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The opinions of the authors published in this journal remain, nevertheless, their own responsibility.

Manuscripts are to be sent to: Prof. Dr. E. SCHOCKAERT, Department SBG, Limburgs Universitaire Centrum, B-3590 Diepenbeek (Belgium).

PREFACE

This 5th Benelux Congress of Zoology, held at the University of Ghent on the 6th and 7th of November 1998, was an excellent congress! The 208 participants, 4 invited speakers, 55 oral contributions and 84 poster presentations, all of excellent quality, prove that "Zoology" is alive and well in the Benelux. Three student presentations were outstanding. Roy Erkens (University of Utrecht) was awarded the prize for the best student oral presentation and Adina Sannen (University of Antwerp) the prize for the best student poster presentation (both awarded by the Royal Belgian Zoological Society). Mathieu Denoël (University of Liege) was awarded the prize from the Royal Dutch Zoological Society for the best poster presented by a Ph.D. student.

The organisation was perfect, and on behalf of all those present, I congratulate and sincerely thank Dr. Ann Huysseune and the whole Ghent crew who made this possible. The organisation and, indeed, the material presented certainly give our younger students a taste of what major international congresses are about.

These proceedings of the 5th BCZ reflect the high quality of some of the contributions. Thanks to the efforts of the guest editor, Dr. Jan Mees and the associate editor Dr. Nikki Watson (who meticulously corrected the English in less than no time), the first manuscripts were delivered to the printer in January. However, without the considerable efforts of the Drukkerij Michiels n.v., it would not have been possible for you to have the proceedings of this congress in your hands less than three months after the congress took place.

Thanks to all who made this superb event possible.

ERNEST SCHOCKAERT,
President
of the Royal Belgian Zoological Society

Invited Contributions

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MOLECULAR EVOLUTION AND THE INCORPORATION OF SITE-TO-SITE RATE VARIATION IN DISTANCE TREE CONSTRUCTION METHODS

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Abstract. The construction of evolutionary trees based on sequence data is not self-evident. Apart from the plethora of methods and software tools to choose from if one wants to infer phylogenetic tree topologies, one has also to be cautious about the sequence data themselves. In this paper, we discuss how systematic errors can be introduced by one of the phenomena that often characterize sequence data, i.e. differences in substitution rates among the different sites of the molecule. Regarding pairwise distance methods, these systematic errors can often be avoided if an appropriate substitution model is applied to the construction of phylogenetic trees. This is demonstrated for a phylogeny based on animal small subunit ribosomal RNA sequences.

Key words : molecular evolution – phylogenetic trees – substitution models – among-site rate variation.

INTRODUCTION

Radical advances in biotechnology and sequencing techniques have led to an explosive growth in the accumulation of biological data. Specifically, new applications of the Polymerase Chain Reaction method and the use of automatic sequencing quickly provide us with a huge amount of sequence data that can be used to study the evolutionary history of organisms, genes, and gene families. However, parallel to the development and improvement of rapid sequencing techniques, advances in computer technologies in particular have been responsible for breakthroughs in our exploration of molecular evolution. Since there is an ever-greater need for faster hardware and new computational tools in order to cope with the exponential growth of sequence data, these advances were and are extremely important for molecular phylogeny to become established. Additionally, modern networks and network facilities such as the World Wide Web (WWW) have made life much easier for molecular biologists and evolutionists. Through the WWW, a wealth of information becomes available with a few mouse clicks or keystrokes : sequence databases

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can be browsed, servers with biological information can be consulted on-line, data can be exchanged rapidly, and software can easily be downloaded and installed.

Because of these advances in computer hardware and software, one should expect the construction of phylogenetic trees – which is highly computer dependent – to have become quite easy. This is only partly true. Although fast and user-friendly programs are now available, the number of different methods for tree topology inference has increased rapidly during the last few years. As a result, people are often bewildered by the vast range of computer algorithms that can be applied to sequence data. Moreover, literature on the construction of phylogenetic trees is extensive and the pros and cons of different methods are frequently debated. On the other hand, the powers and pitfalls of the different algorithms and methods are becoming more and more understood. Lately, much effort goes into the study of specific substitution models that try to explain the evolutionary change of the molecules used for tree construction. If the "true" evolutionary process could be described accurately by a certain mathematical model, trees inferred on the basis of that model would suffer less from systematic errors. One of the recently well-studied phenomena that often cause systematic errors in a tree topology, is site-to-site rate variation in molecules. In this paper, we try to show and explain how differences in substitution rates among sites in the small subunit ribosomal RNA (SSU rRNA) can influence tree topologies that are inferred on the basis of evolutionary distances. For a more general discussion about tree construction methods and models of evolution, we refer to some of the nice reviews that have been written about phylogenetic inference (NEI, 1987; FELSENSTEIN, 1988; SWOFFORD *et al.*, 1996).

PAIRWISE DISTANCE TREES AND SITE-TO-SITE RATE VARIATION

Distance methods for tree construction first fit a tree to a matrix of pairwise evolutionary distances. For every two sequences, the distance is a single value based on the fraction of positions in which both sequences differ, defined as dissimilarity. This dissimilarity is actually an underestimation of the true evolutionary distance, because some of the individual sequence differences are the result of multiple events. Since mutations are fixed in the genes, there is an increasing chance over time of multiple mutations having occurred at the same sequence position. As a result, later mutations can hide previous ones. Therefore, in distance-based methods, the actual number of substitutions is usually estimated by applying a specific evolutionary model that makes assumptions about the nature of evolutionary changes. When all the pairwise distances have been computed for a set of sequences, a tree topology can then be inferred by a variety of methods, the most well-known of which is probably the neighbor-joining method (SAITOU & NEI, 1987).

Correct estimation of the evolutionary distance is crucial and several studies have shown that the use of an unrealistic substitution model can cause serious artifacts in tree topologies (OLSEN, 1987; VAN DE PEER *et al.*, 1993; LOCKHART *et al.*, 1994; DE RIJK *et al.*, 1995; VAN DE PEER *et al.*, 1996a, b). However, since we do not have an exact historical record of the events that took place in the evolution of sequences, it is not obvious how to correctly estimate the evolutionary distance. One of the first models used in the estimation of evolutionary distances is the one of JUKES & CANTOR (1969). This model starts from the

assumptions that all substitutions are independent, that all sequence positions are equally subject to change, that substitutions occur randomly among the four types of nucleotides – in other words, there is no bias in the direction of change –, and that no insertions or deletions have occurred. Based on these pre-assumptions, the authors derived an equation for estimating evolutionary distances from observed dissimilarity (see also Fig. 1):

$$d_{AB} = -\frac{3}{4} \ln \left(1 - \frac{4}{3} f_{AB}\right)$$

(Equation 1)

where f_{AB} is the dissimilarity (fraction of observed differences) between sequences A and B, and d_{AB} is the estimated evolutionary distance (fraction of expected differences) between sequences A and B.

Several other equations were subsequently proposed for the estimation of evolutionary distances. For example, KIMURA (1980) provided a method based on a model of evolution in which transitions and transversions may occur at different rates. Other equations are based on substitution models in which the four different nucleotides are not used in equal proportions (TAJIMA & NEI, 1984), or where a bias in the direction of change is accounted for (TAMURA & NEI, 1993; ZHARKIKH, 1994).

An important drawback of these models is that they ignore differences in substitution rate among the sites of a molecule. However, it has been known for some time that substitution rates differ among sites is nearly all genes. For example, OLSEN (1987) demonstrated that application of the JUKES & CANTOR model to sequences composed of sites with unequal rates could lead to artifacts in tree topology. He proposed a different evolutionary model that assumed a log-normal distribution of substitution rates over the sequence positions. JIN & NEI (1990) followed a similar approach but assumed that substitution rates were gamma distributed. On the basis of this distribution they derived several equations to compute evolutionary distances from observed sequence dissimilarities, using a parameter α that describes the extent of the rate variation. The main problem with applying gamma distances is the estimation of this parameter α, the accuracy of which depends on the estimation method, predefined tree topology, and on the number of sequences used in the estimation (SULLIVAN *et al.*, 1996; YANG, 1996).

Quantitative estimation of the substitution rates or variabilities of nucleotide sites is not obvious. Maximum parsimony estimates can be heavily biased while maximum likelihood estimates may experience computational difficulties (YANG, 1996). Recently, we developed a new method called "substitution rate calibration" for measuring the relative substitution rate of individual sites in a nucleotide sequence alignment on the basis of a distance approach (VAN DE PEER *et al.*, 1993; 1996a). The main advantage of this approach is that nucleotide variability estimates are independent from a given tree topology, contrary to estimates inferred from maximum parsimony or maximum likelihood methods (SULLIVAN *et al.*, 1996), and that they can be based on a large number of sequences (VAN DE PEER *et al.*, 1996c; VAN DE PEER & DE WACHTER, 1997a). The latter point is particularly important since the accuracy of the substitution rate estimate increases with the num-

ber of sequences taken into account. When the substitution rates of the individual nucleotides of the molecule are computed as described previously (VAN DE PEER *et al.*, 1993 ; VAN DE PEER *et al.*, 1996a), an equation can be derived that describes a more realistic substitution model, and that discriminates more selectively between sequence dissimilarity and evolutionary distance (see also Fig. 1) :

$$d_{AB} = p\left[\left(1 - \frac{4}{3} f_{AB}\right)^{-\frac{3}{4}p} - 1\right]$$

(Equation 2)

This equation is similar to the general formula proposed by RZHETSKY & NEI (1994) (with parameter $p = \frac{3}{4}\alpha$) to compute gamma distances.

APPLICATION OF SUBSTITUTION RATE CALIBRATION TO TREE CONSTRUCTION

When substitution rate calibration is applied to tree construction, sequence dissimilarity is converted into evolutionary distance, but for a set of nucleotides mutating with variable rates, the conversion allows for a slower increase in dissimilarity as a function of distance than for a randomly mutating set (see Fig. c). This is achieved using equation 2

Legend to the figure (see opposite page)

Fig. 1. – A. Hypothetical distribution of substitutions in a sequence of 20 nucleotides. It is assumed that the rate of substitution per site is the same for all sites in the sequence. In other words, substitutions (represented by gray squares) occur randomly. In this particular example, 11 substitutions are observed although 20 have really occurred. Several sites have undergone multiple substitutions ; *e.g.* site 4 has mutated 3 times. When the dissimilarity (11/20) is converted into evolutionary distance by using the equation of Jukes & Cantor (1969 ; equation 1, see text) a value of about 1 is obtained. This means that, on average, every site has been substituted once (20 substitutions in a sequence of 20 nucleotides) which is indeed correct.

B. Hypothetical distribution of substitutions in a sequence of 20 nucleotides but here it is assumed that the rate of substitution in site 20 is 100 times that of site 1 (thus assuming a ratio of 1/100). As a result, the majority of substitutions will take place near the end of the sequence. Consequently, the number of observed substitutions will be smaller than in sequences where substitutions occur randomly (see a). In this particular example, the number of observed substitutions is only 8. If the distance is computed according to JUKES & CANTOR (equation 1, see text), the evolutionary distance is about 0.57. Therefore, the evolutionary distance is seriously underestimated, since the "true" evolutionary distance should be close to 1.

C. Graphic representation of the functions describing the relationship between evolutionary distance (expected fraction of substitutions) and dissimilarity (observed fraction of substitutions) when substitutions are assumed to occur randomly (upper curve ; inverse of equation 1, see text), and when substitution rates are assumed to differ among sites (lower curve, inverse of equation 2, see text). See text for details.

with parameter p adapted to the shape of a rate spectrum constructed by grouping alignment positions of similar variability (see VAN DE PEER *et al.*, 1993 ; 1996c). Using the latter approach yielded some significant improvements in tree topology for the evolution of eukaryotic SSU rRNA sequences of different groups of protists (VAN DE PEER *et al.*, 1996a, 1996b ; VAN DE PEER & DE WACHTER, 1997a). In particular, the rate calibration method largely avoids tree distortions due to the presence of sequences with increased evolutio-

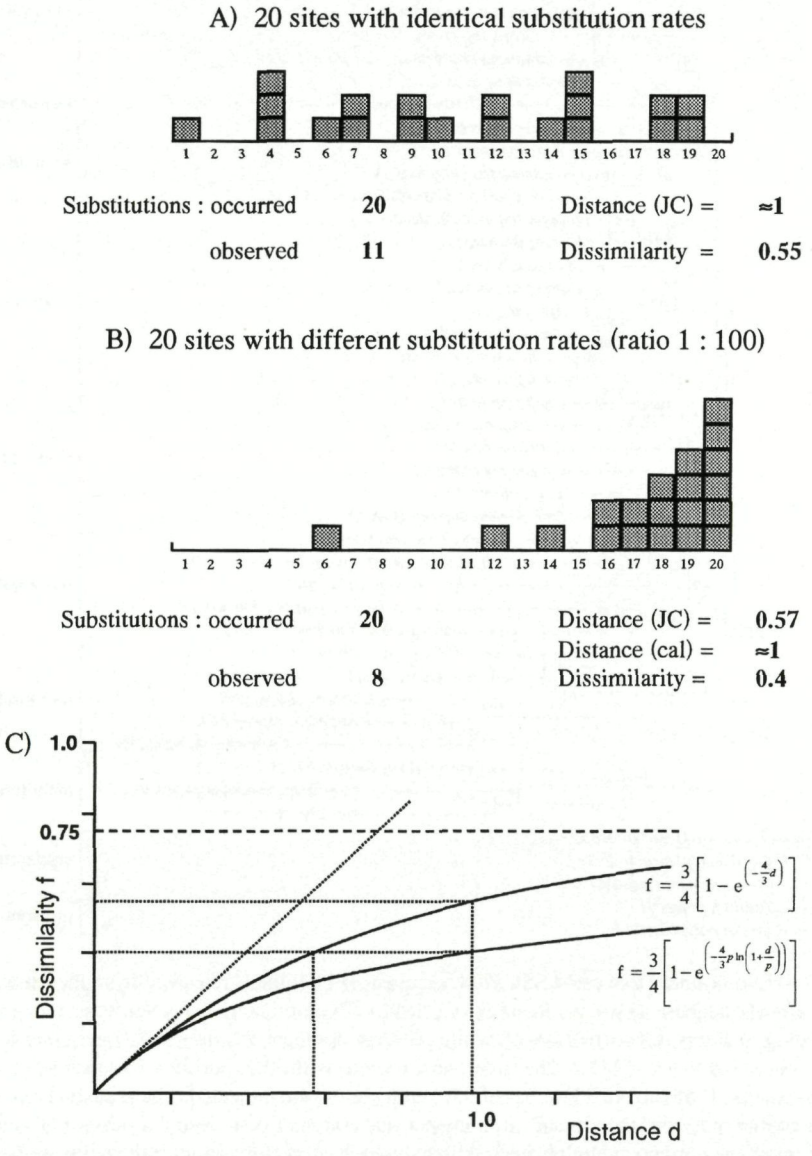

Fig. 1

nary rates. These long-branch distortions are usually caused by the underestimation of large distances with respect to smaller ones if distances are computed assuming equal variability of all nucleotides in a sequence (see Fig. 1c).

