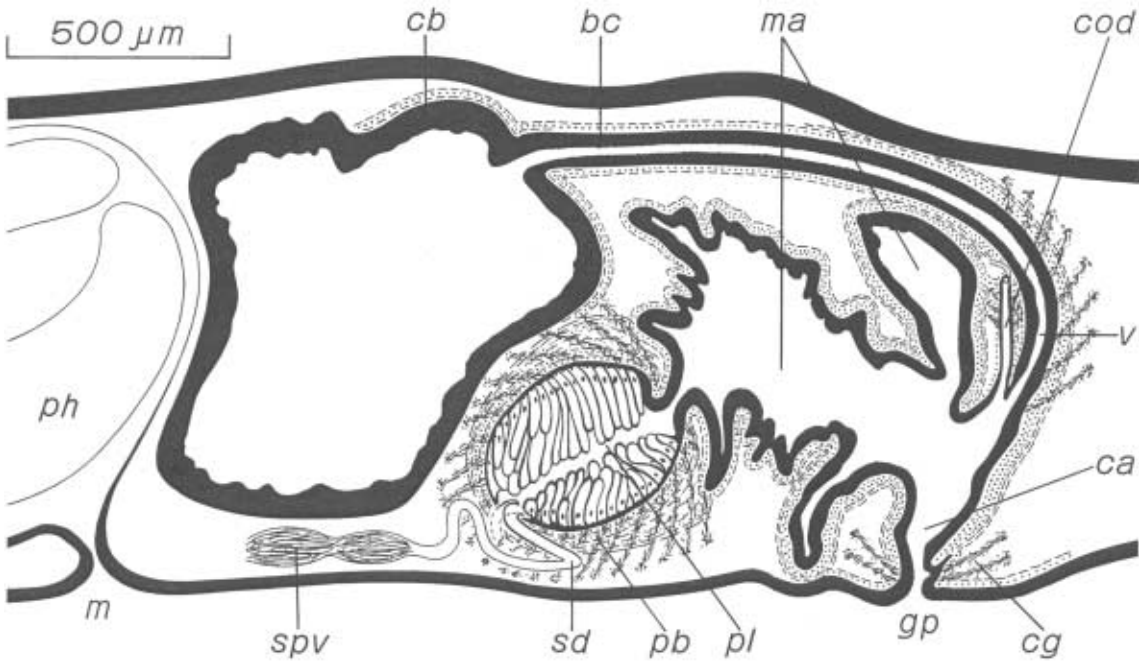

Fig. 4. – *Bdellocephala grubiiformis*. Sagittal view of the copulatory apparatus of a specimen from Dalnee Lake, Kamchatka (Timoshkin's collection). m, mouth; ph, pharynx. For other abbreviations, see Fig. 2.

B. borealis: 2x = 56 and n = 28. Karyotype: 38M + 18SM (NISHITANI et al., 1995; NISHITANI, 1998).

On the status of *Dendrocoelopsis ichikawai*

A short discussion about the molecular data of *Bdellocephala* species from Japan, Kamchatka and Lake Baikal in Russia was given in a previous paper (OKI et al., 1998). In a recent paper by KUZNEDELOV et al. (2000) based upon the 18S rRNA sequence data, a taxonomic revision of the genera, *Bdellocephala* de Man, 1875, and *Dendrocoelopsis* Kenk, 1930, was suggested. They argued that *Dendrocoelopsis* should be revised, and that the generic status of *Dendrocoelopsis ichikawai* Kawakatsu, 1977, should be reconsidered, since their results suggested that *D. ichikawai* belongs to the genus *Bdellocephala*.

We re-examined several sets of serial sections of *D. ichikawai* used in the original description (KAWAKATSU et al., 1977). However, we could not find any unique morphological, anatomical and histological characters thereby *D. ichikawai* should be transferred into the *Bdellocephala*. Notably, *D. ichikawai* shows a well-developed penial papilla, in contrast to the *Bdellocephala* species, and should remain in *Dendrocoelopsis*.

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On the ecology of Acoela living in the Arctic Sea ice

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ABSTRACT. A common meiofauna representative found in the pore system of the arctic sea ice from the Laptev, Barents and Greenland Seas was found to be a species of the Acoela characterized, among other features, by its bright red colour. It was studied by video recording of specimens moving in slices of ice and in 0°C sea water cultures after thawing of the ice. It is evidently well adapted to live and move in the partly very narrow brine channels. It has a great vertical distribution in the ice, but has its highest concentration in the lowermost 15 cm. In ice core samples from the Greenland Sea densities of up to 16,200 specimens per square meter were found. Diatoms were seen being eaten and were also found in the digestive parenchyma of sectioned specimens. Once a young nematode was observed being eaten. A description of the acoel species is given elsewhere.

KEY WORDS: Platyhelminthes, Acoela, arctic, meiofauna, sea ice.

INTRODUCTION

About 150 years ago arctic and antarctic sea ice of brown colour was discovered, the colour of which was found to be due to pennate diatoms and other microscopic algae (EHRENBERG 1841, 1853; HOOKER, 1847). Ciliates in the arctic ice were reported by NANSEN (1906) and USACHEV (1949). In addition to these organisms multicellular animals occur in the brine channels of arctic and antarctic sea ice. These channels form a branched network inside the ice matrix and they are most often very narrow, typically about 200 μm in diameter, but sometimes up to 5 cm in diameter (WEISSENBERGER et al., 1992; EICKEN et al., 1995; own observations). This limits the size of the metazoan inhabitants, which represent meiofaunal species of a number of groups.

In the Greenland Sea pack ice ciliates, nematodes, acoels and crustaceans were found to be dominant among the meiofauna (FRIEDRICH, 1997; GRADINGER et al., 1999). Acoels were found to be a prominent component of the antarctic ice as well; two species (not determined) were found by JANSSEN and GRADINGER (1999). Earlier reports of the occurrence of turbellarians in the sea ice were given by e.g. KERN and CAREY (1983), GRAINGER & HSIAO (1990), GRADINGER et al. (1991) and MELNIKOV (1997).

The most common acoel found in the pore system of the arctic sea ice is a species characterized by its bright red colour (FRIEDRICH, 1997). Its vertical distribution in the sea ice of the Laptev, Barents and Greenland Seas, its movements in the ice and its uptake of food are reported here. In this context some of the earlier results of studies of its ecology, reported in German (FRIEDRICH, 1997), are summarized.

MATERIAL AND METHODS

The material for this study was collected during cruises ARK IX/4 (6/8-5/10 1993) and ARK X/1 (6/7-15/8 1994) with RV "Polarstern" to the Arctic Sea. The material used for the studies of the behaviour of the Acoela in the ice was collected in the Greenland Sea during the 1994 cruise. Sampling stations were erected on relatively flat parts of the ice floes, reachable from the ship or by using a helicopter. Ice cores of 7.5 or 10 cm in diameter were drilled from the surface to the bottom of the ice and immediately cut into 1-10 cm long sections. While in the lowermost parts 1, 2 and 10 cm thick sections were investigated, in the upper segments that usually contain lower organism densities, sections of 20 cm (2x10 cm) were investigated for abundance of the meiofauna. These sec-

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tions were thawed in darkness in 0.2 µm prefiltered sea water at 4°C to avoid osmotic stress (GARRISON & BUCK, 1986). After complete melting of the segments, after about 24h, the animals were filtered from the sea water using a 20 µm sieve. In this paper the designation meiofauna is used for all the multicellular animals retained by this method, whereas the ciliates are not included.

Most of the material was sorted alive in the laboratory of the ship. Bouin´s fluid or formalin solution was used for fixation; samples for taxonomic studies were fixed exclusively with Bouin´s fluid. Some of the largest acoels were embedded in paraffin, serially sectioned, stained with eosin and hematoxylin and mounted on slides for light microscope studies.

Due to their bright red colour acoels occasionally could be observed in the underside of the cores immediately after drilling. To obtain an impression of their mobility in the ice 1–2 cm thick slices of the undersides of those ice cores with visible high densities of acoels were transferred in tight sealed sample containers to the ship laboratory within a few minutes. The slices were cooled down on a tissue cool plate (Reichert-Jung) every time the melting of the ice was visible. By this method the samples could be observed for up to about 15 minutes. The acoels moving in the ice pore system were recorded by using a video camera (JVC) mounted on a dissecting microscope. To record the animals in melted ice cultures an inverted microscope was used (Zeiss Axiovert 135) equipped with a video camera (Sony). Some of the vital studies were performed under the dissecting microscope on material brought to the laboratory in Kiel and kept in culture at 0-1°C in dark/light periods of 12h/12h, using a mixed algal culture as food.