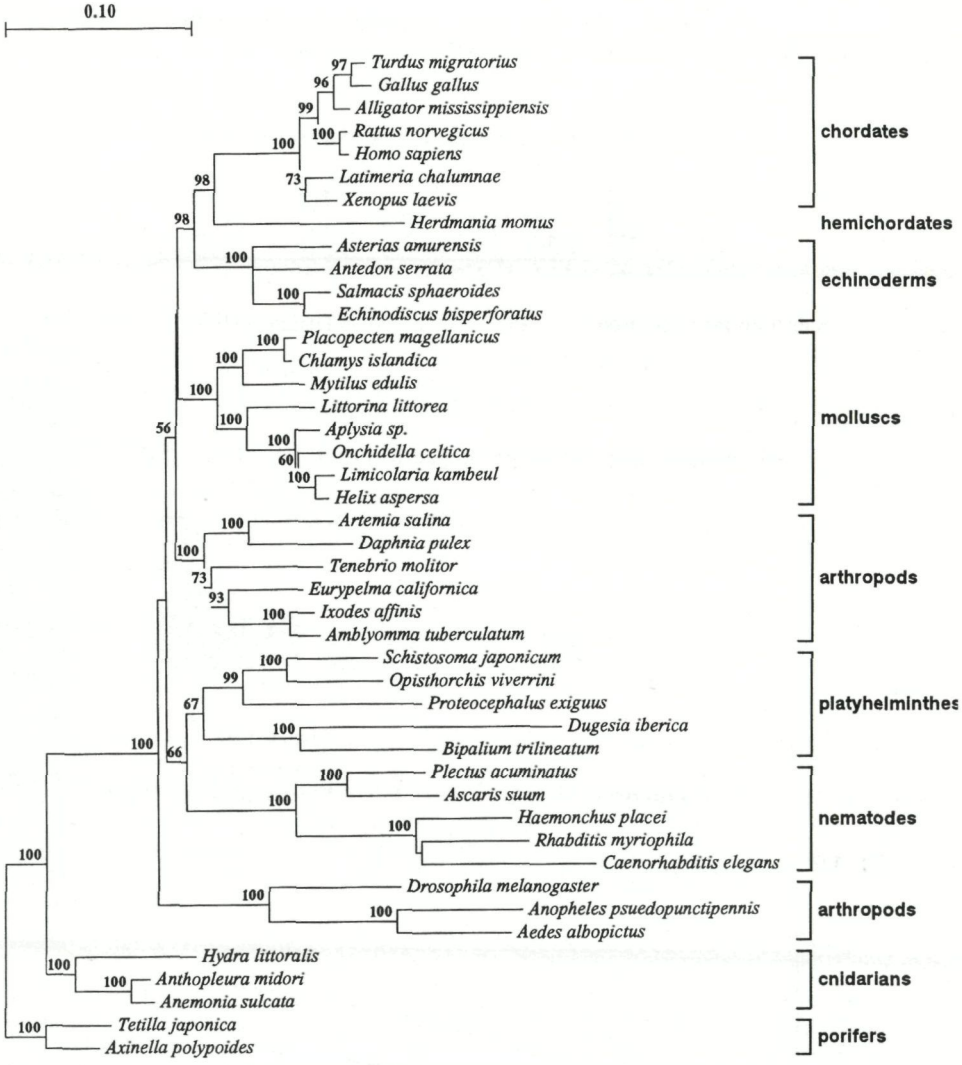

Fig. 2. – Evolutionary tree of 44 SSU rRNA sequences of animals retrieved from the Antwerp SSU rRNA database (VAN DE PEER *et al.*, 1998). Evolutionary distances were computed according to JUKES & CANTOR (1969) while the tree topology was inferred by neighbor-joining (SAITOU & NEI, 1987). The tree was rooted with the porifers. Bootstrap values (FELSENSTEIN, 1985) above 50 % (out of 500 replications) are indicated. The scale on top measures evolutionary distance in substitutions per nucleotide. Taxon designations are placed to the right of the corresponding clusters. All analyses were performed with the software package TREECON for Windows (VAN DE PEER & DE WACHTER, 1997b).

In the following example, rate calibration was applied to a phylogeny of Metazoa on the basis of SSU rRNA. Fig. 2. shows an evolutionary tree of 44 SSU rRNA sequences derived from different animals. The tree was constructed by neighbor-joining (SAITOU & NEI, 1987) on the basis of JUKES & CANTOR (1969) distances. Fig. 3 shows an evolutionary tree of the same set of sequences but with differences in substitution rates among the various sites of the SSU rRNA taken into account. Evolutionary distances were computed as described previously (VAN DE PEER *et al.*, 1993; 1996a) and a "p" value of 0.44 was obtained (see equation 2). Although the same groups of animals can be found in both trees, there are some remarkable differences, the most important of which is the position of the

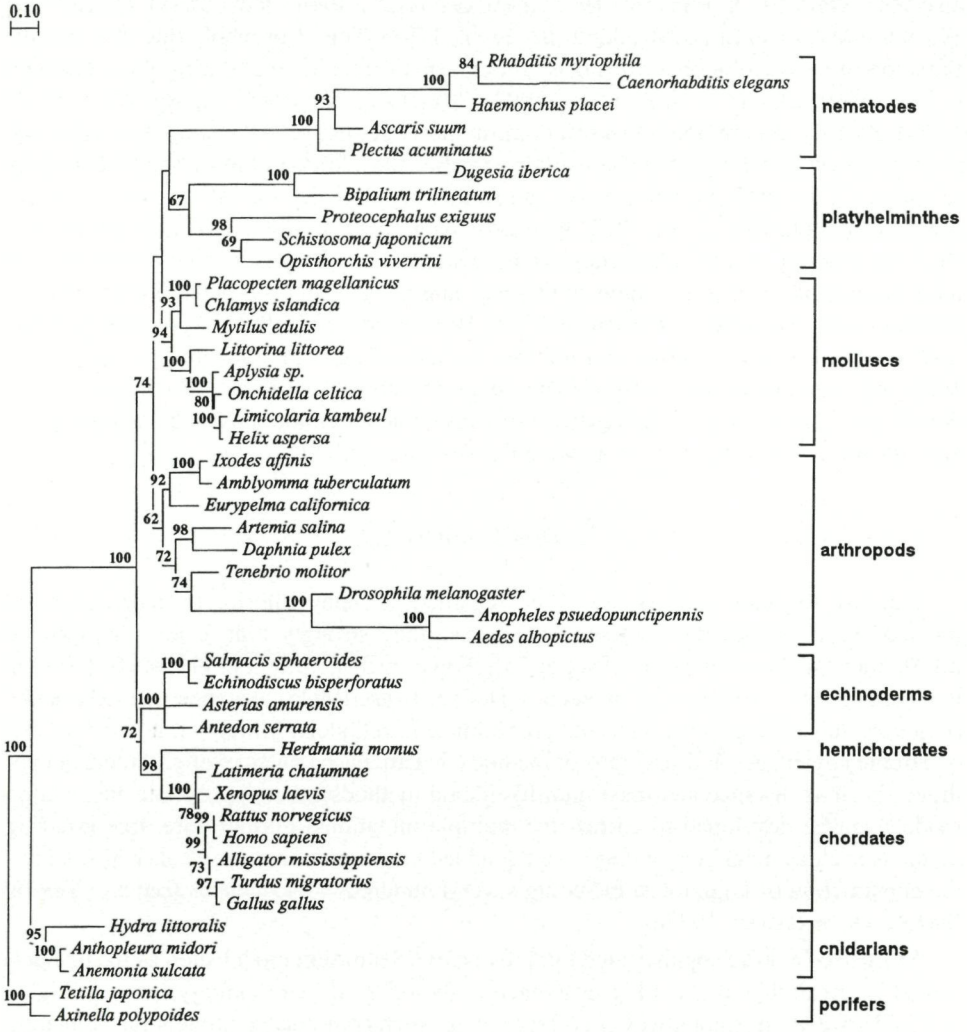

Fig. 3. – Evolutionary tree of the same set of animals as in Fig. 2., but based on "substitution rate calibration". Interpretation is as in Fig. 2. See text for details.

insects *Drosophila*, *Anopheles*, and *Aedes*. As can be seen in the calibrated tree (Fig. 3), these three SSU rRNA sequences are characterized by an increased evolutionary rate. As a result they form long branches in the tree with respect to most other sequences. Nevertheless, when rate calibration is applied, they are clustered as expected: with the other arthropods and more specifically with the other insect *Tenebrio*. Contrarily, in the tree based on JUKES & CANTOR distances, *Drosophila*, *Anopheles*, and *Aedes* seem to form an independent evolutionary lineage and diverge near the base of the tree, which is often characteristic for fast-evolving lineages.

As can be seen in Figs 2 and 3, the phylogenetic relationships and divergence order between the different animal phyla are hard to resolve on the basis of SSU rRNA data (see also *e.g.* ADDOUTE & PHILIPPE, 1993; MACKEY *et al.*, 1996; PAWLOWSKI *et al.* 1996; WINNEPENNINCKX *et al.*, 1996; ABOUHEIF *et al.*, 1998). This is probably due to a massive radiation of new evolutionary lineages within a small time interval during the Cambrium (ERWIN, 1991; ADOUTTE & PHILIPPE; 1993; PHILIPPE *et al.*, 1994; but see WRAY *et al.*, 1996 for a different opinion). As a result, most internodes between the animal taxa are very short and therefore difficult to reconstruct. Possibly, the addition of more sequences representative for the different animal taxa can further stabilize the animal tree, as previously suggested (TURBEVILLE *et al.*, 1992; LECOINTRE *et al.*, 1993). Alternatively, the problem of short internodes in animal phylogeny can be tackled by combining different genes into one long alignment. In this so-called multigenic approach, even complete (mitochondrial) genomes can be compared (CUMMINGS *et al.*, 1995, OTTO *et al.* 1996). Additionally, information such as the gene order and inferred number of gene rearrangements can then be taken into account in the study of evolutionary relationships (BOORE & BROWN, 1994; BOORE *et al.*, 1995). For a more general discussion about animal phylogenies taking into account site-to-site rate calibration, we refer to WINNEPENNINCKX *et al.* (1998)

DISCUSSION

Pairwise distance methods are often regarded as being inferior to character-based methods such as maximum parsimony because they strongly reduce the phylogenetic information of the sequences. However, as shown in this study and elsewhere (VAN DE PEER *et al.*, 1993; 1996b, VAN DE PEER & DE WACHTER, 1997a), distance methods can be of great value as long as the distances are estimated accurately. Moreover, it is indeed just one of the advantages and strengths of methods that are based on an explicit model of evolution (such as distance and maximum likelihood methods), that appropriate substitution models can be developed to correct for multiple mutations. Furthermore, tree inferring methods such as neighbor-joining have the added bonus of being very fast, which allows the construction of large trees, including several hundreds of sequences (see *e.g.* VAN DE PEER & DE WACHTER, 1997a).

The use of a more sophisticated (and realistic) substitution model such as the one presented here can thus make a big difference in the inferred tree topology (see *e.g.* Figs 2 and 3). However, it is not always necessary to use such complicated models for estimating evolutionary distances. As can be seen in Fig. 1c, the effect of using different models of evolution can be quite extensive for large evolutionary distances, but for small distances

(<0.25), the effect is often only marginal. Moreover, if closely related sequences are being analyzed by distance methods, it is even better to use a simpler model such as the one of JUKES & CANTOR, because of the lower variance compared to more sophisticated methods (SWOFFORD *et al.*, 1996).

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Belg. J. Zool. - Volume 129 (1999) - issue 1 - pages 17-42 - Brussels 1999

BIODIVERSITY OF THE GENUS *CONUS* (FLEMING, 1822): A RICH SOURCE OF BIOACTIVE PEPTIDES

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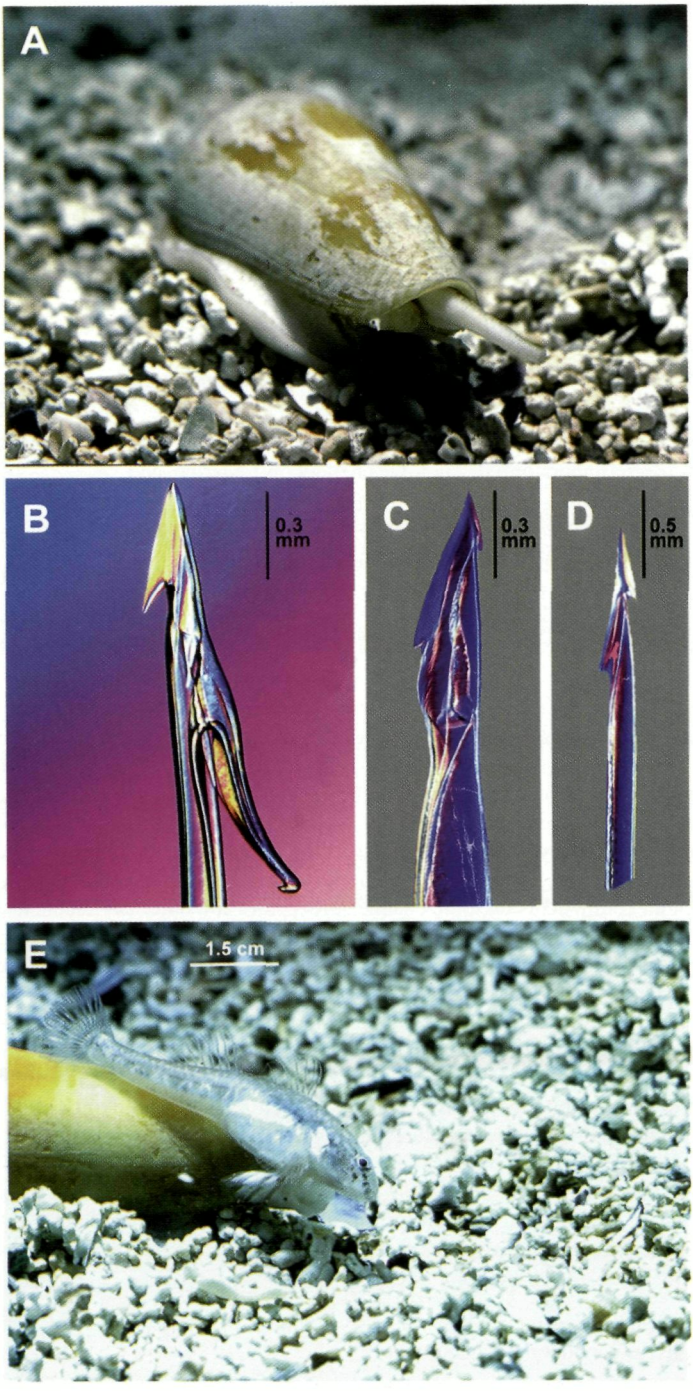
Abstract. In this paper, we present an overview of the biodiversity of both marine snails of the large genus *Conus* and their venoms. After a brief survey of Conidae malacology, we focus on the high degree of biodiversity of this genus, its specific biogeography as well as its habitat, and the relatively strict diet of its members. The venom of Conidae species contains a large number of peptides that can interact selectively with key elements of the peripheral and central nervous systems of vertebrates and invertebrates. Emphasis is on summarizing our current knowledge of the specific actions of venom components on ionic channels, receptors and other key elements of cellular communication. The peptides isolated from venoms, called conotoxins, form different families according to both their primary structure and their specific pharmacological targets. Three families encompassing the μ-, μO- and δ-conotoxins target voltage-sensitive sodium channels but with different modes of action or tissue selectivity. Another important class of conotoxins is the ω-conotoxin family which acts on voltage-sensitive calcium channels. The α-conotoxin family is represented by several peptides blocking muscular or neuronal nicotinic acetylcholine receptors. Finally, a blocker of potassium channels is presented as well as two conotoxins acting on the N-Methyl-D-Aspartate receptor. Primary structures and cysteine frameworks of all these conotoxins are shown and compared. At the end of the review, we report the contribution of molecular biology to identification of new conotoxins having original pharmacological properties. In conclusion, conotoxins have received increasing attention from physiologists, pharmacologists, biochemists and physicians because of their selectivity as well as their pharmacological and therapeutic potential.