RESULTS

A dominating acoel species

All worms of turbellarian shape in the samples were found to belong to the Acoela. This was evident from features of their general morphology, including a typical statocyst somewhat behind the front end, and from studies of the sectioned material under a light microscope. In life, most of them were of a bright red colour and were so similar to each other in appearance that we came to the conclusion that they belong to one and the same species. In one station in the Greenland Sea five white specimens were found. They were not studied further and may represent another species of the Acoela. This means that all the information given below refers to the bright red acoels.

As far as we know this species found in the arctic sea ice is not described in the literature, but videoprints of it have been published by FRIEDRICH (1997). Since this species occurred frequently in all investigated regions and makes up a significant part of the meiofaunal biomass of the arctic sea ice (GRADINGER et al., 1999) it will be described in spite of the fact that we did not find any fully mature specimens – in fact, most of the specimens were small, less than 0.5 mm, while the largest ones were about 1.2 mm. The description will be published separately.

Occurrence and distribution in the ice

The red acoel was found in ice cores in the Laptev, Barents and the Greenland Seas; median ice thickness 133 cm, 197 cm, and 270 cm, respectively (FRIEDRICH, 1997). It has a great vertical distribution, as is shown in Fig. 1. The acoels were found at a maximum distance of

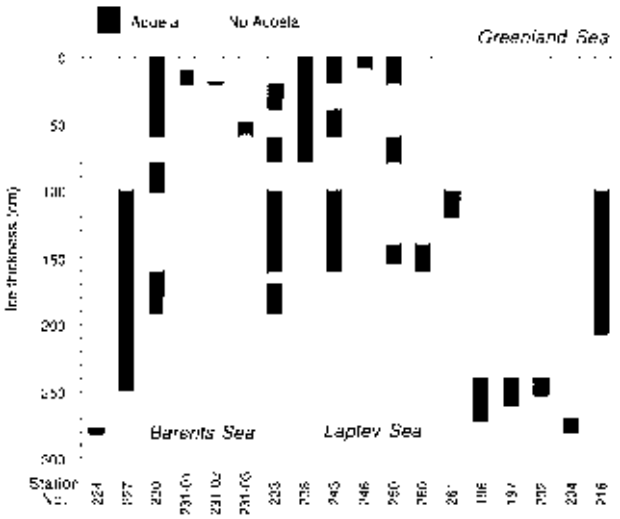

Fig. 1. – Vertical distribution of the red acoel in the sea ice of Barents Sea, Laptev Sea and Greenland Sea. Only cores from which all segments were investigated are shown. An exception is the core from station 227, where only the segments 1-20, 50-70, 100-120, 150-170, 200-220 and 240-252 cm were investigated. In one station (231) three cores were taken and fully investigated.

180 cm from the bottom of the ice. However, their highest concentration was found in the lowermost 15 cm with up to 293 specimens/l ice (Station 240, Laptev Sea), 258 specimens/l (Station 230, Barents Sea) and 222 specimens/l (Station 204, Greenland Sea). In the lowermost 15-30 cm up to 37 specimens/l were found (Station 196, Greenland Sea), but in the levels above 30 cm from the bottom the densities found did not exceed 22 specimens/l.

In the relatively old and thick ice of the Greenland Sea integrated densities of up to 16,200 specimens/m^2 ice (Fig. 2) were found with a median value of 2,260 specimens/m^2. In the younger and thinner ice of the Laptev Sea the abundance of the acoels was lowest, with up to 5,350 specimens/m^2, while in the Barents Sea ice up to 9,870 specimens/m^2 with a median density of 660 specimens/m^2 were found. In Fig. 2 the percentage of the total meiofauna abundance that is made up by the specimens of the red acoel is indicated, showing its high variability from 0 to 100%. In the core where the red acoel was found to have its highest density it made up 35% of the total meiofauna.

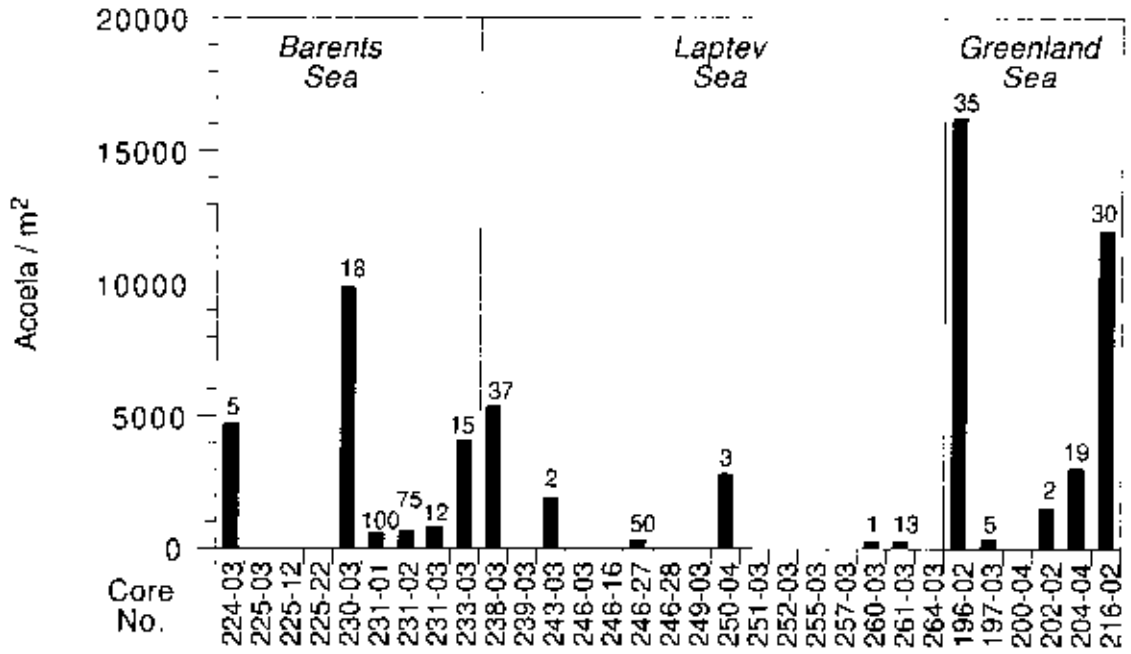

Fig. 2. – Abundance of Acoela in the sea ice cores from Barents Sea, Laptev Sea and Greenland Sea. All cores in which the abundance of meiofauna was studied are included, also those in which no acoels were found. The red acoel made up the total number of acoels in all cores except for 204:4, in which 5% of the specimens were white and thus may be of another species. The percentage of the total meiofauna made up by the acoels (in each station where acoels were found) is indicated by the figures above the columns.

Movements in the ice pore system

The specimens of the red acoel (Fig. 3) were observed to move through the brine channels in different directions, vertically as well as horizontally. As was demonstrated in our video recordings, they often seemed to be hindered by narrow passages, but after a while they succeeded in getting into a broader channel by pressing themselves through openings with a diameter smaller than the one they have when swimming in a relaxed way. When no hindrance had to be passed, they were observed to cover distances of a few cm within few minutes.

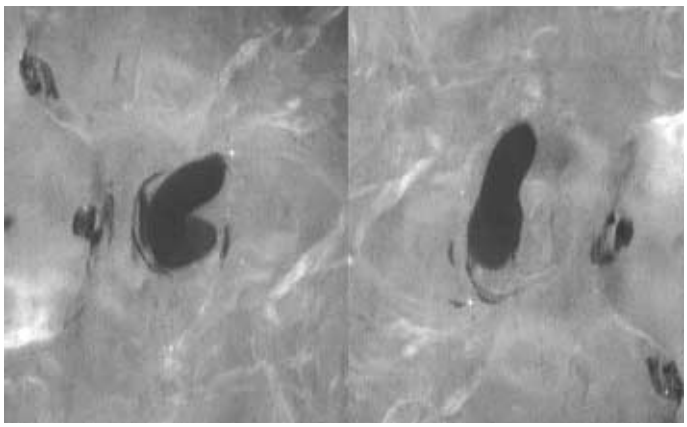

Fig. 3. Videoprints of living specimens of the red acoel in the ice, made from freshly cut ice cores on board of RV "Polarstern" by stereomicroscopy. As the magnifications were frequently changed, no exact scale bars can be given, but the length of each worm is approximately 1 mm.