Key words : Conidae ; Conus malacology ; Conus venoms ; μ-conotoxins ; δ-conotoxins ; ω-conotoxins ; α-conotoxins ; κ-conotoxins ; conantokins.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THE BIODIVERSITY OF CONIDAE

The Conidae (FLEMING, 1822) is a family of prosobranch gastropod molluscs which, together with the Turridae and the Terebridae, make up the Conoidea superfamily (genus and species names are according to COOMANS *et al.* (1979-1986), RÖCKEL *et al.* (1995) and RICHARD, (1990)). These animals paralyse their prey with specialized mouth parts that inject venom via highly modified radular teeth.

The more abundant species of Conidae can easily be found on the infra-littoral level of the inter-tropical zone. Their elegance and the large variety of colours of their shells make them very popular among amateurs, while for the researcher they represent experimental material that is now being used ever more frequently. A vast number of samples have been accumulated and there is a wealth of literature devoted to the Conidae, making its members ideal and original models both for the study of evolutionary biogeography and for the development of pharmacological applications based on the knowledge of how cone venom functions.

A BRIEF SURVEY OF CONIDAE MALACOLOGY AND CONCHOLOGY

In its natural environment, the only visible part of the cone is very often its inhalent siphon, although sometimes the sheath of the proboscis bearing the ocular peduncle, lying immediately below the siphon and sticking out from the anterior end of the animal, can also be seen (Fig. 1A). Cones crawl over the substrate using a muscular foot that is largely hidden by the shell. The posterior end of the animal has a small, nail-shaped infolding operculum. The colours of the siphon, the proboscis and the foot vary widely between different members of the family, but are extremely uniform within many species, an example being the red tones of *Conus imperialis*. In some cases, however, colours are very poor indicators of specific rank.

Like many neogastropods, cones are dioecious, their reproduction involving internal fertilisation after mating between the two sexes. The fact that the female has a seminal receptacle and that sexual partners gather at certain seasons (KOHN & PERRON, 1994 – personal observation) makes it possible, or even likely, that in numerous species the female is inseminated by several different males. The female can lay from a few hundred to several million eggs that are contained in egg capsules (KORN, 1994). Capsules are in the shape of a flat purse, and are placed by the female under blocks of coral or rock to protect them. The first stage of larval development occurs inside these capsules. For species with a

Legend to the figure (see page 18)

Fig. 1. — (A). *Conus consors,* a piscivorous species collected in Chesterfields Islands and acclimated in aquarium. Radula tooth morphology from *Conus consors* (piscivorous species). – (B). *Conus imperialis* (vermivorous species). – (C) and *Conus textile* (molluscivorous species). – (D). Note the presence in (B) of a long harpoon, posteriorly-directed with a curved tip. – (E). *Conus consors* stinging a fish by using the « harpooners » strategy.

« direct development », the young larvae look almost like miniature adults when they leave the capsules. They can be distinguished by the small protoconch shell with a small number of spirals and with roughly the shape of a conical cap with the top bent forward, as can be seen, for example, on *Conus magellanicus* (POINTIER *et al.*, 1987). Sometimes the larvae go through a planktonic stage of development of varying length. In Conidae, this stage may last from a few days (species probably undergoing lecithotrophic development) to weeks or months (species with larvae that feed in the plankton) : in the latter case, at the top of the adult shell (or teleoconch), a multi-spiral protoconch is present which is larger (has more spirals) than that of the species with direct development. *Conus catus* is an example of a cone which has a planktonic developmental stage, lasting in its case for approximately three weeks. Very little is presently known about the growth of cones, in contrast to the many families of tropical molluscs (RICHARD, 1982, 1986). Data that until now have not been published put the life span of a few species of cones living on reef flats in French Polynesia at between ten and twenty years.

It is essentially only the last whorl of the teleoconch that can be used to observe the shell characteristics ; the upper whorls, hidden by the last whorl, form the spire. In its general aspect, the shell of a cone can be turbinate (*e.g. C. monile, C. thomae, C. bayani*), conical (*C. pertusus, C. dalli, C. pretiosus*), biconical (*C. arcuatus, C. excelsus, C. cancellatus*), obconical (*C. lenavati, C. hirasei, C. sugimotonis*) or even fusiform (*C. glans, C. coccineus, C. nucleus*). The spire, which can have various numbers of whorls (such as *C. kintoki*, with a flattened spire, or *C. milneedwardsi*, with a very high spire) may have a convex (*C. rolani, C. bulbus*), straight (*C. sulcocastaneus, C. dayriti*) or concave profile (*C. armadillo, C. schepmani*). The last whorl may be totally smooth (*C. dusaveli, C. eburneus*) or it may have many ribs (*C. proximus, C. raoulensis*) ; its texture may also vary substantially between members of the same species, as is the case with *C. muriculatus* and *C. mucronatus*. The shoulder can be either smooth and angular (*C. striatus, C. thalassiarchus*), rounded (*C. omaria, C. zebra*) or scalloped (*C. imperialis, C. marielae*). The aperture is either narrow and covers the whole length of the peristome (*C. coelinae, C. shikamai*) or is vase-shaped toward the anterior (*C. geographus, C. tinianus*) ; on the inside it is lined with a columella that is usually smooth but it can also have a fold at its base that varies in size but is constantly present in a small number of species (*C. angasi, C. lozeti, C. luciae*). In some species the periostracum is rather thin and transparent, and the underlying polychromatic patterns can be seen through it (*C. textile, C. plinthis*), but in most species it is thick and relatively opaque, more or less totally hiding the coloration of the shell (*C. leopardus, C. virgo*). During the last few years the siphonal canal (anterior) and the anal canal (posterior) have been studied with a view to providing additional distinctive elements for the specific rank.

A HIGH DEGREE OF BIODIVERSITY

The first Conidae (genus *Conorbis*) appeared right at the beginning of the Eocene period, almost 60 million years ago. They evolved from an ancestral group belonging to the same family as earlier Strombidae ; the direct ancestor of the cones was more than likely a member of the Turridae family. During that period, the world's ocean was uni-

formly warm, with surface water temperatures of at least 22°C at low latitudes. However it was during the Lutetian period that the family began to expand, with the appearance of *Conospira, Cryptoconus, Hemiconus, Mamiconus;* even the *Lithoconus* group appeared during this period. It is possible to collect a large number of species dating from this period in France, particularly in the Paris basin (*C. calvimontensis, C. diversiformis, C. granatinus, C. glabratus*) and also in Normandy (*C. douvillei*). A little later, during the Miocene period (Helvetian), during which time many of the modern groups of cones appeared, the first fish-eating cones made their appearance with the diversification of the *Chelyconus* group. *C. aldrovandi, C. pelagicus* and *C. ponderosus* were among the cones that existed at that time. In Europe, the diversity of the family seemed to decline somewhat during the Pliocene (*C. antiquus, C. brocchii* and *C. striatus* are from this period), before expanding rapidly again during the Pleistocene. Nevertheless, compared with other families of molluscs, very few Conidae fossils have been found, primarily because their favoured habitats are seldom fossilized.

Today, the Conidae family is prospering in its natural environment, particularly in the Indo-Pacific province and in the southern Atlantic. The ease with which scientists can now travel and obtain material, the improved access to the scientific literature and to standard specimens, together with the ability to go prospecting in new areas (an example being the trawling of the bathyal zone) are some of the reasons why the last few decades have seen such large increase in the descriptions of new taxa (genus, sub-genus and species). Many scientists and several extremely competent and enlightened amateurs have attempted, with varying degrees of success, to propose an up-to-date cone taxonomy (COOMANS *et al.*, 1979 to 1986; KOHN, 1963 to 1992; WALLS, 1979; RICHARD, 1990). The work of Alan KOHN, professor at the University of Seattle (USA), is undoubtedly among the most interesting, and the recent book by RÖCKEL, KORN & KOHN (1995) is an almost exhaustive survey of what is known at the present time about the taxonomy of cones from the Indo-Pacific province, down to the specific rank, based on morphological and biometric criteria of the shell. Although this book is now an obligatory starting point for all the taxonomists of this family, it is not wholly satisfying, and work is in progress that will attempt to improve cone taxonomy and make it congruent with the evolution of the family.

It has been estimated that there are at least 600 extant species, with at least 700 species and sub-species divided into about thirty « groups », which are currently under study to see if they correspond to monophyletic taxa. It is already well established that a large number of these taxa correspond at most to sub-genus rank. The table below indicates the « working groups » that RICHARD (1990) defined, and shows a few examples of present-day species that belong in each group; most of the groups are either species groups or sub-genera.

AFRICONUS GROUP : *C. borgesi, C. cuneolus, C. grahami, C. irregularis.*

ASPRELLA GROUP : *C. aculeiformis, C. comatosa, C. elegans, C. insculptus.*

CHELYCONUS GROUP : *C. achatinus, C. aemulus, C. catus, C. ermineus.*

COCHLICONUS GROUP : *C. centurio, C. emarginatus, C. gradatus, C. poormani.*

CONASPRELLA GROUP : *C. acutangulus, C. baileyi, C. memiae, C. nereis.*

CONUS GROUP : *C. araneosus, C. bandanus, C. marmoreus, C. nocturnus.*

CYLINDER GROUP : *C. abbas, C. gloriamaris, C. telatus, C. textile.*

CYLINDRELLA GROUP : *C. marielae, C. moluccensis, C. sibogae, C. sulcocastaneus.*
DARIOCONUS GROUP : *C. crocatus, C. magnificus, C. omaria, C. pennaceus.*
DAUCICONUS GROUP : *C. ferrugineus, C. planorbis, C. striatellus, C. swainsonii.*
DENDROCONUS GROUP : *C. betulinus, C. figulinus, C. genuanus, C. glaucus.*
EMBRIKENA GROUP : *C. pergrandis, C. potusmarumai, C. stupa, C. stupella.*
ENDEMOCONUS GROUP : *C. boucheti, C. dayriti, C. otohimeae, C. spirofilis.*
FLORACONUS GROUP : *C. anemone, C. peronianus, C. spectrum, C. tinianus.*
GASTRIDIUM GROUP : *C. cuvieri, C. eldredi, C. geographus, C. tulipa.*
HERMES GROUP : *C. artoptus, C. nussatella, C. viola, C. violaceus.*
LAUTOCONUS GROUP : *C. californicus, C. taslei, C. unifasciatus, C. ventricosus.*
LEPORICONUS GROUP : *C. cylindraceus, C. mitratus, C. nucleus, C. tenuistriatus.*
LEPTOCONUS GROUP : *C. amadis, C. ammiralis, C. locumtenens, C. splendidulus.*
LITHOCONUS GROUP : *C. eburneus, C. leopardus, C. litteratus, C. tessulatus.*
PHASMOCONUS GROUP : *C. janus, C. neptunus, C. ochroleucus, C. pretiosus.*
PIOCONUS GROUP : *C. consors, C. fischoederi, C. magus, C. richeri.*
PROFUNDICONUS GROUP : *C. lani, C. profundorum, C. scopulicola, C. smirna.*
PUNCTICULIS GROUP : *C. arenatus, C. pulicarius, C. vautieri, C. zeylanicus.*
REGICONUS GROUP : *C. aulicus, C. auratinus, C. aureus, C. auricomus.*
RHIZOCONUS GROUP : *C. capitaneus, C. miles, C. mustelinus, C. namocanus.*
RHOMBUS GROUP : *C. imperialis, C. zonatus.*
STEPHANOCONUS GROUP : *C. archon, C. cedonulli, C. mappa, C. regius.*
STRIOCONUS GROUP : *C. barthelemyi, C. gauguini, C. gubernator, C. striatus.*
TEXTILIA GROUP : *C. bullatus, C. cervus, C. dusaveli, C. vicveei.*
TURRICONUS GROUP : *C. exelsus, C. gemmulatus, C. sowerbii, C. subaequalis.*
VARIOCONUS GROUP : *C. cepasi, C. fuscolineatus, C. naranjus, C. nobrei.*
VIRGICONUS GROUP : *C. berdulinus, C. coelinae, C. kintoki, C. martensi.*
VIRROCONUS GROUP : *C. chaldaeus, C. coronatus, C. ebraeus, C. miliaris.*
XIMENICONUS GROUP : *C. paraguana, C. perplexus, C. tornatus, C. ximenes.*

A RELATIVELY STRICT DIET

The Conidae are relatively strict carnivores. Based on the study of KOHN (1959), they can be put into three categories : vermivorous (*C. coronatus, C. imperialis, C. lividus, C. striatellus, ...*), which feed principally on annelids and polychaetes : Eunicidae, Terebellidae, Sabellidae ; piscivorous (*C. catus, C. ermineus, C. geographus, C. consors, ...*) which feed on fish ; and molluscivorous (*C. textile, C. dalli, C. retifer, C. auratinus*), which feed on molluscs such as Strombidae, Cymatiidae and other Conidae (RICHARD personal observations). LIM (1969) estimated that 65% of cones were worm-eaters, 18% were fish-eaters and 16% mollusc-eaters (figures rounded off). More recent

studies have tended to confirm these findings. At least two types of identification keys have been proposed, one of which is based on diet. The first relies on the morphology of the radular teeth, which have now been described in numerous species (ROLAN & RAYBAUDI MASSILIA, 1994) and which seem to be highly adapted to predator preference (ENDEAN & RUDKIN, 1965) and to the strategy used by *Conus* to envenomate their prey (LE GALL et *al*, 1999). The second is based on the presence or absence of a few specific shell characteristics (LIM, 1969). Nevertheless, there are a few exceptions that do not fulfil these conditions and certain observations show that the existence of a fourth category, including omnivorous cones with a more varied diet (*C. californicus, C. pictus, ...*), should be considered. Finally, several species are known to frequently stray from their diet, *C. regius* (considered to be vermivorous) being a well-known example.

Cones first paralyse their prey by firing a harpoon-like radular tooth (Fig. 1B-D), a veritable poisoned dart through which the venom is injected. The tooth is fired by the protrusible proboscis, which is then used to ingest the prey. These darts have their origin in the transformation of the radular ribbon which, during the course of evolution, may have lost its lateral and median teeth while the outside teeth grew longer, became hooked and jagged. They are well separated and stored, ready for use, in a radular sac at the back of the pharynx.

In fact, the venomous device of the Conidae consists of four organs (Fig. 2): the venom gland, the venom duct, the radular sac and the pharynx-proboscis complex.

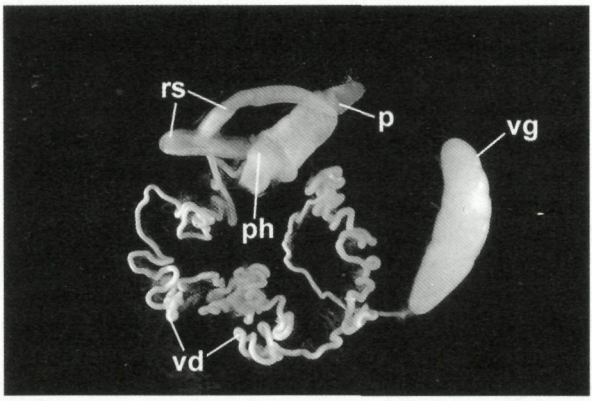

Fig. 2. – Venom apparatus of Conidae. It comprises the proboscis (p), the pharynx (ph), the radula sac (rs), the venom gland (vg) and the venom duct (vd). The role of these various components is detailed in the text.

The venom gland, otherwise known as the gland of Leiblin, is the largest organ of the venomous apparatus. Histological observations have confirmed the mechanical function of this gland, which was once considered to be responsible for secreting the poison. A transverse section shows that the internal structure has three layers: two layers of polygonal cells between which lies a fibrous ring about one hundred microns thick that acts as

a kind of skeleton, reinforcing the cohesion of the outer layer. The crescent shape gland is whitish, lies at right angles to the axis of the cone's body, slightly to the left side, and with the concave side facing forward.

The venom duct is the main organ of the venomous apparatus. It is a long yellowish tube, 4 to 6 cm long and a few hundred micrometers in diameter, wound into a ball. It emerges from the back of the pharynx, on the right side, just behind the muscular ring that forms the base of the rostrum (this description corresponds most closely to *C. lividus*). A section through this duct shows a large luminous area filled with an abundance of venom in the form of strings of coloured granules, surrounded by a thin epithelium of cuboidal secretory cells lying on a fibrous base. The wall of the tube comprises an intermediary layer of smooth ring muscles and an outer layer of longitudinal muscles. It is, as HERMITTE pointed out as far back as 1946, not just a simple duct for the transport of venom to the envenomation apparatus but the organ where the venom is made.

The radular sac consists of a short arm whose front end emerges on the right side of the pharynx, and a long, curved arm that starts at the middle of the short arm and continues toward the right front edge of the interior cavity.

The odontoblasts at the bottom of the radular sac are responsible for synthesizing the teeth. The teeth are initially chitinous and flexible but then become hard during their migration from the long to the short arm (MARSCH, 1977). En route to the short arm, the radular teeth are organised into two parallel and longitudinal rows, with their sharp ends pointing toward the bottom of the sac. Once inside the short arm, the mature and rigid teeth now face towards the opening into the pharynx. They are held there by a flexible and transparent ligament comprising a cylindrical stack of acellular strips attached to the base of the tooth. The teeth, ready for use, are in fact thin calcified sheets, from a few tens of micrometers to one or two centimeters long, rolled up to form cylinders and flared at the edges. This configuration allows them to accumulate venom.

The morphology of the radular teeth can vary enormously between species and is strictly related to diet (ENDEAN & RUDKIN, 1965), indicating a high degree of functional adaptation. The teeth of the vermivorous species are generally smaller and simpler (straighter, with fewer, simpler barbels) (Fig. 1C). Some piscivorous *Conus* tether their prey before engulfing them, and are named « harpooners » (Fig. 1E). In contrast to the vermivorous and molluscivorous species, these piscivorous species have the most complex radular tooth morphology, which in all likelihood they require to perforate the fish tegument (see Fig. 1B).

The proboscis-pharynx complex is at the front end of the Conidae venomous apparatus. The end of the short arm of the radular sac emerges on the right side of the pharynx. It then continues toward the front as a pre-pharynx, surrounded by a long protrusible proboscis. The latter is itself contained within a rostrum, which is a kind of sheath bearing among other things the ocular peduncles.

It would appear that, during an attack, the animal invaginates the end of its proboscis down as far as the front opening of the short arm of the radular sac to load a tooth. The tooth is then ejected in the direction of the prey by a rapid devagination of the proboscis. Histological sections of the proboscis show that the wall is covered with a thick layer of

muscle-type cells, which are all sheathed in a thick tegument of transversally striated fibres. This structure gives the organ its phenomenal capacity for extension and contraction.

The toxicity of cones has been known for a very long time. As early as 1705, RUMPHIUS reported the death of a native woman on the island of Banda (the Molucca islands) after she was stung by a *Conus textile*. Sir Edward Belcher was himself the victim of a *Conus aulicus* while collecting specimens of marine molluscs during the famous scientific expedition on the H.M.S. Samarang; luckily, the sting was not fatal. The first accidents in eastern Polynesia reported in the literature were due to *Conus tulipa*, a species that is abundant on certain atolls of the Tuamotu Archipelago.

Although all cones are capable of stinging the imprudent collector working on sunken reefs, the sting of most species only results in slight pain (*C. eburneus, C. virgo,* and the vermivorous species in general). Cones that provoke the most serious consequences are *C. geographus, C. omaria, C. striatus, C. tulipa, C. textile and C. magus*, *i.e.* mainly fish-eating, followed by mollusc-eating cones.

BIOGEOGRAPHY

The geographic range of a species depends on its capacity to adapt to imposed external factors, and especially on its means of dissemination. A majority of Conidae species have a high rate of reproduction but are rather stenotopic in relation to ecological factors and have a relatively short larval life, although some species do have planktonic periods lasting about a month (*C. coronatus*, for example). As a result, none of the Conidae species is cosmopolitan or even circumtropical. Pan-provincial species do, however, exist and poorly distributed species can be found in all of the great oceanic subdivisions of the globe, although the situation varies according to the biogeographical province. In the Indo-Pacific, the number of planktonic species is 4 to 5 times higher than that of non-planktonic species, whereas in the southern Atlantic the ratio is inverted between the two categories of larval development (it is doubled in the case of the Caribbean and the ratio is even greater in the case of West Africa).

For the Indo-Pacific, at least 50 of the 330 species (number rounded off) occur throughout this biogeographical region. These species (including *C. catus, C. chaldaeus, C. ebraeus, C. lividus*) represent a percentage of the specific wealth in Conidae that increases as one leaves the Philippines (25%) in the direction of Madagascar (50%) or French Polynesia (75%). This corresponds to an axis of impoverishment in species, more pronounced to the east than to the west, going away from the Philippines. As a result, almost all of the species of Conidae in western Polynesia are pan-Indo-Pacific or endemic.

Generally speaking, less than fifty species of Conidae have a distribution zone which covers all of the biogeographical province to which they belong. More than half of the others are species that are distributed over several biogeographical regions, not necessarily adjoining, of the same province. In addition, each biogeographical region has species of Conidae that are present solely in that region.

Furthermore, each region has a collection of various numbers of macroendemic species. The distribution of these species can cover the whole region (*C. taeniatus*, in the north-west of the Indian Ocean and the Red Sea), or just an archipelago (*C. julii*, in the Mascarene Islands). More often than not, however, endemism depends on geographical insularity. Some archipelagos have numerous endemic species and/or sub-species, such as the Marquesas Islands, which are the islands furthest away from a continent. Some of these micro-endemic species are restricted to a group of islands (*C. gauguini* on the Marquesas Islands (RICHARD & SALVAT, 1973)), to one island (*C. magellanicus*, in Guadeloupe) or even to a single bay (*C. nobrei*, in Angola). The table below provides a few examples of endemic species in several regions of the inter-tropical zone :

PANAMANIAN REGION : *C. archon, C. bartschi, C. purpurascens, C. vittatus*
CARIBBEAN REGION : *C. cardinalis, C. cedonulli, C. granulatus, C. jucundus*
BRAZILIAN REGION : *C. clerii, C. carioca, C. scopulorum, C. selenae*
SENEGALESE REGION : *C. adansonii, C. cloveri, C. mercator, C. taslei*
PERSIAN REGION : *C. ardisiaceus, C. melvilli, C. milesi, C. stocki*
CALEDONIAN REGION : *C. boucheti, C. lamberti, C. lienardi, C. luciae*
POLYNESIAN REGION : *C. encaustus, C. marchionatus, C. marielae, C. vautieri*
SOUTH AFRICAN REGION : *C. infrenatus, C. natalis, C. pictus, C. tinianus*

EACH CONE HAS ITS OWN HABITAT

We are now beginning to have a good insight into the lifestyle of the Conidae that live in shallow water, particularly those that live in the coral reefs of Melanesia and western Polynesia, where one of the authors of this article has been on numerous missions. In these areas, cones are to be found on dead coral substrates, such as slabs (*C. balteatus, C. ebraeus, C. miliaris*), on madrepora clumps (*C. circumcisus, C. magnificus, C. omaria*), on rough detrital accumulations (*C. coffeae, C. flavidus, C. imperialis*) or in sandy basins (*C. arenatus, C. bullatus, C. tessulatus*). Certain species have very specific habitats, such as *C. legatus*, which lies buried in the sand with *Halimeda*, on the outer slopes of the Society Islands, or *C. miles*, which lives nestled in the grass situated behind the algal crest, on the outer reefs of the Tuamotu atolls.

In other regions of the reef environment, vast stretches of the sea bed sediment have been colonised by different species : *C. ammiralis, C. aulicus, C. generalis ;* in the thick mass of grass, *C. ammiralis* seems to have been replaced by *C. pseudocedonulli* (observations made in the Amirante Islands, which form part of the Seychelles islands).

The rocky coasts also have their own species of Conidae, which take up residence under blocks of rock (*C. ardisiaceus, C. monachus*), in mud (*C. artopus, C. viola*) or among algae (*C. klemae, C. orion*). Some members of the family have even settled in mangroves (*C. trigonus*).

The above examples correspond to situations very frequently observed during field missions, but they are not exhaustive. Every cone has at some time been observed outside of its zone of predilection. Generally speaking, the majority of species (at least 60%) are considered to be part of the epifauna. In French Polynesia, for example, about twenty

species of cone from a total of seventy, *i.e.* less than half, are part of the endofauna. However, for paleogeographical reasons, this region is particularly poor in endofauna in the lagoons of its atolls.

Each zone of the reef also has its dominant, characteristic and exclusive species. In French Polynesia, *C. coronatus* and *C. quercinus* can be more easily found on the fringing reef, whereas *C. pulicarius* and *C. ratus* are more abundant on the barrier reef. To find *C. catus* and *C. retifer*, it is best to look on the outer reefs of atolls where the conglomerates lie on slabs. *C. moreleti* can be found in the depressions under rocky outcrops on the outer slopes. On the outer slopes, below low tide mark, numerous discoveries are still to be made. It is from this poorly accessible zone that the few rare samples of *C. adamsonii, C. aurisiacus* and *C. luteus* come.

The ocean islands of the central Pacific are surrounded by steep slopes. However Australia, for example, has a continental shelf with numerous species of endemic Conidae, including *C. rufimaculosus, C. sculetti* and *C. wallangra.*

Even further down, light is now being shed on the Conidae fauna of the bathyal level, particularly around New Caledonia (*C. boucheti, C. estivali, C. kanakinus, C. luciae*), in the south-west of the Indian Ocean (*C. patens, C. gradatulus, C. caillaudi*) and along the coast of Brazil (*C. capricorni, C. candidus, C. selenae*). The samples trawled in the New Caledonia region are so abundant that they have enabled a bionomic diagram to be proposed for a bathymetric gradient that goes from a depth of 100 to 700 m. (RÖCKEL *et al.*, 1995). Of the 17 species of cones trawled below 450 m., eight were captured alive : *C. alisi* (460 m., max. depth), *C. boucheti* (500 m.), *C. ichinoseana* (490 m.), *C. loyaltiensis* (480 m.), *C. luciae* (485 m.), *C. pergrandis* (509 m.), *C. profundorum* (500 m.) and *C. teramachii* (675 m.). At these depths, none of the species found so far are cosmopolitan. However *C. orbignyi* has been trawled in the Mozambique channel and near Madagascar (*C. orbignyi elokismenos*), off Indonesia and the Philippines (*C. orbignyi orbignyi*), and off New Caledonia (*C. orbignyi coriolisi*) and it forms, together with *C. emersoni* (American Pacific), *C. macgintyi* and *C. mazei* (southern Atlantic), a group of circumtropical species.

CONIDAE VENOMS

The venoms of cone snails contain short peptides called conotoxins. Each cone produces a mixture of conotoxins that have multiple biological activity (OLIVERA, 1997 ; LE GALL, 1999). In general these peptides have a highly conserved structure that will be reviewed according to the molecular targets involved in their specific actions.