Uptake of food

When the specimens of the red acoel were observed in culture dishes they were often seen to engulf diatoms. When a specimen, while gliding by ciliary motion, is passing over a diatom resting on the bottom of the dish it forms a furrow from the mouth opening (which is situated on the ventral side near the middle of the body) to near the front end. The engulfing can be described as a sucking movement, which evidently engages much of the ventral muscle layers of the animal. At the same time the acoel often contracts its sides so it becomes broader both in front of and behind the mouth. When it has passed over the place, the diatom is no longer seen in the dish, so evidently the engulfing is successful. Often several diatoms were observed being engulfed during a time period of one to two minutes. That diatoms are really eaten is evident also from studies of the sectioned material in which diatoms were found in the digestive parenchyma. The red acoel may also feed on other members of the meiofauna. Once a specimen was observed to engulf a young nematode. Though the latter was bending its body in different directions, the acoel succeeded in swallowing it.

DISCUSSION

A lot of observations speak in favour of the red acoel being very well adapted for a life in the brine

channel system of the arctic sea ice. (1) Its occurrence in sea ice over a large area of the Transpolar Drift System of the Arctic Sea, the Laptev Sea, northern Barents Sea and Greenland Sea, (2) its great vertical distribution in the sea ice, (3) that the specimens move so easily in the brine channel system, (4) that they evidently to a great extent feed on diatoms, a rich food resource in the brine channels, (5) that they can live for long periods (at least 1.5 years) in cultures kept at a temperature of about 0°C (FRIEDRICH, 1997), and (6) that they tolerate salinities from 5 to 65‰ and temperatures down to -6°C (FRIEDRICH, 1997). The typical vertical distribution of the Acoela is very similar to the chlorophyll distribution, which is also highest in the bottom of the ice (GRADINGER et al, 1999) providing a rich food supply for the acoels. In addition the brine channels become smaller in diameter in the upper parts of the ice (MELNIKOV, 1997), thus limiting the space for the organisms. However, the flexible body and cilia coverage on their surface seems to enable at least some specimens to reach the higher levels of the floes.

Whether the acoels reproduce sexually in the brine system is not yet shown, but the occurrence of all size dimensions from a lot of very small specimens to some very big ones indicate that they live at least a very great part of their lives in the ice.

The extreme conditions under which the meiofaunal organisms live in the brine channel system of the arctic and antarctic sea ice may explain the low number of turbellarian-shaped species. Also in the antarctic sea ice only one red and one white species (neither of them determined) of the Acoela were found (JANSSEN and GRADINGER, 1999). In addition to the acoels a species (not determined) of the Macrostomida has been reported from arctic ice by MELNIKOV (1997).

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Further monthly records (1994 to 2000) of size and abundance in a population of the "Australian" flatworm, *Australoplana sanguinea alba* in the U.K.

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ABSTRACT. Collections of the "Australian" flatworm, *Australoplana sanguinea alba*, have been made in one garden in the UK three times a week from February 1995 to September 2000. All specimens seen were placed in 70% alcohol, one jar each month. Flatworms were counted but not collected for some months from March 1992. The first flatworm was seen in December 1991. Rainfall, soil moisture and air and soil temperature (and latterly, the depth of the water table) were recorded. All specimens (5121) were weighed and measured. For some years the maturity of each specimen was also determined. Fewest flatworms are found in July and most in November. Seasonal variation is probably related to soil moisture content. Relatively low rainfall in the winter of 1995-6 apparently led to low numbers of flatworms the following winter. Average size of flatworms is smallest in July and largest in March. Apparent hatchlings are most abundant August to October. 80% of the specimens were mature. Immature flatworms form the majority only between July and October. Specimens as small as 9.5 mm long are mature. Only four egg capsules have been seen over eight years. Flatworms have been observed undergoing fission and it is suspected that fission may be the main method of reproduction in this population. Body weight varies with the square of body length (exponent = 2.093).

KEY WORDS: Platyhelminthes, Tricladida, *Australoplana sanguinea var. alba*; *Geoplana sanguinea*; reproduction; growth; allometry.

INTRODUCTION

The "Australian" flatworm, *Australoplana sanguinea* var. *alba* (Jones, 1981) was probably introduced into the British Isles sometime around the early 1970's, and is now distributed widely, mainly in the south and west of Great Britain (JONES & BOAG, 1996). It feeds exclusively on earthworms and is thus perceived as undesirable by gardeners and farmers.

In December 1991 two specimens were found in a domestic garden near Southport, the first at that site. Numbers of flatworms were recorded at regular intervals over 1992 and sporadically in 1993 and 1994. From February 1995 to the present (September 2000) flatworms have been systematically collected, weighed and measured, along with rainfall and soil and air temperature records. Soil moisture is presumed to be the most significant factor in determining the number of flatworms collected. At any time, this is likely to be the result of recent rainfall and current temperature (and plant transpiration). Thus flatworm numbers are compared with rainfall (including totals of previous months) and air and soil temperature, as well as with soil moisture. JONES et al (1998) reported a partial analysis of the data based on collections to December 1997. This paper reports on collections of flatworms to September 2000 and further analyses the data.

MATERIAL AND METHODS

The garden covers an area about 198 m^2 of which about 70 m^2 is covered by lawn and paving, the rest is cultivated as flower borders or for vegetables. Paving stones are inset or round the edge of the lawn, and planks were placed on bare soil in the cultivated part of the garden. The soil is sandy and drains rapidly, though the water table is only 1 to1.5 m below the surface, varying seasonally. Flatworms were collected from beneath the same paving stones and planks three times a week. All flatworms seen were collected and placed in a jar of 70% industrial methylated spirits, one jar per calendar month (except for 11 specimens found between July and October

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1996 which were preserved together). In December 1997 illness prevented collection for the second half of the month – the number of flatworms has been doubled for inclusion. Daily records are kept of rainfall (from December 1995) and maximum and minimum air temperature. Rainfall records for previous years at Southport (5 km distant) were kindly provided by Sefton Borough Council. From December 1995, soil temperature at 10 cm depth and soil moisture (using a proprietary soil moisture meter with an arbitrary scale of 0-10 of unknown manufacture obtained from a garden centre) were also recorded on each collection date. From May 1999, the depth of the water table in a well in the centre of the garden has been recorded.

All specimens (5121) were individually weighed and length and width (to 0.5 mm accuracy) measured. JONES et al (1998) showed that preserved weight is 91.4% of live weight. Specimens from February 1995 to February 1999 were individually examined externally for the presence of the gonopore. Ones with no gonopore visible were cleared in cedarwood oil to see if there was any development of the copulatory apparatus. An arbitrary scale of maturity, 0-3, was used: 0 = immature; copulatory organs (and sometimes the pharynx) absent due to recent fission or breakage. 1 = immature; no development of the copulatory organs visible after clearing. 2 = partially mature; copulatory organs visible after clearing, but gonopore not open. 3 = mature; gonopore open.

RESULTS

Flatworm numbers – temporal variation

Flatworm numbers clearly fluctuate seasonally. In most years, and on average, numbers are highest in November (Figs1 & 2), averaging 215±122 SD (7 yr data). Numbers decline from November to July when they are at their lowest, averaging 3.29±4 SD (8 yr data). Numbers climb rapidly through the autumn months (Fig. 2). September appears to be the most variable month. Flatworm numbers are obviously correlated with rainfall (however totalled) and negatively correlated with temperature (Table1; Fig. 1).

TABLE 1

Spearman's rank correlation coefficient of the number of flatworms each month against the given factor. * - P = 0.05-0.01; ** - P <0.01. N = 89 months unless otherwise stated.