Conotoxins acting on sodium channels : the μ-, μO- and δ-conotoxins

Sodium channels are transmembrane proteins that play a fundamental role in membrane excitability. At least six different toxin receptor binding sites have been identified on the sodium channel-protein (reviewed by CATERALL, 1986 ; GORDON, 1997). From a general standpoint two families of conotoxins have been reported to interact with voltage-dependent sodium channels : the μ-conotoxins, which block the channels by binding to

their receptor-site 1, and the δ-conotoxins, which mainly modify channel inactivation by binding to their receptor-site 6.

The μ-conotoxins from the venom of piscivorous Conus

The μ-conotoxins, first purified from the venom of *C. geographus*, μ-GIIIA, μ-GIIIB and μ-GIIIC (CRUZ *et al.*, 1985) are basic peptides comprising 22 amino-acid residues folded by three disulfide bridges and include an amidated C-terminal (Table 1). Electrophysiological experiments, performed on sodium channels purified from rat muscle or brain and incorporated into lipid bilayers, revealed that μ-GIIIA reversibly inhibits muscle sodium channels, in a voltage-dependent manner, without affecting neuronal sodium channels (CRUZ *et al.*, 1985). The specificity of action of μ-conotoxins on muscle sodium channels was further confirmed by MOCZYDLOVSKY *et al.* (1986), who reported that the toxins have no affinity for neuronal sodium channels, but instead inhibit the specific binding of ^{3}H-saxitoxin and ^{3}H-tetrodotoxin on receptor-site 1 of muscle sodium channels.

TABLE 1

Sequences of μ-conotoxins that block voltage-gated sodium channels

μ-conotoxins	Sequences*
Piscivorous *Conus*	
C. geographus	
μ-GIIIA	R D C C T O O K K C K D R Q C K O Q R C C A
μ-GIIIB	R D C C T O O R K C K D R R C K O M K C C A
μ-GIIIC	R D C C T O O K K C K D R R C K O L K C C A
C. purpurascens	
μ-PIIIA	Z R L C C G F O K S C R S R Q C K O H R C C
Framework	CC C C CC
Molluscivorous Conus	
C. marmoreus	
μO-MrVIA	A C R K K W E Y C I V P I I G F I Y C C P G L I C G P F V C V ^
μO-MrVIB	A C S K K W E Y C I V P I L G F V Y C C P G L I C G P F V C V ^
Framework	C C CC C C

* See references in text.

Following the chemical syntheses of μ-conotoxins (CRUZ *et al.*, 1989), these conopeptides proved to be tools of particular interest both for studying synaptic transmission

mechanisms at the vertebrate neuromuscular junction (SOSA & ZENGEL, 1993), and to functionally discriminate between the different types of voltage-dependent sodium channels, *i.e.* muscle or neuronal (CRUZ *et al.*, 1989).

Recently, a novel polypeptide (μ-PIIIA) was isolated from the venom of *C. purpurascens* (SHON *et al.*, 1998a). As reported for μ-conotoxins, μ-PIIIA comprises 22 amino-acid residues folded by three disulfide bridges (see Table 1). Although μ-PIIIA was shown to block muscle sodium channels by binding to their receptor-site 1, its action was not reversible. Moreover, in contrast to previously reported μ-conotoxins, μ-PIIIA also reversibly inhibited rat brain type II sodium channels expressed in *Xenopus* oocytes. This result raises questions about the specificity of μ-conotoxins, purified from piscivorous cone snails, on muscle sodium channels.

Here again, the conotoxin μ-PIIIA provides a good tool to pharmacologically differentiate between different sub-types of voltage-dependent sodium channels : (1) the sodium channels from skeletal muscle sensitive to μ-PIIIA and μ-GIIIA, (2) the neuronal sodium channels (type II) that are sensitive to μ-PIIIA and resistant to μ-GIIIA, and (3) the neuronal μ-PIIIA and μ-GIIIA-insensitive sodium channels (see SHON *et al.*, 1998a).

The μ- and μO-conotoxins from the venom of molluscivorous Conus

The recent characterization of two μ-conotoxins, μ-PnIVA and μ-PnIVB, purified from the venom of the molluscivorous cone snail *C. pennaceus* (see Table 1), introduces another specificity in the blocking action of μ-conotoxins (FAINZILBER *et al.*, 1995a). Indeed, these toxins specifically act on mollusc neuronal sodium channels without altering mammalian neuronal sodium channels, *i.e.* those of either bovine chromaffin cells or rat brain synaptosomes.

Finally, two conotoxins, the μO-conotoxins MrVIA and MrVIB, have been purified from the venom of the molluscivorous cone *C. marmoreus*. These toxins differ from those described above firstly, because they are composed of 31 amino-acid residues (instead of 22, see Table 1), and secondly, because they do not bind to the receptor-site 1 of sodium channels. In particular, μO-MrVIA blocks rat brain type II sodium channels expressed in *Xenopus* oocytes, but neither affects nor modifies the specific binding of ^{3}H-saxitoxin to rat brain membranes or to *Torpedo* electric organ (FAINZILBER *et al.*, 1995b ; MCINTOSH *et al.*, 1995, TERLAU *et al.*, 1996a). Therefore, μO-MrVIA inhibitory action on the neuronal sodium channel occurs through the binding to a receptor-site that is different from site 1.

The δ-conotoxins from the venom of molluscivorous Conus

The identification of the receptor-site 6 of voltage-dependent sodium channels was made possible by using a novel conotoxin purified from the venom of *C. textile* named δ-TxVIA, (FAINZILBER *et al.*, 1994). This hydrophobic peptide of 27 amino-acid residues is folded by three disulfide bridges (Table 2) (HILLYARD *et al.*, 1989 ; FAINZILBER *et al.*, 1991). Specific binding studies, involving an iodinated derivative of δ-TxVIA, revealed that δ-TxVIA interacts with high affinity, and in a voltage-independent manner, with the

receptor-site 6 of rat brain sodium channels. It should be noted that δ-TxVIA was reported to have no toxic activity when injected into mammals (ZLOTKIN *et al.*, 1996; SHICHOR *et al.*, 1996). Until now, there has been no clear explanation to account for this discrepancy.

TABLE 2

Sequences of δ-conotoxins that inhibit sodium channel inactivation

δ-conotoxins	Sequences*
Piscivorous *Conus*	
C. nigropunctatus	
δ-NgVIA	SK CF SOGTF CGI KOGL CCS VR CF SLF CI SFE
C. purpurascens	
δ-PVIA	E A CY AOGTF CGI KOGL CCSE F CLP GV CF G
Molluscivorous *Conus*	
C. textile	
δ-TxVIA	W CK QS GE MC NLL DQN CC DGY CI VL V CT
δ-TxVIB	W CK QS GE MC NVL DQN CC DGY CI VF V CT
C. gloriamaris	
δ-GmVIA	VK P CR KE GQL CDP I F QN CC RGW NC VL F CV^
Framework	C C CC C C

* See references in text.

The δ-conotoxins from the venom of piscivorous Conus

In contrast to δ-TxVIA, the two δ-conotoxins, δ-NgVIA and δ-PVIA, purified from the venom of *C. nigropunctatus* and *C. purpurascens* respectively (see Table 2), were reported to have a toxic activity when injected into mammals (FAINZILBER *et al.*, 1995c; SHON *et al.*, 1995). In addition, δ-PVIA and δ-NgVIA were shown to suppress and/or slow sodium channel inactivation in rat hippocampal cells and in mollusc neurons respectively (TERLAU *et al.*, 1996b; FAINZILBER *et al.*, 1995c). Although both δ-PVIA and δ-NgVIA were reported to inhibit the specific binding of δ-TxVIA on rat and mollusc neuronal membranes, δ-NgVIA is supposed to bind to sodium channels on a receptor-site different from site 6.

Conotoxins acting on calcium channels: the ω-conotoxins

Because calcium ions play a crucial role in the regulation of various cell functions including neurotransmitter release, enzyme activation, axonal growth, muscle contraction, membrane excitability and gene expression, voltage-dependent calcium channels have

been studied in great detail. The understanding of the physiological function of calcium channel sub-types, notably those named N, P/Q, has been made possible by using toxins isolated from the venom of various cones. Indeed, the ω-conotoxins purified from the venom of piscivorous cones, due to their high specificity, have been essential tools to characterize the different sub-types of calcium channels in nerve cells and chemical synapses (see for reviews, CRUZ & OLIVERA, 1986; MC CLESKEY *et al.*, 1987; RIVIER *et al.*, 1987; MYERS *et al.*, 1990; OLIVERA *et al.*, 1994; MILJANICH & RAMACHANDRAN, 1995).

The first ω-conotoxin that was isolated and purified from the venom of *C. geographus* was named ω-GVIA (OLIVERA *et al.*, 1984). Then, two other ω-conotoxins were directly purified from the venom of the cone *C. magus*: ω-MVIIA and ω-MVIIB (OLIVERA *et al.*, 1987) and two others were characterized (ω-MVIIC and ω-MVIID) from cDNA gene sequences, extracted from the venom duct (KOCH *et al.*, 1990; MONJE *et al.*, 1993). Other ω-conotoxins have been isolated: ω-SVIA and ω-SVIB from the venom of *C. striatus*, ω-TVIA from the venom of *C. tulipa* and ω-RVIA from the venom of *C. radiatus* (MILJANICH *et al.*, 1991; RAMILO *et al.*, 1992). All these ω-conotoxins are basic peptides composed of 24 to 29 amino-acid residues folded by three disulfide bridges (Table 3) (NISHIUCHI *et al.*, 1986).

TABLE 3

ω-conotoxins that block voltage-gated sodium channels

ω-conotoxins	Sequences *	*Conus* species
ω-GVIA	C K S O G S S C S O T S Y N C C R S C N O Y T K R C Y	*C. geographus*
ω-MVIIA	C K G K G A K C S R L M Y D C C T G S C R S G K C	*C. magus*
ω-MVIIB	C K G K G A S C H R T S Y D C C T G S C N R G K C	
ω-MVIIC	C K G K G A P C R K T M Y D C C S G S C G R R G K C	
ω-MVIID	C Q G K G A S C R K T M Y N C C S G S C N R G R C	
ω-SVIA	C R S S G S O C G V T S I C C G R C Y R G K C	*C. striatus*
ω-SVIB	C K L K G Q S C R K T S Y D C C S G S C G R S G K C	
ω-TVIA	C L S O G S S C S O T S Y N C C R S C N O Y S R K C	*C. tulipa*
ω-RVIA	C K P O G S O C R V S S Y N C C S S C K S Y N K K C G	*C. radiatus*
Framework	**C C CC C C**	

* See references in text.

The conotoxin ω-GVIA exerts a specific action on N-type calcium channels. Indeed, it irreversibly blocks calcium channels in various mammalian neuronal preparations (FELDMAN *et al.*, 1987; REGAN *et al.*, 1991). As a result of its specific action, ω-GVIA inhibits neurotransmitter release at the frog neuromuscular junction, as revealed by the blockade of nerve-evoked end-plate potentials, the spontaneous miniature end-plate potentials being unaffected by the toxin (KERR & YOSHIKAMI, 1984; KOYANO *et al.*, 1987). Surprisingly, ω-GVIA was reported to have no effect on the mammalian neuromuscular junction (ANDERSON & HARVEY, 1987). The ω-MVIIA also blocks N-type calcium channels but, in contrast to ω-GVIA, its effects are reversible (OLIVERA *et al.*, 1987).

More recently, the conotoxins ω-MVIIC and ω-MVIID were reported to block, with a high affinity, P/Q-type calcium channels in Purkinje cells of the mammalian cerebellum, as well as (although with a lower affinity) the N-type calcium channels (HILLYARD *et al.*, 1992 ; MONJE *et al.*, 1993 ; KRISTIPATI *et al.*, 1994). As a consequence, ω-MVIIC inhibits neurotransmitter release at the mammalian neuromuscular junction, as revealed by the reduction and blockade of nerve-evoked end-plate potentials (SUGIURA *et al.*, 1995).

Therefore, it appears that the variability of intercysteine residues of ω-conotoxins (see Table 3) is responsible for the specificity of action of these neurotoxins on the different presynaptic calcium channel sub-types. Thus, ω-conotoxins are considered as essential tools, firstly to pharmacologically separate the distinct calcium channel sub-types, and secondly, to identify new ones.

TABLE 4

α-conotoxins that inhibit muscular nicotinic acetylcholine receptors

α-conotoxins	Sequences*
C. geographus	
α-GI	E C C N P A C G R H Y S C
α-GIA	E C C N P A C G R H Y S C G K
α-GII	E C C H P A C G K H F S C
C. magus	
α-MI	E C C N P A C G R H Y S C G K
α-SI	I C C N P A C G P K Y S C
α-SIA	Y C C H P A C G P K Y S C
C. ermineus	
α-EI	R D O C C Y H P T C N M S N P Q I C
Framework	**CC C C**
C. purpurascens	
αA-PIVA	G C C G S Y O N A A C H O C S C K D R O S Y C G Q
C. ermineus	
αA-EIVA	G C C G P Y O N A A C H O C G C K V G R O O Y C D R O S G G
αA-EIVB	G C C G K Y O N A A C H O C G C T V G R O O Y C D R O S G G
Framework	**C C C C C C**

* See references in text.

Conotoxins acting on nicotinic acetylcholine receptors: the α-conotoxins

Among the prominent conotoxins found in cone venom, the α-conotoxins define a set of peptides sharing a similar cysteine pattern and pharmacological target: the nicotinic acetylcholine receptors (nAChRs). Unlike the α-neurotoxins from snake venoms (comprising 60 to 80 amino-acids), the α-conotoxins are small peptides of 12 to 30 amino-acid residues, usually folded by two disulfide bridges and showing a characteristic cysteine pattern (—CC—-C—-C—) (Tables 4 and 5). Due to their small peptide length, these conopeptides can be easily obtained by chemical synthesis. Moreover, the preparation of specific derivatives is relatively straightforward since α-conotoxins have many chemical groups that can potentially be modified. As a consequence of the high variability in the sequence of α-conotoxins from one species to another (OLIVERA, 1996), a great number of these toxins have been characterized. They present a high specificity for the different types of nAChRs, *i.e.* peripheral nAChRs of skeletal muscles and central nAChRs of neurons, and even for the different receptors sub-types.

TABLE 5

α-conotoxins that inhibit neuronal nicotinic acetylcholine receptors

α-conotoxines	Séquences*
Pisvivorous Conus	
C. magus	
α-MII	G C C S N P V C H L E H S N L C
Vermivorous *Conus*	
C. imperialis	
α-ImI	G C C S D P R C A W R C
Framework	CC C C

* See references in text.

The α-conotoxins acting on peripheral nAChRs

The α-conotoxin GI, isolated from *C. geographus* (GRAY *et al.*, 1981), MI from *C. magus* (MCINTOSH *et al.*, 1982), SI from *C. striatus* (ZAFFARALLA *et al.*, 1988) and EI from *C. ermineus* (MARTINEZ *et al.*, 1995) target peripheral nAChRs that are composed of $\alpha_2\beta\gamma\delta$ subunits. Some of these conotoxins recently aroused interest due to their ability to specifically inhibit one of the two acetylcholine binding sites of nAChRs. Indeed, in mammals, the α-conotoxins MI and GI have been shown to specifically target the α/δ binding site compared to the α/γ binding site of muscle nAChRs (KREINKAMP *et al.*, 1994; GROEBE *et al.*, 1995). However, in the fish *Torpedo marmorata*, these toxins preferentially bind to the α/γ site (HANN *et al.*, 1994; GROEBE *et al.*, 1995). Site-directed mutagenesis of nAChRs

of mouse skeletal muscles led to the identification of three amino-acids that differ between δ and γ subunits and that are involved in the binding of α-conotoxins (SINE *et al.*, 1995).

The conotoxins αA-EIVA and αA-EIVB, purified from the venom of *C. ermineus*, as well as the conotoxin αA-PIVA purified from the venom of *C. purpurascens*, are polypeptides comprising 25 to 30 amino-acid residues folded by three disulfide bridges (Table 4) (HOPKINS *et al.*, 1995 ; JACOBSEN *et al.*, 1997). As reported for the α-conotoxin SI, the two conotoxins αA-EIVA and αA-EIVB block nAChRs of mammalian skeletal muscle by binding indifferently to their two sites, *i.e.* α/δ and α/γ.

The α-conotoxins acting on neuronal nAChRs

Various α-conotoxins are high-affinity ligands for neuronal nAChRs (Table 5) which are composed of α (α_2 to α_9) and β (β_2 to β_4) subunits. It is worth noting that the number of nAChR subunits, identified to date, allows the targeting of a great diversity of nicotinic receptors (by multiple combination of the subunits) in various tissues. The α-conotoxin ImI, purified from *C. imperialis* (MCINTOSH *et al.*, 1994), selectively targets the α_7 subunit, which forms an homomeric nAChR. In contrast, nAChRs composed of $\alpha_3\beta_2$ subunits are potently blocked by the α-conotoxin MII isolated from the venom of *C. magus* (CARTIER *et al.*, 1996). The α-conotoxins PnIA and PnIB, both purified from *C. pennaceus*, block *Aplysia* neuronal nAChRs (FAINZILBER *et al.*, 1994). More recently, the α-conotoxin EpI, isolated from *C. episcopatus*, was characterized as a specific inhibitor of $\alpha_3\beta_2$ and $\alpha_3\beta_4$ nAChRs (LOUGHAN *et al.*, 1998).

Finally, α-conotoxins have been reported as useful tools for phylogenetic discrimination between vertebrate nAChRs (ZAFFARALLA *et al.*, 1988). In addition, these conotoxins have proved to be very effective in probing nAChRs by photoaffinity labeling with considerable accuracy (MYERS *et al.*, 1991, 1993). Therefore, it appears that α-conotoxins represent useful tools for probing the surface of the acetylcholine receptor. Both the work so far carried out on α-conotoxins and the wide variety of cone species give great promise for potential applications of these conotoxins. The discovery of new, highly specific ligands will improve the understanding of the pharmacology, physiology and structure-activity relationships of nAChRs.

Conotoxins acting on potassium channels : the κ-conotoxin

The conotoxin κ-PVIIA, purified from the venom of *C. purpurascens*, is a polypeptide comprising 27 amino-acid residues folded by three disulfide bridges and including an amidated C terminal (Table 6) (TERLAU *et al.*, 1996b). Electrophysiological studies revealed that κ-PVIIA reversibly blocks the Shaker-type of potassium channels expressed into *Xenopus* oocytes, without affecting either the $Kv_{1.1}$ or the $Kv_{1.4}$ type of potassium channels in rat brain (SHON *et al.*, 1998b). The conotoxin κ-PVIIA appears thus to be a specific tool to study the Shaker type of potassium channels. The conotoxins κ-PVIIA and δ-PVIA act in synergy for rapid prey immobilization, as required by the « harpooners » strategy used by *C. purpurascens*.

TABLE 6

Sequence of the κ-conotoxin that blocks the voltage-gated potassium channel

κ-conotoxin	Sequence*
κ-PVIIA	C R I ONQK C F QHL DD C C S R K C NRF NK C V
Framework	**C C CC C C**

* See references in text.

Conotoxins acting on N-methyl-D-aspartate receptors: the conantokins

The conantokins are a family of conotoxins including conantokin-G, purified from the venom of *C. geographus* (McINTOSH *et al.*, 1984; OLIVERA *et al.*, 1985), and conantokin-T, isolated from the venom of *C. tulipa* (HAACK *et al.*, 1990). These toxins have been reported to block N-methyl-D-aspartate receptors and, as a consequence, to inhibit calcium influx into central nervous system neurons.

Conantokins comprise 4 to 5 γ-carboxyglutamate residues (Table 7). These residues have been suspected to play an essential role in the formation, in the presence of calcium ions, of stable α-helices, which are necessary for the physiological action of conantokins. It is worth noting that conantokins do not contain cysteine residues.

TABLE 7

Conantokins present in the venom of C. geographus *and* C. tulipa

C. geographus	
Conantokine-G	G E γ γ L Q γ N Q γ L I R γ K S N
C. tulipa	
Conantokine-T	G E γ γ Y Q K M L γ N L R γ A E V K K N A

* See references in text.

SEARCHING FOR NEW CONOTOXINS

Using molecular biology techniques, it has been possible to search in the venom duct of Conidae species for specific ADN coding for prepropeptides, which are precursors of mature toxins. Such prepropeptides have three structural segments: a signal sequence, a propeptide region and the mature toxin region. For a given family (for example the α-conotoxin family), the C-terminal end of the prepropeptide (signal sequence) is well

conserved, whereas the N-terminal part (coding for the mature toxin) is hypervariable (WOODWARD *et al.*, 1990). The conserved signal sequence may play an addressing role towards a determined region of the endoplasmic reticulum where the prepropeptide may undergo post-traductionnal modifications. Such modifications have been characterized in conotoxins, and include C-terminal amidation, glutamate carboxylation, prolyl hydroxylation (STONE *et al.*, 1982), tryptophan bromination (JIMENEZ *et al.*, 1996, 1997) and tyrosine sulfatation (LOUGHNAN *et al.*, 1998). The signal sequence could also help the specific formation of the disulfide bridges leading to the mature toxin. Indeed, a linear peptide containing 6 cysteines can fold into 15 different isomers. Notably, only one form possessing biological activity is found in the venom. Although the role of the propeptide is at present unknown, it may help the formation of disulfide bridges. The mature toxin region is hypervariable in its sequence within a conserved cysteine framework. The variability of the residues between the cysteines produces a great variety of toxin sequences. Thus, with a conserved structure (same cysteine framework), the toxin may exhibit a specificity for either sodium, calcium or potassium channels. This biochemical strategy can be summarized as follows: one common structure for diverse physiological activities.

In conclusion, the genus *Conus* represents an almost inhexaustible source of bioactive products because of its species richness, each species demonstrating an original set of conotoxins. Thus, the venoms of Conidae present a challenge both due to the number of toxins they contain and their pharmacological diversity. These conotoxins, by their selective pharmacological action, are of great interest for neuroscientists requiring selective tools to study nervous function. Moreover, conotoxins can have multiple applications, for example the probing of ionic channels with fluorescent toxins or mapping of binding sites between toxins and given ionic channels or receptors. In this way, conotoxins have already greatly improved our understanding of the function of some voltage-sensitive ionic channels. By way of peptide synthesis, most of the conotoxins are now available in large quantities for widespread use. Some conotoxins such as ω-conotoxins have major therapeutic potential. Indeed, the ω-conotoxin MVIIA is used in chronic neuropathic and malignant pain to relieve patients resistant to opioid treatments. To date, venoms from a dozen *Conus* species have been partially studied. Given the diversity of the genus *Conus* and the number of conotoxins found in the venom from each species, the search for new bioactive compounds in Conidae species offers great promise.

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CAN ANCIENT COLOUR POLYMORPHISMS EXPLAIN WHY SOME CICHLID LINEAGES SPECIATE RAPIDLY UNDER DISRUPTIVE SEXUAL SELECTION?

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Abstract. It is not sufficiently understood why some lineages of cichlid fishes have proliferated in the Great Lakes of East Africa much more than anywhere else in the world, and much faster than other cichlid lineages or any other group of freshwater fish. Recent field and experimental work on Lake Victoria haplochromines suggests that mate choice-mediated disruptive sexual selection on coloration, that can cause speciation even in the absence of geographical isolation, may explain it. We summarize the evidence and propose a hypothesis for the genetics of coloration that may help understand the phenomenon. By defining colour patterns by hue and arrangement of hues on the body, we could assign almost all observed phenotypes of Lake Victoria cichlids to one of three female («plain», «orange blotched», «black and white») and three male («blue», «red-ventrum», «red-dorsum») colour patterns. These patterns diagnose species but frequently co-occur also as morphs within the same population, where they are associated with variation in mate preferences, and appear to be transient stages in speciation. Particularly the male patterns occur in almost every genus of the species flock. We propose that the patterns and their association into polymorphisms express an ancestral trait that is retained across speciation. Our model for male colour pattern assumes two structural loci. When both are switched off, the body is blue. When switched on by a cascade of polymorphic regulatory genes, one expresses a yellow to red ventrum, the other one a yellow to red dorsum. The expression of colour variation initiates speciation. The blue daughter species will inherit the variation at the regulatory genes that can, without new mutational events, purely by recombination, again expose the colour polymorphism, starting the process anew. Very similar colour patterns also dominate among the Mbuna of Lake Malawi. In contrast, similar colour polymorphisms do not exist in the lineages that have not proliferated in the Great Lakes. The colour pattern polymorphism may be an ancient trait in the lineage (or lineages) that gave rise to the two large haplochromine radiations. We propose two tests of our hypothesis.

INTRODUCTION

Why cichlid fishes have proliferated in the Great Lakes of East Africa (FRYER & ILES, 1972) much more than anywhere else in the world, and more than any other group of freshwater fish is not sufficiently understood. Morphological and molecular estimates of the phylogeny of the East African species flocks suggest that the species that now live in the three

main lake systems (Lake Victoria, Lake Tanganyika, Lake Malawi) have also evolved in their confines, and that, in the case of Lakes Victoria and Malawi, the entire flocks are derived from one or a few closely related ancestral species (LIPPITSCH, 1993 ; MEYER, 1993 ; NISHIDA, 1997). Explanations for the outstanding species richness, as well as for variation in richness between these and other lakes, and between different cichlid lineages in the lakes must be sought in lake- and lineage-specific variation in the relative rates of speciation and extinction. Understanding the relative contributions of the four possible factor combinations is important for understanding diversification in cichlids, and may contribute more generally to the understanding of causes of variation in animal species diversity.

Geological evidence strongly suggests that gross and net rates of speciation are extraordinarily high in some of the Great Lakes. The three Great Lakes differ considerably in age. Lake Tanganyika is the oldest, estimated at 9-12 My (COHEN *et al.*, 1993). Malawi is estimated at 1-2 My (FRYER & ILES, 1972). The basin of Lake Victoria is estimated at 0.25-0.75 My (FRYER, 1996), but the lake most likely dried up 200,000 years ago (MARTENS, 1997), and seems to have dried up again in the late pleistocene and filled up again only 13,200 (BEUNING *et al.*, 1997) to 12,400 (JOHNSON *et al.*, 1996) years ago (also STAGER *et al.*, 1986). Contradicting the expectation that species numbers increase over time, the younger lakes contain considerably more endemic cichlid species than the old Lake Tanganyika. Recent figures of known species stand at about 200 for Lake Tanganyika (SNOEKS *et al.*, 1994), compared with more than 500 for Lake Malawi (KONINGS, 1995), and about 500 for Lake Victoria (SEEHAUSEN, 1996). Notwithstanding that some biologists retained doubts that Lake Victoria had been entirely dry as recently as in the late pleistocene (FRYER, 1997), it is beyond doubt that, if the basin was not entirely dry, only a shallow though extensive swamp can have persisted, possibly with seasonal pools. Whether or not some endemic cichlid species survived the drought to spawn the modern species flock, speciation must have been truly explosive in Lake Victoria. Recent field and experimental work points to a possible explanation. Supporting a hypothesis based on earlier studies of Lake Malawi cichlids, mate choice-mediated disruptive sexual selection on coloration seems a suitable mechanism to cause speciation even in the absence of geographical isolation. In this paper we summarize the evidence and propose a hypothesis for the genetics of coloration that may help understand the phenomenon.

MATE CHOICE, SEXUAL SELECTION AND SPECIATION IN CICHLIDS

Most of the species-rich East African cichlid lineages possess polygynous mating systems in which females invest heavily in parental care (mouthbrooding) while males do not contribute to parental care. Sexual selection holds particular promise to explain elevated speciation rates in such situations (WEST-EBERHARD, 1983). The strongly asymmetric investment in parental care is conducive to sexual selection upon male secondary sexual characters. As predicted, many polygynous cichlids have sexually strongly dimorphic breeding coloration and sexually dimorphic courtship behaviour, and the most species-rich lineage of cichlids is polygynous (DOMINEY, 1984). These are the haplochromines with more than 1200 known species (KONINGS, 1995 ; TURNER, 1996 ; SEEHAUSEN, 1996 ; KAUFMAN *et al.*, 1997), that make up the entire endemic species flock of Lake Victoria and 99% of the endemic species in Lake

Malawi. Ecological field work and population genetical studies conducted in the late 70s and early 80s on two haplochromine taxa in Lake Malawi suggested the possibility of sympatric speciation via colour polymorphism associated with coloration-based mate selection (HOLZBERG, 1978; MARSH *et al.*, 1981; MCKAYE *et al.*, 1982, 1984).