	Spearman's Rho
Rain, same month	0.284**
Smoothed (*4253H, twice*) rainfall	0.330**
Rain previous 2 months total	0.542**
Rain previous 3 months total	0.551**
Daily maximum air temperature (n = 58)	-0.733**
Soil temperature (n = 58)	-0.679**
Soil moisture (n = 58)	0.601**
Water table (n = 17)	-0.533*

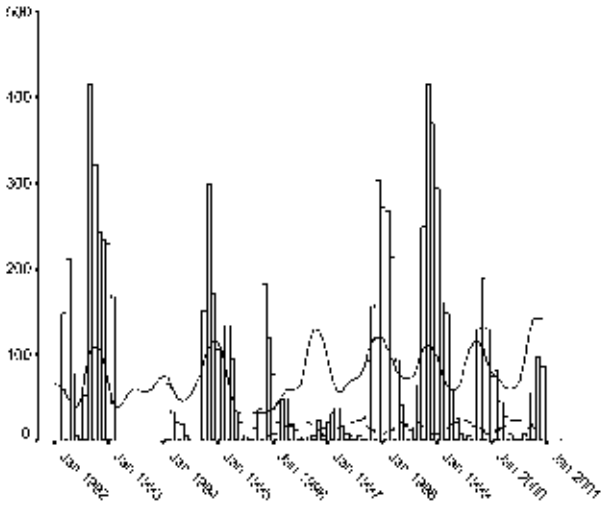

Fig. 1. – Number of flatworms (bars), smoothed rainfall (mm, continuous line) and maximum daily air temperature (°C, dashed line) each month. From March 1992 to January 1995 flatworm numbers were recorded but not collected (except from March 1993 to January 1994 and from July to September 1994 – no data). After February 1995 flatworms were collected. Monthly rainfall has been smoothed using *4253H, twice* smoother in SPSS.

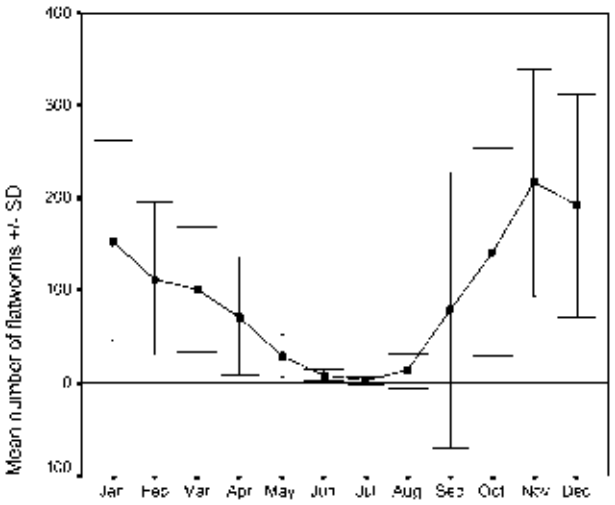

Fig. 2. – Mean (±confidence interval) monthly (a) worm numbers and (b) rainfall (mm). All data included from 1992.

Flatworm numbers vary between years (Fig. 1). The autumn-winter of 1992-3 and of 1998-9 showed the highest numbers. Over the autumn-winter of 1996-7 numbers were extremely low. In most years there was a clear autumn-winter peak in rainfall (Fig. 1). However, rainfall over the autumn-winter of 1996-7 was not particularly low, but that of previous summer, autumn and winter, 1995-6, was (Fig. 1) and 1995 was the hottest summer for 200 years. There is a striking parallel between the low rainfall of autumn-winter 1995-6 and the low flatworm numbers of the following autumn-winter, 1996-7.

Monthly size-frequency

For all years combined, flatworms are heaviest in March (Fig. 3). Weight is particularly variable between May and September. Weight-frequency for each month (all years combined) is shown in Fig. 4.

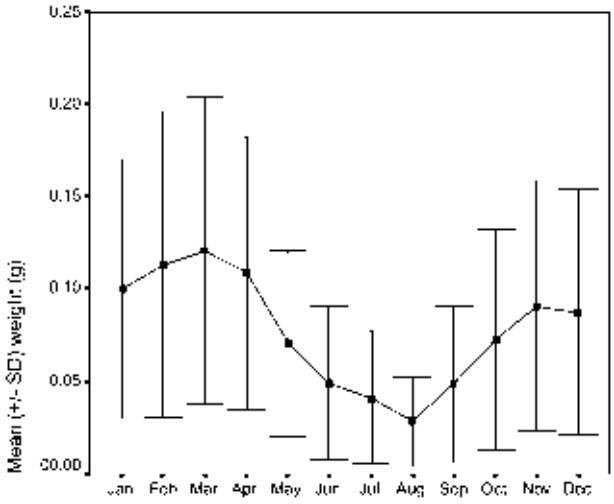

Fig. 3. – Mean (± 95% confidence interval) weight (g) of collected flatworms from February 1995 to September 2000.

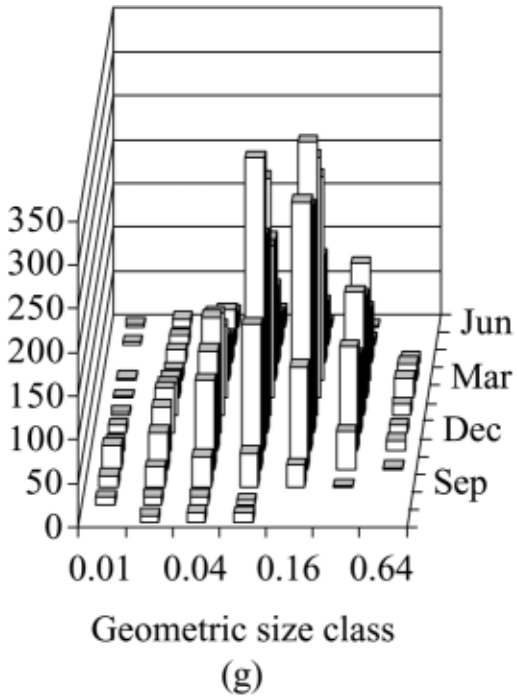

Fig. 4. – Weight frequency (geometric size class) of flatworms collected for each calendar month. All data from February 1995 to September 2000 pooled.

Maturity

Fig. 5 illustrates the proportion of mature specimens for each month from February 1995 to February 1999. Specimens as small as 9.5 mm long are mature. 80% of the specimens examined possessed an open gonopore and were judged to be mature. Partially mature and immature specimens appear to be in the majority August to October 1995, August to October 1997 and July 1998 (1996 data was pooled over these months). This suggests that partially mature and immature specimens may predominate between July and October.

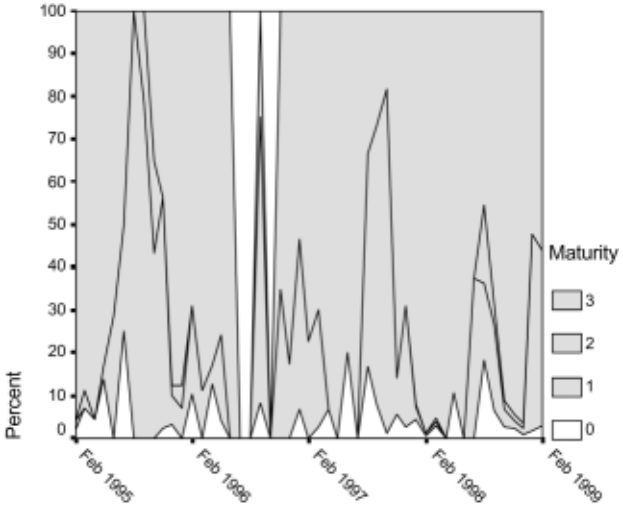

Fig. 5. – The proportion of mature specimens in each monthly collection between February 1995 and February 1999. See *Materials and Methods* for explanation of the assessment.

Allometry

Fig. 6 shows the relationship between body length and weight. Correlation equations are given in the legend. Body width varies against body length as follows: width = 1.415 (±0.033 SE) + 0.0089 (±0.001 SE) length, r = 0.761.

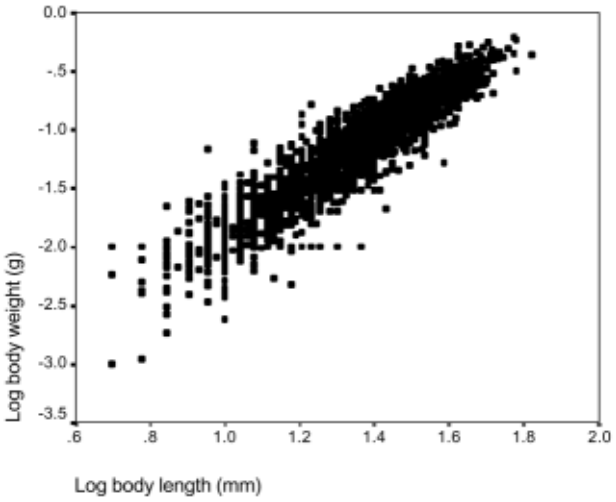

Fig. 6. – Scattergraph of $\log_{10}$ body length (mm) against $\log_{10}$ body weight (g). Regression equation: log weight = 2.093(±0.012 SE) log length – 3.961(±0.016 SE).