The absence of intermediate colour morphs, and the lack of evidence for the predicted gradations in the degree of morph differentiation and isolation (MCKAYE *et al.*, 1982) may have contained the impact of these findings. Most authors continued to propagate various allopatric speciation models to explain the evolution of cichlid species flocks. Specifically, speciation in separate lake basins and in marginal lagoons (satellite lakes) has been proposed to explain the origin of the haplochromine species flock in Lake Victoria (FRYER & ILES, 1972; GREENWOOD, 1974; KAUFMAN & OCHUMBA, 1993; MEYER, 1993; FRYER, 1996; but see HOOGERHOUD *et al.*, 1983). We had argued that there are biological arguments for intralacustrine origin of species diversity, derived from distribution patterns and ecological specializations among the recent fauna (SEEHAUSEN, 1996: 269ff). This view received support (KAUFMAN *et al.*, 1997) after new geological evidence for a very recent desiccation (complete or incomplete), in combination with new data on the shape of the lake basin became available, that made a particularly strong case for speciation within one water body. Core evidence from the deepest part of the lake, together with seismic evidence, suggests a several millenia-long total desiccation of the lake that ended 13,000 to 12,400 years ago (JOHNSON *et al.*, 1996; BEUNING *et al.*, 1997). The implications for the palaeoclimate (nearly 50% of the rainfall in the region stems from lake-derived moisture) make it unlikely that satellite lakes could have persisted during the drought (JOHNSON *et al.*, 1996). Basin morphology rules out the existence of more than one basin when the lake filled up again (JOHNSON *et al.*, 1996). Palaeohydrological evidence rules out that lake level fluctuations since the refill, potentially causing allopatric speciation in satellite lakes, could account for any major part of the 500+ speciation events (BEUNING *et al.*, 1997). The largest satellite lake today (Lake Nabugabo) contains merely 5 endemic cichlid species despite the fact that it has been isolated from Lake Victoria for at least a third of the time that was available for the formation of the modern species flock of Lake Victoria (GREENWOOD, 1965). The case of the haplochromines in Lake Victoria, therefore, is a challenge to the conventional view (MAYR, 1963; PATERSON, 1985) that speciation occurs as a byproduct of population differentiation in geographical isolation. Although not of quite so recent origin, the species flock of Lake Malawi poses a similar challenge (TURNER, 1994). The water level of Lake Malawi has fluctuated much over the millenia but also in this case, the basin morphology rules out the possibility that the lake had ever been split into isolated subbasins (for sympatric origin of cichlid species flocks elsewhere see also SCHLIEWEN *et al.*, 1994).

However, given the lack of empirical demonstration of the mechanics of sympatric speciation in cichlid fish, many researchers remained reluctant to consider sympatric modes of speciation in cichlid species flocks. This has recently began to change. Speciation models, tailored to match the case of the haplochromine cichlids, suggest the possibility of rapid sympatric speciation under sexual selection (TURNER & BURROWS, 1995; PAYNE & KRAKAUER, 1997; VAN DOORN *et al.*, 1998). Patterns in species diversity and intraspecific polymorphisms, support this possibility now more strongly than before because evidence has been produced for gradations in the degree of isolation and differentiation of colour morphs,

from situations in which they are conspecific colour morphs to situations in which the same phenotypes behave as reproductively isolated species. Experimental studies of mate choice in polymorphic populations and among sympatric species, and genetics of polymorphisms, begin to make the mechanics of speciation visible, as we shall summarize:

NEW TESTS OF DOMINEY'S SEXUAL SELECTION AND HOLZBERG'S POLYMORPHISM HYPOTHESES

The hypothesis that colour and mate preference diversification are associated with speciation (DOMINEY, 1984) received support from comparative studies of patterns in colour variation and species diversity. A study of Lake Malawi haplochromines revealed that the evolution of male nuptial coloration is neither phylogenetically nor ecologically constrained (DEUTSCH, 1997). A study that used a consensus tree of the greater East African cichlid radiation, and information on coloration and ecology from a large number of species, revealed that male nuptial coloration has likely arisen under sexual selection and is evolving in frequent association with speciation in cichlid clades with a polygynous mating system (SEEHAUSEN *et al.*, 1999).

A systematic study combining anatomical and ecological evidence with a large data set on the geographical distribution and variation in coloration of more than 100 species of haplochromines from rocky habitats in southern Lake Victoria revealed patterns that are consistent with the hypothesis of HOLZBERG (1978) that colour diversification is the first step in speciation (SEEHAUSEN, 1996; SEEHAUSEN *et al.*, 1998a): (1) Closely related (= congeneric) species with identical geographical distribution usually have conspicuously different body coloration (usually red or yellow versus blue) but usually differ little in ecology. (2) Closely related species with different geographical distributions but considerable overlap, often differ only in fin coloration and do not differ much in ecology either. (3) Sympatric and allopatric species that are not closely related differ or do not differ in coloration but usually differ distinctly in ecology. (4) There are forms living sympatrically that differ in body coloration but differ neither anatomically nor ecologically. Breeding in captivity in four of such cases has demonstrated that these are conspecific colour morphs that are not, or only incompletely isolated (*Neochromis omnicaeruleus*, Seehausen & Bouton, 1998: four male and two female colour morphs obtained from breeding with a monomorphic pair; *N. greenwoodi,* Seehausen & Bouton, 1998, *Pundamilia pundamilia*, Seehausen & Bouton, 1998, *P. nyererei*, Witte-Maas & Witte, 1985: two male colour morphs obtained in single clutches). Field work has shown that what are interbreeding colour morphs in some localities can be isolated sibling species in other localities (SEEHAUSEN, 1997; SEEHAUSEN *et al.*, 1997).

Three predictions regarding mechanisms, yielded by the hypothesis that colour diversification is the first step in speciation, have been tested on a subset of the same Lake Victoria haplochromines, and partly also on Lake Malawi haplochromines. All three were supported by all tests done to date: (1) It has been shown that coloration affects intraspecific mate choice, such that colour polymorphism is associated with mate preference polymorphism, possibly exerting disruptive sexual selection on coloration (SEEHAUSEN, VAN ALPHEN & LANDE, unpubl.). (2) There is direct interspecific mate choice among closely related species (HOLZBERG, 1978; MARSH, *et al.*, 1981; SEEHAUSEN, 1997; KNIGHT *et al.*,

1998) in which coloration is important (SEEHAUSEN & VAN ALPHEN, 1998). (3) Closely related species are reproductively isolated only by mate choice (SEEHAUSEN *et al.*, 1998b).

If conspicuousness of, and diversity in coloration evolve under sexual selection, diversification of coloration is possible only where light conditions make colour variation visible. Three predictions regarding patterns, yielded by this hypothesis were also supported by tests: The underwater light regime (band width of the spectrum) is the ecological variable that explains most of the variation in the number of species that coexist at isolated rocky islands in Lake Victoria, most of the variation in the number of sympatric colour morphs within a species, and most of the variation in the distinctiveness of hue difference between sympatric species (SEEHAUSEN *et al.*, 1997). Hence, there is now strong support both for the hypothesis of speciation by sexual selection (DOMINEY, 1984) and for the hypothesis of speciation without geographical barriers via colour polymorphism (HOLZBERG, 1978).

SHORTCOMINGS OF THE SEXUAL SELECTION HYPOTHESIS

Recent reviews of the fish speciation literature showed that speciation rates of polygynous cichlids in large lakes did not seem generally different from those in monogamous cichlids or other fish taxa. Speciation rates of freshwater fish were found to be generally higher in lakes than in rivers, which may be due to frequent disruptive natural selection in lacustrine environments, which can lead to sympatric ecological speciation (SKULASON & SMITH, 1995; SCHLUTER, 1996). The haplochromines in Lakes Victoria and Malawi were the only striking exception (MCCUNE, 1997; SEEHAUSEN, 1999a, b). They speciated by one to several orders of magnitude faster than all other fish groups for which information was available, including other polygynous cichlids. As a direct consequence, close to two thirds of the currently known more than 2000 cichlid species belong to the haplochromine tribe, while the remaining species are distributed over 14 other tribes. This strongly implies effects of lineage-specific properties on speciation rates. Beyond those, environment-specific properties are strongly implicated too: haplochromines have undergone diversification only in some of the many lakes in which they occur (SEEHAUSEN *et al.*, 1997), and the riverine haplochromine sister taxa to the lacustrine species flocks have not undergone more diversification than other riverine cichlid lineages. African rivers harbour about 50 haplochromine species (GREENWOOD, 1979).

Hence, the explanation for the unique species richness of haplochromine species flocks has to be sought in lineage-specific properties that unfold their impact on diversification only under some extrinsic conditions. Mate choice based on coloration could be this property because the impact of sexual selection on coloration depends on visual conditions and predation regimes (ENDLER, 1991), and because both differ profoundly between lakes and rivers, and differ also between different lakes. Sympatric speciation due to disruptive sexual selection on coloration is predictably even faster than sympatric speciation due to disruptive natural selection because the mating system is directly under disruptive selection (WU, 1985; TURNER & BURROWS, 1995; PAYNE & KRAKAUER, 1997). When ecological opportunity is available – and the particular anatomy of cichlids makes it that it very often is (GALIS & DRUCKER, 1998) – speciation by disruptive sexual selection will proba-

bly speed up the adaptive radiation process by rapidly generating sympatric genetically isolated incipient species that can respond more quickly than can interbreeding resource utilization morphs to the disruptive natural selection. Because ecological differentiation that is sufficient for coexistence can be less discrete than ecological differentiation that would cause assortative mate choice (which is required for ecological speciation), species packing is expected to become more dense in taxa that speciate by disruptive sexual selection, provided that signal space (*e.g.* colour visibility and perception) does not limit signal diversification. Beyond causing speciation in sympatry, disruptive sexual selection has a role in maintaining reproductive isolation in sympatry between populations that have diverged in sympatry or allopatry. It is, therefore, suitable to explain the high speciation rates in haplochromines in lacustrine environments. Yet, alone it is insufficient because several other cichlid lineages (*Oreochromis* Günther, 1889, Ectodini, Tropheini) that share the same mating and parental care system with the Lake Victoria and Malawi haplochromines (polygynous ; female mouthbrooding), and also have sexually dimorphic coloration, do not share the high speciation rates with haplochromines. Moreover, the haplochromine species flocks in Lakes Victoria and Malawi live side by side with three other haplochromine lineages that are stunningly species poor: *Serranochromis* Regan, 1920, *Pseudocrenilabrus* Fowler, 1934 and *Astatoreochromis* Pellegrin, 1903†. Only one *Serranochromis* species lives in Lake Malawi, only one *Astatoreochromis* species and only one *Pseudocrenilabrus* species live in Lake Victoria.

A GENETICAL HYPOTHESIS DERIVED FROM PATTERNS OF COLOUR VARIATION

We propose that ancestral genetical colour polymorphisms exist in the rapidly radiated lineage(s), that are not present in others, and that this difference causes the difference in propensity to speciate under sexual selection. To describe colour patterns and categorize intra- and interspecific variation in female and male Lake Victoria haplochromines, we inspected more than 10,000 and photographed more than 1000 individuals of 120 species and an additional 11 female and 24 male colour morphs (females of 108 species and colour morphs, males of 138 species and colour morphs, subsequently referred to as « morphs »). More than 300 photos of most « morphs » have been published (SEEHAUSEN, 1996). Brightness and saturation in male coloration varied greatly between conspecific populations (SEEHAUSEN *et al.*, 1997) but colour pattern (arrangement of colours on the body) varied much less (SEEHAUSEN *et al.*, 1998a).

Defining colour patterns by hue and arrangement of hues on the body, hence not considering the melanophore systems (SEEHAUSEN *et al.*, 1999), we could assign 105 of the 108 female « morphs » to one of three female patterns, and 124 of the 136 male « morphs » to one of three male nuptial patterns (Table 1, Fig. 1). The female colour patterns were: (i) « Plain »: This is the most common colour pattern in females (96 « morphs »). It is a cryptic brownish pattern that varies in lightness, possibly depending on the background colour in the environment. (ii) « Orange blotched » (OB): This is bright and conspicuous, consisting of dark brown blotches on bright orange or pink (8 «morphs»). (iii) « Black and white » (WB): This is similarly bright and conspicuous and consists of black blotches on brownish to whitish (6 « morphs »). « OB »

and « WB » patterns also occur occasionally in males. The common male nuptial colour patterns were: (i) « Blue »: blue is the only hue on the entire flanks, including the head (66 « morphs »). (ii) « Red-ventrum »: the anteroventral region of the body (gill cover, chest and ventrum) is yellow, orange or red, the remainder of the flanks is yellow-green, blue or grey (35 « morphs »). (iii) « Red-dorsum »: the anterodorsal region of the body (head and dorsum) and the dorsal fin are yellow, orange or red, the remainder of the flanks is yellow-green or blue (26 « morphs »). The extension of the yellow-red patches in « red-dorsum » and « red-ventrum » is very variable. Even entirely yellow or red individuals can be assigned because the brightness center of the yellow-red coloration is distinctly anteroventrally or anterodorsally, and the dorsum or ventrum respectively is uncoloured. Both colour pattern trimorphisms, but that of the males in particular, are common also in various genera of Lake Victoria haplochromines that inhabit other habitats and were not included in this study.

TABLE 1

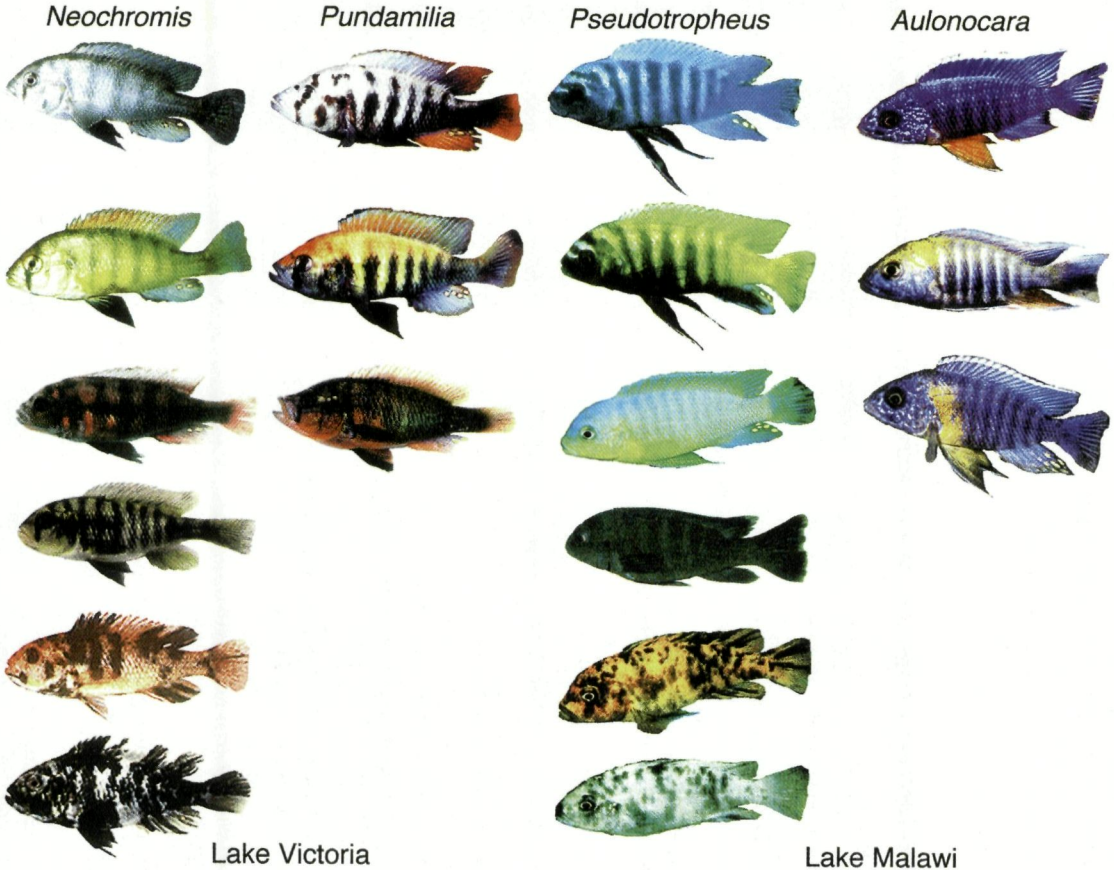
Distribution of female and male colour patterns over species of rock-dwelling haplochromines from Lake Victoria

All species are discussed in Seehausen (1996), many of which are still undescribed. Female patterns: P « plain », OB « orange blotched », WB « black and white blotched», Y « yellow »; male patterns: B « blue », Rv « red-ventrum », Rd « red-dorsum », others are written out in full. Melanic phenotypes that could not be assigned to a colour pattern are given as « Melanic ». Overall $n > 10{,}000$.