DISCUSSION

There is a clear autumn-winter peak in flatworm numbers each year. The population grows from a seasonal low in July to a seasonal maximum in November. From 1992 to 1997, though the data are incomplete, seasonal maximum numbers declined each year. We suggested then (JONES et al. 1998) that the flatworm population might have reached a low level after an initial population explosion, a feature common with introduced species. Numbers since 1997 have been high each winter (Fig. 1) which tends to negate that suggestion.

The low flatworm numbers of autumn-winter 1996-7 followed the low rainfall of the previous autumn-winter (Fig. 1). There has not been another such a dry winter over the recording period, but it is suggested that the latter was a major cause of the former. Subsequent winters have been as wet or wetter than usual and flatworm numbers have been high.

The regular collection of flatworms might itself affect numbers. However, in recent years, numbers have remained high in winter months even though they have been regularly collected over nearly six years. Clearly the reproductive capacity of the flatworm is capable of keeping pace with regular collection.

JONES et al (1998) concentrated on rainfall as the factor likely to be most significant in affecting the number of flatworms found, though acknowledging that other factors such as the summer rise in temperature and soil moisture deficit were probably involved. The initial data suggested that flatworm numbers correlated best with the previous 2 months rainfall (JONES et al., 1998). The longer data set presented here shows that flatworm numbers correlate positively with rainfall (with a slight lag, the peak in flatworm numbers is a month or two after maximum rainfall, Fig. 1) and soil moisture, and negatively with soil and air temperature (Table 1). Surely a combination of moisture and temperature is the ultimate factor in determining how many flatworms are found under objects resting on the soil surface. The low numbers in May, June, July and August are probably the result of surface dryness and lowered water table resulting from increased temperature, transpiration and reduced rainfall.

Mean size of flatworms is minimal in August (though variable) and then increases (with a slight decline in December) to a maximum in March (Figs 3 & 4). Thus growth of flatworms takes place over autumn, winter and early spring. The appearance of relatively large specimens, as well as small ones, in September and October (Fig. 4) suggests that flatworms may have been residing at some depth in the soil over the dryer period. Numbers of small individuals (probable hatchlings) are at a maximum between July and October (Fig. 4), even though absolute numbers are small, and immature specimens are most abundant between July and October (Fig. 5). All this suggests that juveniles appear in the population roughly between July and October. This provides some confirmation of the suggestion by JONES et al. (1998) that the flatworms may breed and lay egg capsules in the late Spring (April, May, June) as soil dries out, and that they hatch as conditions (soil wetness) improve in October. This is despite the fact that over the entire period of observations only four egg capsules have been found. (On 31 July 2000 a hatching egg capsule, only the fourth capsule to be found at the site, was found, photographed and preserved.) If egg capsules are being laid, then clearly most are not being laid near the surface or more would have been recorded. Asexual fission has been observed occurring during the winter when conditions are benign. JONES et al (1998) proposed that a cycle of reproduction occurs: sexual when conditions deteriorate, asexual when conditions are benign. MATHER & CHRISTENSEN (1996) found that egg capsules of this species were abundant at a location in New Zealand.

The data have been collected over eight and a half years. Any one year's data, though giving some indications, is unlikely to show typical cycles, particularly so soon after the initial infestation in December 1991. The data illustrate value of a long term and continuing data set in population studies.

The weight of most organisms showing isometric growth increases in proportion to the cube of length, giving a log:log slope (exponent) of 3. The log:log slope in this case is 2.093 (Fig. 6) which, though statistically different from 2, indicates that body weight increases as the square of length rather than the cube. This is to be expected in flattened animals such as these. As the slope is 2.093 rather than 2, a slight dorso-ventral thickening may take place as size increases. In Platyhelminthes generally, the lack of a circulatory system particularly limits growth in the third dimension. Body width in this species varies as 0.088 of the body length.

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SHORT NOTES

Chromosomes of bipaliid land planarians from the vicinity of Nagasaki in Kyûshû, Southern Japan (Platyhelminthes, Tricladida, Terricola)

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Nagasaki Prefecture is located in the north-western part of Kyûshû, Southern Japan; it consists of peninsulas and many islands on the East China Sea (Nagasaki City: lat. 32º44´N, long. 129º53´E; alt. 26 m; average air temp., 15.6ºC; total rainfall, 1,967 mm/y). Eight species of bipaliid land planarians were collected in the vicinity of Nagasaki City. They consist of three identified and five unidentified species. Their external appearance and collection records will be given here, together with tentative karyological data. For examination of chromosome numbers and karyotypes, our standard method was employed (Oki et al., 1980, 1991).

1. *Bipalium nobile* Kawakatsu et Makino, 1982. Nagasaki City (Nameshi: 22 VI, '98); Nishisonogi-gun (Iôjima: 28 IV, '98). Typical appearance was observed (120–300 mm long and 5-10 mm wide). Chromosome no.: 2x = 10, with a karyotype of 2m + 2m + m & sm + 2sm + 2sm (Nameshi).

2. *Bipalium multilineatum* Makino et Shirasawa, 1983. Nagasaki City (Bunkyô: 24 and 29 IV, '98); Nishisonogi-gun (Kinkai, 2 sts. 25 VI, '96 / 28 VII '99; Nagayo: 30 VI, '97 / 7 V, '98). Typical appearance was observed (40–150 mm long and 2-3 mm wide). Chromosome no.: 2x = 10, with karyotypes of 2m + sm & st + 2sm + 2sm + sm & st (Bunkyô), 2m + 2sm + 2st + 2sm + 2sm (Kinkai) and 2m + sm & st + 2st + 2st + sm & st (Nagayo). No sexual animal is known in this fissiparous species.

3. *Bipalium kewense* Moseley, 1878. Nagasaki City (Kakidô: 4 VII, 25 X, '96; Bunkyô: 26 IX, '96); Nishisonogi-gun (Nagayo: 17 XI, '97 / 13 IV, 8 X, '98). Typical appearance was observed (60–200 mm long and 3–5 mm wide). Chromosome no.: 2x = 18, with karyotypes of 2m + 2m + 2m + 2st + 2sm + 2sm + 2sm + 2sm + 2sm (Bunkyô and Kakidô) and 2m + 2m + 2m + 2sm + m & st + 2st + 2st + 2sm + 2sm (Nagayo).

4. *Bipalium* sp. Nagasaki-1. Isahaya City, Nagasaki Pref. (26 XI, '97). A lunate or semilunate head moderately large (70 mm long and 4 mm wide); dark brown above with one mid-dorsal and two lateral stripes; with a pair of indistinct, dark lateral stripes on the ventral side. Chromosome no.: 2x = 10, with a karyotype of 2m + 2m + 2m + 2m + 2sm.

5. *Bipalium* sp. Nagasaki-2. Shimabara City, Nagaski Pref. (14 VI, '98). A lunate head well developed (70 mm long and 6 mm wide); dark grayish brown above with one mid-dorsal and two marginal stripes; with a pair of indistinct, lateral stripes on the ventral side. Chromosome no.: 2x = 10, with a karyotype of 2m + 2sm + 2sm + 2m + m & sm.

6. *Bipalium* sp. Nagasaki-3. The same locality as *B*. sp. Nagasaki-2 (5 XI, '97). A semilunate head well developed (80 mm long and 5-7 mm wide); light yellowish brown above with one broad mid-dorsal and two thin marginal stripes; with a pair of indistinct, lateral stripes on the ventral side. Chromosome no.: 2x = 10, with a karyotype of 2m + 2m + m & sm + 2m + 2m.

7. *Bipalium* sp. Nagasaki-4. Nagasaki City (Bunkyô: 24 X, '97, 16 II, '99). A lunate head moderately developed (30 mm long and 2-4 mm wide); dark brown above with one blackish mid-dorsal stripe; without stripes on the ventral side. Chromosome no.: 2x = 10, with a karyotype of 2m + m & sm + 2sm + 2m + 2m.

8. *Bipalium* sp. Nagasaki-5. Shimabara City, Nagasaki Pref. (Benten: 20 & 29 X, '96); Kitatakaki-gun (Takakichô: 14 III, '98). A semilunate head moderately large (200 mm long and 10-15 mm wide); blackish brown to black above; with a pair of dark, indistinct stripes on each lateral side of the creeping sole. Chromosome no.: 2x = 12, with a karyotype of 2m + 2st + 2m + 2sm + 2m + 2m.

Photographs of live specimens and idiograms of these eight bipaliid species can be found in a preprint paper by Kawakatsu et al. (2000).

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Note. Species of *Bipalium* Stimpson, 1857, and *Novibipalium* Kawakatsu, Ogren et Froehlich, 1998, cannot be separated on the basis of external morphology. Thus, it is possible that the five unidentified *Bipalium* species may include *Novibipalium* species (KAWAKATSU et al., 1998).

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Land planarians (Platyhelminthes, Tricladida, Terricola) as indicators of man-induced disturbance in a South Brazilian rainforest

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SLUYS (1998) (1) proposed the usefulness of terrestrial flatworms as bioindicators of the state of soil and forest conservation. He suggested that by comparing Terricola diversity between disturbed and undisturbed ecosystems, the forest conservation status could be inferred. He also proposed this taxon as a good indicator of areas of high general biodiversity (2).The present study aimed to answer two main questions: **a)** is land planarian diversity affected by human disturbance? **b)** are there groups of land planarian that might indicate such disturbance?

The study site, the National Forest of São Francisco de Paula, Southern Brazil, was originally covered by a mixed subtropical rainforest with araucaria pine (*Araucaria angustifolia*). We selected four habitats based on increasing levels of disturbance: subtropical rainforest with araucaria (NA), subtropical rainforest with selective araucaria logging (N), reforestation with araucaria (A), and reforestation with the allochtonous *Pinus elliottii* (P). In each habitat we selected four transects, and in all the resulting 16 transects were conducted 2 surveys per month throughout a year. We calculated the Shannon-Wiener diversity index, *H*' (3) for each habitat. The Morisita's index, C_λ, (3) was used to estimate the similarity between habitat species composition. These indexes were compared to detect significant differences between diversity indices of pairs of habitats (*t* test: 4). The *G* test was applied to detect differences in species composition among the four transects of the same habitat and among the four habitats (4). We used the software program PC-ORD, version 3.18 (5), for the hierarchical cluster analysis of the transects and for species and transects ordination (detrended correspondence analysis, DCA).

We found 402 individuals, 23 of which could not be identified. The other 379 specimens belong to 28 species (Table 1). The greatest number of individuals was recorded in (A) (47.2% of the observed total), and the lowest number of individuals (14%) in (P). *Geoplana ladislavii* Graff, 1899 (32.4% of the total) and *Geoplana* sp. 1 (18.7%) were the most abundant species in the four habitats. The diversity indexes, *H*', of the habitats could be arranged in the following ascending order: H'_P=2.849; H'_A=2.856; H'_{NA}=3.272; H'_N=3.467. The highest similarity index among habitats was C_λ=1 039, between (NA) and (N); the smallest one, C_λ=0 823, between (N) and (P). We detected significant differences between diversity indexes (*t* test) for the following pairs of habitats: (N) and (A), and (N) and (P) [$p<0.01$ for both pairs]. Significant differences were found in the specific composition of the four habitats [G=179.569, p=0.000], and of the transects of two habitats, (NA) [G=67.896, p=0.015] and (P) [G=51.090, p=0.010]. We did not find significant differences in the specific composition of the other two habitats, (N) [G=60.755, p=0.102] and (A) [G=58.552, p=0.141]. The ordination and the hierarchical cluster analysis of the species composition of each transect showed the existence of three main groups. The *G* test confirmed this transect grouping. The main axis of the DCA corresponds with a decreasing disturbance gradient, with best preserved transects/habitats located on one side and the most disturbed ones on the other. We identified two groups of species correlated with the main axis: the first group is composed by species, significantly and positively correlated with that axis: *Geoplana* sp. 2, *Geoplana* sp. 7, and possibly Geoplanidae 3 and *Notogynaphallia* sp. 2., with a marginally significant level (p=0.054 and p=0.057, respectively). Species belonging to this group occurred almost exclusively in non-disturbed habitats and may be indicating less altered forest. The second group is composed of species significantly and negatively correlated with that axis: *Choeradoplana* sp. 1, *Geoplana* sp. 3, *Notogynaphallia marginata*: Marcus, 1951 and *Xerapoa* sp. 1. These species showed a preference for disturbed areas and may represent indicators of more disturbed habitats. Our results suggest that terrricolan distribution is dependent on habitat disturbance. The low species abundance and richness in (P) may be explained by its small complexity and paucity of refuges. Contrarily, there seems to exist a high number of favorable microhabitats for terricolans in (A), probably because of the less compacted soil and the great number of araucaria fallen logs. Despite the low number of individuals observed in (NA) and (N), the flatworm diversity

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TABLE 1

Abundances of species of geoplanids (Platyhelminthes: Tricladida: Terricola) in four habitats of the National Forest of São Francisco de Paula, RS, Brasil. (A): reforestation with araucaria pine; (N): subtropical rainforest with selective araucaria pine logging; (NA): subtropical rainforest with araucaria pine, *Araucaria angustifolia*; (P): Reforestation with *Pinus elliottii*.

Species	**(A)**	**(N)**	**(NA)**	**(P)**	**TOTAL**
Geoplana ladislavii Graff, 1899	73	16	17	17	123
Geoplana sp. 1*	37	10	16	8	71
Geoplana sp. 2*	11	13	13	-	37
Choeradoplana sp. 1*	5	3	7	7	22
Geoplana sp. 3*	4	4	4	8	20
Geoplana sp. 4	17	-	-	-	17
Notogynaphallia marginata Marcus, 1951	6	1	2	5	14
Geoplana sp. 5*	3	3	4	2	12
Geoplanidae 1**	5	3	-	-	8
Geoplana sp. 6	1	3	3	1	8
Choeradoplana sp. 2*	4	1	-	1	6
Geoplanidae 2**	1	2	3	-	6
Notogynaphallia sp. 1*	5	-	1	-	6
Geoplanidae 3	-	4	1	-	5
Notogynaphallia marginata Graff, 1899	2	-	1	-	3
Notogynaphallia sp. 2*	2	1	-	-	3
Pasipha sp. 1*	-	-	2	-	2
Choeradoplana sp. 3*	-	2	-	-	2
Notogynaphallia sp. 3*	-	2	-	-	2
Geoplanidae 4	2	-	-	-	2
Xerapoa sp. 1	-	-	-	2	2
Notogynaphallia sp. 4*	-	1	1	-	2
Geoplanidae 5**	-	-	1	-	1
Pasipha sp. 2	-	-	1	-	1
Geoplana sp. 7	-	1	-	-	1
Geoplana pavani? Marcus, 1951	1	-	-	-	1
Geoplana sp. 8	-	-	-	1	1
Geoplanidae 6	-	-	-	1	1
not identified	4	11	5	3	23
TOTAL	183	81	82	56	402
Specimen proportion	47.2	18.8	20.3	14.0	100.0
Species richness	17	17	16	11	28
Diversity index (*H'*)	2.856	3.467	3.272	2.849	-

* sp. nov.
** gen. nov., sp. nov.

was high, probably reflecting a larger variation in habitat characteristics.

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A species complex in the genus *Notogynaphallia* (Platyhelminthes, Tricladida, Terricola)

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GRAFF (1899) (1) was the first to describe, based on material from Taquara, state of Rio Grande do Sul, Brazil, a land planarian with an elongated body, parallel margins and yellowish dorsum with five dark longitudinal stripes. The worms were identified as *Geoplana marginata* Schultze et Müller, 1857. The author also studied a seven-striped worm from the same locality, naming it *Geoplana marginata* var. *abundans*. GRAFF illustrated the copulatory apparatus of the species with a penis papilla, and a long seminal vesicle with the efferent ducts entering into its proximal end. However, he did not specify the external aspects of the sectioned specimen. Later, worms with five dark longitudinal stripes on a yellowish background, from the states of Rio de Janeiro (2, 3) and São Paulo (4), were studied. RIESTER (2) considered his material conspecific with GRAFF's, as did MARCUS (4) who interpreted the penis papilla of GRAFF's specimen as a temporary structure, as was verified in his own material. E.M. FROEHLICH (3) concluded that her material was conspecific with RIESTER's and different from MARCUS' and GRAFF's species. She separated the two species renaming RIESTER's *G. caissara*. C.G. FROEHLICH (5) showed that GRAFF's specimens could not be *G. marginata* Schultze et Müller, but did not rename Graff's species. He also analysed two seven-striped worms from Rio Grande do Sul, the anatomy of which was not in agreement with GRAFF's drawing, naming it *G. abundans*. The anatomy of the species, however, was not described. Thus, the five-striped GRAFF material, as well as MARCUS' material, remained without a well-defined taxonomic status. When OGREN & KAWAKATSU (6) erected the genus *Notogynaphallia* to Geoplaninae without a penis papilla, the male atrium with folded walls, and the female canal entering dorsally into the atrium, *G. marginata* sensu Graff and *G. abundans* were included.

On studying the geoplanid fauna of the National Forest of São Francisco de Paula, Rio Grande do Sul, 50 km from Taquara, we verified the occurrence of three morphospecies with elongated bodies, parallel margins and yellowish dorsa with five dark longitudinal stripes. Despite these shared characters the morphospecies could be distinguished by the width of the stripes and pattern of distribution. In the present work we study these morphospecies as well as the anatomy of *N. abundans*.

The National Forest of São Francisco de Paula is located between 29º23' and 29º27'S, and 50º23' and 50º25'W, at an altitude of ~930 m. Specimens of *Notogynaphallia abundans* were from Salvador do Sul, Novo Hamburgo and São Leopoldo, state of Rio Grande do Sul, Brazil. In the laboratory, the external aspects were observed from live and fixed animals. They were killed using boiling water and fixed with neutral formaldehyde. The ratio of the height of cutaneous musculature to the height of the body (mc:h index) was calculated by the method of C.G. FROEHLICH (7).

The four species constitute a complex presenting a long prostatic vesicle, a folded and usually very long male atrium, a female atrium ending in a dorsally or dorso-anteriorly directed proximal diverticulum (vagina), and a long common glandular oviduct approaching dorso-anteriorly. The species, besides the details of the external morphology, can be mainly distinguished by the following anatomical characters: thickness of the cutaneous musculature (mc:index), position of the ovary and first testes relative to the body length, exit of the oviduct tubes in relation to the surface of the ovaries, rising of the oviducts in relation to the gonopore, posterior limit of the serial testes, site of the efferent duct entrance into the prostatic vesicle, morphology of the prostatic vesicle, and morphology of the male and female atria.

The study of the external morphology and the anatomy of *Notogynaphallia* sp. 1 and the comparison with the 23 described species of the genus lead us to conclude it is a new species. The external aspects and the anatomy of *Notogynaphallia* sp. 2 are concordant with those of *N. marginata* sensu Marcus, despite some variations of the relative width of both the paired stripes. *Notogynaphallia* sp. 3 has the same external morphology as *N. marginata* sensu Graff, besides a very similar anatomy of the copulatory apparatus. Thus, the study confirmed our presumption that *N. marginata* sensu Graff and *N. marginata* sensu Marcus are different species, so both will be renamed. *N. abundans* was validated as a distinct species and its anatomy will be described.

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A review of the genus *Stylostomum* Lang, 1884 (Platyhelminthes, Polycladida) and the description of a new species

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In 1884 LANG (1) created the genus *Stylostomum* to distinguish other cotylean polyclads with forward-directed male copulatory complexes, where the mouth and the male gonopore open in common to the exterior. He described a new species, *Stylostomum variabile* and included *Planaria ellipsis* Dalyell, 1853 and *Stylochus roseus* Sars, 1878 in the new genus.

HALLEZ (2, 3) added three species to the genus and HEATH & MCGREGOR (4) another one. *S. lentum*, from the Pacific coast of North America (the *S. californicum* in the introduction and in their key to California species is an error and should read *S. lentum*). BOCK (5) placed *S. variabile*, and *S. roseum* in synonymy with *S. ellipsis*. He was uncertain about *S. sanguineum* and silent on *S. punctatum, S. antarcticum* and *S. lentum*. HYMAN (6) considered that there were five valid species of *Stylostomum: S. ellipse* Lang, 1884, *S. lentum* Heath & McGregor, 1912, *S. frigidium* Bock, 1913, *S. hozawai* Kato, 1939 and *S. maculatum* Kato, 1944. Later on, two more species were added by MARCUS (7) and by HOLLEMAN (8). Table 1 compares the major characteristics of the species now considered valid.

In his Monograph on Polyclad Turbellaria PRUDHOE (9) defined the genus *Stylostomum* to include those Cotylea that have the male apparatus directed anteriorly, with the male gonopore and mouth *either* separated or united in a common antrum, no anterior branch of the intestinal trunk over the pharynx, with marginal tentacles more or less inconspicuous. This definition combines characters of the genus *Stylostomum* with those of the genus *Acerotisa*, with the mouth close to the male gonopore, based on MARCUS' fig. 109 of *S. felinum*, which shows a thin fold of tissue separating the mouth and the male gonopore. This thin fold can, however, be considered a preservation artefact. In sagittal sections of all *Stylostomum* species a fold can be seen, separating the male antrum from the pharyngeal pocket until they form a common chamber. It appears from MARCUS' diagram that the specimen he studied could have been flattened and distorted, extending the fold of tissue so that it appears to divide the common antrum giving the appearance that the mouth and male gonopore are positioned very close together.

FAUBEL (10) maintained *Acerotisa* as a separate genus with the mouth and male gonopore separate but in close proximity. He proposed a new genus, *Parastylostomum*, for *S. hozawai* and *S. maculatum*, lacking pronounced tentacles with a few eye-spots arranged in tentacular clusters; mouth and male gonopore in common; prostatic duct enters the ejaculatory duct postero-ventrally. The position of the prostatic vesicle, lateral or ventral, with respect to the seminal vesicle, and the entrance of the prostatic duct with the ejaculatory duct can, however, not be considered characters of generic importance; rather they are species characters.

The diagnosis of the genus *Stylostomum* is hereby amended to read:

Euryleptidae of small to moderate size and oval form; smooth dorsal surface; tentacles reduced to small stumps or wanting; cerebral and marginal eye clusters, often eyes few in number; main intestine with up to six pairs of branches, which do not anastomose; a frontal median branch of the intestine may be present; mouth and male gonopore open in common to the exterior; male copulatory apparatus arranged beneath the pharyngeal cavity; free prostatic vesicle positioned either dorsal, medial or ventral to the seminal vesicle; penis armed with a tubular pointed stylet; female apparatus with two wide elongated uteri; uterine vesicles may be present.

The species of the genus *Stylostomum* occur in boreal and temperate realms north of 36° N and south of 36° S. *S. ellipse* shows a distribution in both the eastern North Atlantic cold-temperate and the western South Atlantic cold-temperate provinces. It has been reported from Spitzbergen to the Mediterranean, Cape Town, South Africa, Falkland Islands, South Georgia and Tierra del Fuego. *S. frigidum* is the other species that shows a discontinuous distribution, occurring in the Gulf of Ancud, Chile and Observatory Bay, Kerguelen. All other species are known only from their type localities. This distribution suggests that the genus *Stylostomum* is restricted to cool or cold waters. However, this is speculative until more is known of the polyclad faunas of more regions of the world.

Stylostomum spanis n. sp.

Twenty five specimens of a new *Stylostomum* species were collected on the South Island of New Zealand in

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TABLE 1

Characteristics of the valid and the new species of the genus *Stylostomum*

Species	Color	Marginal tentacles	Marginal eyes	Cerebral eyes	Intestine	Seminal vesicle	Prostatic vesicle	Penis stylet	Uterus
S. ellipse Lang, 1884	Variable in translucent body	Small	Two groups of 13-16 at base and within tentacles	Two groups of 12-13, one pair anterior to the brain	4-6 pairs of lateral branches which do not anastomose	Large elliptical beneath the prostatic vesicle	Slightly elongated above the seminal vesicle	Tubular, pointed	Paired
S. felinum Marcus, 1954	Gray	Small blunt	Two groups of 13-16	Two groups of 6-7.	Anterior median branch	Ventral, round, curves dorsal-anteriorly to form the ejaculatory duct	Elongate, oblong above the seminal vesicle	Tubular, pointed, on a small penis papilla	Paired, without uterine vesicles
S. frigidum Bock, 1931	Preserved slightly yellowish	Small blunt	90-100	Two groups of 36-38	Anterior median branch	Elongate	Large oblong above the seminal vesicle	Tubular, pointed, on a long slender penis papilla	
S. hozawai Kato, 1939	Translucent; orangish due to injested food	Absent	Two clusters of 7	Two groups of 4 to 5	Lateral branches that do not anastomose	Large elongate, ventral to the main intestine and posterior to the prostatic vesicle	Elliptical, below and anterior to the seminal vesicle; extracapsular glands; prostatic duct enters the ejaculatory ventrally	Pointed, in a long and deep penis sheath	Paired
S. lentum Heath & McGregor, 1912	Orange to lighter near the margin with minute white specks on the entire dorsal surface	Short rudimentary	Two clusters of about 80 each	Two long groups of 50 eyes each	Lateral branches that do not anastomose	Retort shaped anterior to the base of the pharynx	Spherical, immediately in front of the seminal vesicle dorsal to the ejaculatory duct	Very short penis, enclosed in a penis sheath. Lacks penis stylet	Paired
S. maculatum Kato, 1944	Ground color milky white with sparsely scattered large brown spots	Absent	Two groups of 3 each	Two groups of 2 eyes each with a pair of ventral eyes	Anterior median branch	Large elliptical partially anterior to the base of the pharynx	Oval, lateral to the seminal vesicle. Prostatic duct enters the ejaculatory duct from below	Present	Paired, without uterine vesicles
S. sanjuania Holleman, 1972	Translucent white on the margin shading to light yellow middorsally	Absent	Two groups of 5-11	Two groups of 3-4	Lateral branches only	Oval, ventrally and at the posterior margin of the prostatic vesicle	Elliptical, dorsal and anterior to the seminal vesicle	Slender, tubular, in a moderate penis sheath	Paired, without uterine vesicles
S. spanis, n.sp.	Translucent with intestinal branches orangish	Absent	Two clusters of 17-36	Two clusters of 17-36	4 pairs of lateral branches; first pair immediately branches; medial branches extend anteriorly; no anastomoses	Large, elliptical, ventral and posterior to the prostatic vesicle	Oval, above and anterior to the seminal vesicle	Small slender penis with a long stylet	Paired

1970 and 1971 intertidally under rocks and on *Macrocystis pyrifera* holdfasts.

The body is an elongate oval with the largest specimen measuring 10 mm long and 5 mm wide when moving. Marginal tentacles are wanting. The sucker is located in the middle of the ventral surface. The body is translucent white with orangish branches of the intestine, which fade upon standing. There are white maculae scattered on the dorsal surface. The cerebral eyes are in two clusters numbering between 15 and 35 in each cluster. Two small groups of cerebral eyes numbering three or four are slightly anterior to the main clusters of cerebral eyes. The marginal eye clusters contain between 17 and 36 small eyes. The digestive system is typical of the genus with the mouth located anteriorly and the cylindrical pharynx opening to the main intestine. The main intestine has four pairs of lateral branches. The posterior lateral branch is at the level of the sucker with the other three pairs originating between the sucker and the cerebral eye clusters. At the origin of the first lateral branch the main intestine divides into two branches, which extend forward. The lateral branches of the intestine do not anastomose. Sagittal sections show that the spermiducal vesicles enter into the oval seminal vesicle, which is positioned below and at the posterior ventral margin of the oval prostatic vesicle. The ejaculatory duct and the prostatic duct unite and pass into the slender, armed penis. The penis is surrounded by a penis sheath. The large male antrum opens in common with the mouth to the exterior. The female gonopore is located posterior to the base of the pharynx. The vagina ascends from a ciliated female antrum and expands into a cement pouch surrounded by cement glands. The vagina continues dorsally then turns posteriorly enlarging and dividing into the paired uteri.

The *Stylostomum spanis* differs from S. *ellipse, S. felinum, S. frigidum* and *S. lentum* in lacking tentacles. In *S. sanjuania* and the *S. Spanis* the prostatic vesicle is located dorsal to the penis and seminal vesicle, whereas in *S. hozawai* the prostatic vesicle is located ventral to the seminal vesicle and in *S. maculatum* it is lateral to the ejaculatory duct of the seminal vesicle. The elliptical prostatic vesicle of *S. sanjuania* differs from the oval prostatic vesicle of the *S. Spanis*. The number of marginal and cerebral eyes of the new species differ from *S. sanjuania*, 17 to 36 versus five to 11 marginal eyes in each cluster and 15 to 35 versus three to four cerebral eyes in each cerebral cluster.

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ABSTRACT

Endemic *Bdellocephala* (Platyhelminthes, Tricladida, Paludicola) from Lake Baikal: diversity, morphology and taxonomy

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Lake Baikal is the deepest ancient lake in the world, with the greatest faunistic biodiversity (TIMOSHKIN, 1997). Tricladida belongs to one of the most exciting groups of Baikal animals that are characterized by quantitative and qualitative diversity. All 37 Baikal planarian species and 13 of the 14 genera are endemic (PORFIRIEVA, 1977). *Bdellocephala* de Man, 1875 is the only non-endemic genus of Baikal planarian. Until recently researchers attributed four species and seven subspecies to this genus: *B. angarensis* Gerstfeldt, 1858 with five subspecies (*B. angarensis angarensis* (Gerstfeldt, 1858), *B. a. olivacea* (Korotnev, 1912), *B. a. melanocinerea* (Korotnev, 1912), *B. a. subrufa* (Korotnev, 1912), and *B. a. cotyloides* Rubtsov, 1928), *B. baicalensis* (Zabusov, 1903) with two subspecies (*B. baicalensis baicalensis* Livanov, 1962 and *B. b. subniger* Porfirieva, 1970), *B. bathyalis* Timoshkin et Porfirieva, 1989, and *B. hypervesiculina* Livanov, 1962. It is PORFIRIEVA's opinion (1977) that representatives of ancestral-*Bdellocephalla* were the "stem"-forms for most specialized and unusual giant species of Baikal planarians, while at the same time *Bdellocephala* proper produced several flocks of species. Baikal representatives of the genus colonized all depth zones from the water's edge to the abyssal (a schematic pattern of the vertical zonality after KOZHOV, 1962); maximal depth of *Bdellocephala* occurrence is 1313 m (PORFIRIEVA, 1977). *B. bathyalis* is a giant form with the morphological features typical of abyssal and cave-dwelling animals: growth of the body size (length up to 12 cm), complete reduction of eyes, and partial or full depigmentation (compared to colorful littoral forms). At the depth of 160-180 m (supraabyssal) we found *B. roseocula* sp.n. This finding is especially interesting and illustrates the correlation between depth and form; the species possesses all intermediate features and semi-reduced pink eyes consisting of fragmentary pigmented cells in contrast to the whole black eyes of the shallow-water forms. While studying the morphology of species within the genus, we found that the structure of a musculo-cutaneous sac is very conservative for *Bdellocephala*, and its ventral side consists of five muscle layers: outer circular, outer longitudinal (one to two layers), diagonal, inner longitudinal, and inner circular. The dorsal side shows four muscle layers: outer circular, outer longitudinal (one to two layers), diagonal, and inner longitudinal. We found the structure of the outer pharyngeal wall in all Baikal species, except *B. baicalensis*, to be different from that of *B. annandalei* Ijima et Kaburaki, 1916, *B. brunnea* Ijima et Kaburaki, 1916, *B. punctata* (Pallas, 1774), and *B. parva* Zabusova, 1936, their inner muscle layer consisting of longitudinal muscles on the side of the pharyngeal pouch, whereas it consists of alternating longitudinal and circular fibres in most of the Baikal species. According to our phylogenetic hypothesis, ancestors of the present Baikal *Bdellocephala* penetrated the Lake at least twice; the branch to which *B. baicalensis* being the closest to the Japanese-Kamchatka *Bdellocephala* fauna. After a taxonomical revision, we identified three new species and two endemic subspecies of *Bdellocephala*. Consequently, Baikal fauna of *Bdellocephala* comprises eight species and four subspecies. *B. a. subrufa, B. a. cotyloides,* and *B. hypervesiculina* are attributed to species inquirenda.

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