Species	*females*	*males*
Neochromis greenwoodi Seehausen & Bouton, 1998	P, OB	B, Rv, Rd
Neochromis omnicaeruleus Seehausen & Bouton, 1998	P, OB, WB	B, Rv, Rd
Neochromis 'unicuspid scraper'	P, OB	B
Neochromis 'yellow anal scraper'	P, OB	Rv
Neochromis 'orange anal picker'	P, OB	B
Neochromis rufocaudalis Seehausen & Bouton, 1998	P	B
Neochromis gigas Seehausen & Lippitsch, 1998	P	B
Neochromis 'short head nigricans'	P	B
Neochromis 'red tail giant scraper'	?	B
Neochromis 'black tail giant scraper'	?	B
Neochromis 'eastern blue scraper'	P	B
Neochromis 'long black'	P	B, Rv
Neochromis 'Bihiru scraper'	P	B
Neochromis 'large eye nigricans'	P	?
Neochromis 'pseudoblack'	P	Melanic
Mbipia mbipi Seehausen, Lippitsch & Bouton, 1998	P	B
Mbipia 'short scraper'	P	B
Mbipia 'large eye black'	P	B
Mbipia 'red anal blue'	P	B, Rd
Mbipia lutea Seehausen & Bouton, 1998	P	Rv
Mbipia 'red carp'	P	Rv
Mbipia 'orange carp'	P	Rv
Pundamilia 'Ukerewe'	P	B
Pundamilia azurea Seehausen & Lippitsch, 1998	P	B
Pundamilia 'pink anal'	P	B

Species	*females*	*males*
Pundamilia 'red rim anal'	P	B
Pundamilia 'Bwiru'	P	B
Pundamilia 'red anal'	P	B
Pundamilia pundamilia Seehausen & Bouton, 1998	P	B, Rv
Pundamilia 'big blue'	P	B, Rv, Rd
Pundamilia 'red head'	P	Rv
Pundamilia 'red flank'	?	Rv
Pundamilia 'all red'	?	Rv, Rd
Pundamilia nyererei (Witte-Maas & Witte, 1985)	P	Rd, Melanic
Pundamilia igneopinnis Seehausen & Lippitsch, 1998	P	Rd, Melanic
Pundamilia 'lemon fin'	P	Rd
Pundamilia 'orange dorsal'	P	Rd
Pundamilia macrocephala Seehausen & Bouton, 1998	P	B
Pundamilia 'blue deepwater'	P	B
Pundamilia 'yellow deepwater'	P	Rd
Pundamilia 'slender deepwater'	P	Rd
Pundamilia 'orange anal'	P	Melanic
Pundamilia 'small mouth'	P	Melanic
Pundamilia 'all black'	?	Melanic
Lithochromis 'yellow chin'	P	B
Lithochromis 'Ukerewe'	?	B
Lithochromis 'black Ukerewe'	P	B
Lithochromis 'long snout'	P	B
Lithochromis rubripinnis Seehausen, Lippitsch & Bouton, 1998	P	B, Rd
Lithochromis 'scraper'	P	B, Rd
Lithochromis 'pseudoblue'	P	B, Rd
Lithochromis xanthopteryx Seehausen & Bouton, 1998	P, OB	Melanic, Rd
Lithochromis 'orange'	P	Rd
Lithochromis 'Gana'	?	Rd
Lithochromis rufus Seehausen & Lippitsch, 1998	P	Rv
Haplochromis 'blue obliquidens'	P	B
Haplochromis lividus Greenwood, 1956	P	Rv
Haplochromis 'orange chest silvery scraper'	P	Rv
Haplochromis 'purple yellow'	P	Rd
Haplochromis 'red back scraper'	P	Rd
Ptyochromis 'striped rock sheller'	?	B
Ptyochromis 'Zue sheller'	P	B
Ptyochromis sauvagei (Pfeffer, 1896)	P, WB	B, Rv
Ptyochromis xenognathus (Greenwood, 1957)	P	B, Rv
Ptyochromis 'deep water rock sheller'	P	B, Rd
Ptyochromis 'red giant sheller'	P	Rv
Ptyochromis 'red rock sheller'	P	Rv
Macropleurodus bicolor (Boulenger, 1906)	P, WB	Rv
Paralabidochromis chilotes (Boulenger, 1911)	P, WB	B, Rv
Paralabidochromis 'short head chilotes'	P	Rv
Paralabidochromis cf. chromogynos (Greenwood, 1959)	WB	?

Species	*females*	*males*
Paralabidochromis 'pointed jaw chromogynos'	?	B
Paralabidochromis 'fleshy lips'	P	B
Paralabidochromis 'long teeth'	WB	?
Paralabidochromis 'short snout scraper'	P	B
Paralabidochromis 'blue short snout scraper'	P	B
Paralabidochromis 'red short snout scraper'	P	Rv
Paralabidochromis 'elongate short snout scraper'	P	Rv
Paralabidochromis 'rock macula'	Y	Rv
Paralabidochromis 'rockkribensis'	Y	Rv
Paralabido. plagiodon (Regan & Trewavas, 1928)	P	Rv
'Haplochromis' cyaneus Seehausen, Bouton & Zwennes, 1998	P	B
'Haplochromis' 'Zue rockpicker'	P	B
'Haplochromis' 'rockpicker'	P	B
'Haplochromis' 'elongate rockpicker'	?	B
'Haplochromis' 'orange anal picker'	P	B
'Haplochromis' 'sky blue picker'	?	B, Rd
'Haplochromis' 'pseudorockpicker'	P	B, Rd
'Haplochromis' flavus Seehausen, Zwennes & Lippitsch, 1998	P	Rv, B
'Haplochromis' 'red pseudorockpicker'	?	Rv
'Haplochromis' 'chessboard picker'	?	Rv
Psammochromis riponianus (Boulenger, 1911)	P	B
Psammochromis 'blue sharp snout'	?	B
Psammochromis 'rock riponianus'	P	Grey
Psammochromis saxicola (Greenwood, 1960)	P	Grey
Psammochromis aelocephalus (Greenwood, 1959)	P	Rv
Psammochromis 'red Zebra'	?	Rv
Psammochromis 'Ruti-Psammo'	P	?
Psammochromis 'striped crusher'	P	?
Psammochromis 'yellow giant crusher'	?	Rv
Lipochromis cryptodon (Greenwood, 1959)	?	B
Lipochromis 'velvet black cryptodon'	OB	B
Lipochromis 'blue microdon'	?	B
Lipochromis cf. melanopterus (Trewavas, 1928)	P, OB	Rv
Lipochromis 'nyererei paedophage'	?	Rd
Lipochromis 'matumbi hunter'	P	Grey
Harpagochromis serranus (Pfeffer, 1896)	P	B
Harpagochromis 'big blue hunter'	P	B
Harpagochromis howesi (Van Oijen, 1992)	P	B
Harpagochromis 'orange rock hunter'	P	Rv
Harpagochromis cavifrons (Hilgendorf, 1888)	Blotched	?
'Astatotilapia' nubila (Boulenger, 1906)	P	B
'Astatotilapia' 'incurved dorsal head profile'	P	B
'Astatotilapia' 'large brownae'	P	B
'Astatotilapia' 'black long snout'	P	Melanic
'Astatotilapia' 'black cave'	?	Rd
'Haplochromis' 'brown narrow snout'	?	Rd
'Haplochromis' 'orange belly'	?	Rv
'Haplochromis' 'pale egg dummy'	?	Grey
'Haplochromis' 'stone'	P	B, Rd


Neochromis
Pundamilia
Pseudotropheus
Aulonocara
Lake Victoria
Lake Malawi

Colour patterns that are very similar are common also among Lake Malawi haplochromines, in particular in the Mbuna species flock including the species of the genus *Aulonocara* Regan, 1922 (Fig. 1 ; photos in KONINGS, 1995, *e.g.* : *Pseudotropheus tropheops* Regan, 1922 species complex on pp. 30, 31 ; *P. zebra* (Boulenger, 1899) species complex on pp. 48, 49, 54, 55 ; *Cynotilapia* Regan, 1922 species on pp. 106-107 ; *Petrotilapia* Trewavas, 1935 species on pp. 72, 73 ; *Labeotropheus* Abel, 1927 species on pp. 27, 131), with two differences : (1) colour patches that are red in Lake Victoria are generally yellow in Lake Malawi ; (2) the genetical basis of WB-like female coloration in the Lake Malawi species flock is likely to be different from that of WB coloration in the Lake Victoria flock. Heterozygous females look similar in both cases but homozygous females in Lake Victoria become almost black, while homozygotes in Lake Malawi seem to become white. Two studies that quantified interspecific hue variation in male Mbuna, though not considering distribution of hue patches on the body, found a strikingly bimodal distribution of hue in colour space, where most species were dominated by blue or yellow (MCELROY *et al.*, 1991 ; DEUTSCH, 1997). Functional explanations for the haplochromine colour patterns are given elsewhere (SEEHAUSEN, 1999b).

Experimental and systematic studies have demonstrated that in Lake Victoria haplochromines, all these colour patterns that can diagnose species frequently co-occur in one population (SEEHAUSEN, 1996 ; SEEHAUSEN & BOUTON, 1996 ; SEEHAUSEN *et al.*, 1998a ; and unpublished data mentioned above in the section « new tests... »). In 18 of the studied species, populations were polymorphic for male nuptial colour pattern, three additional species had different male nuptial colour patterns in different populations, and populations in 10 species were polymorphic for female colour pattern (Table 1). As summarized above, laboratory studies demonstrated that sympatric occurrence of several male nuptial colour patterns is associated with variation in female mate preferences within (SEEHAUSEN, 1999a ; SEEHAUSEN, VAN ALPHEN & WITTE, unpubl.) and between species (KNIGHT *et al.*, 1998 ; SEEHAUSEN and VAN ALPHEN, 1998), and sympatric occurrence of female colour pat-

Legend to the figure (see page 52)

Fig. 1. – Representatives of the male and female colour patterns from two ecologically different genera of the Lake Victoria species flock and two ecologically different genera of the Lake Malawi species flock. Columnwise from left to right and from top to bottom.

– Column 1 (*Neochromis*, algae scrapers from Lake Victoria) : *N. omnicaeruleus* «blue» male, *N. omnicaeruleus* «red-dorsum» male, *N.* spec. «long black» «red-ventrum» male, *N. omnicaeruleus* « plain » female, *N. omnicaeruleus* «OB» female, *N. omnicaeruleus* «WB» female.

– Column 2 (*Pundamilia*, macroinvertebrate and plankton eaters from Lake Victoria) : *P. pundamilia* «blue» male, *P. nyererei* «red-dorsum» male, *P.* spec. «red head» «red-ventrum» male.

– Column 3 (*Pseudotropheus* Regan 1922, algae scrapers from Lake Malawi) : *P. zebra* «blue» male, *P.* spec. «zebra gold» «yellow-dorsum» male, *P.* spec. «aurora yellow » « yellow-ventrum» male, *P. zebra*, « plain » female, *P. estherae* Konings 1995 «OB» female, *P. callainos* Stauffer & Herf, 1992 «WB» female.

– Column 4 (*Aulonocara*, macroinvertebrate eaters from Lake Malawi) : *A. stuartgranti* Meyer & Riehl 1985 «blue» male, *A. jacobfreibergi* (Günther, 1889) «red-dorsum» male, *A. stuartgranti* «red-ventrum» male. The photos of Lake Malawi cichlids are reproduced with permission from KONINGS (1995).

terns is associated with variation in male mate preferences within (SEEHAUSEN, VAN ALPHEN & LANDE, unpubl.) and between species (HOLZBERG, 1978; KNIGHT & TURNER, pers. comm.). The degree of isolation and differentiation between colour morphs varies between localities in Lake Victoria, ranging from little to distinct differentiation with complete isolation (SEEHAUSEN, 1997; SEEHAUSEN *et al.*, 1997, SEEHAUSEN, VAN ALPHEN & LANDE, unpubl.).

Studies of colour variation in relation to geographical distribution, conducted in Lake Victoria, show that when closely related species are sympatric in their entire distribution range, they more often than closely related species with different distribution ranges exhibit alternative colour patterns, where one has «blue» males and the other one «red-dorsum» or «red-ventrum» males, or where one has «OB» or «WB» females and the other one does not (SEEHAUSEN, 1999b). Altogether, this implies that the two commonly observed and widely distributed colour polymorphisms that we describe, can be transient stages in sympatric speciation by disruptive sexual selection. As far as published information allows conclusions, this hypothesis is also supported by the distributions of colour patterns over closely related sympatric species among the Mbuna of Lake Malawi (KONINGS, 1995[1]), and by population genetical studies on one sibling species complex the species of which differ in male nuptial colour patterns (MCKAYE *et al.*, 1982), and another one the species of which differs in female colour patterns (MCKAYE *et al.*, 1984).

The stereotypic recurrence of colour patterns and their stereotypic associations into polymorphisms in various genera of Lake Victoria haplochromines, and even in the Mbuna of Lake Malawi, is peculiar. It is difficult to imagine that it would have evolved over and over again in all genera of endemic Lake Victoria cichlids and Lake Malawi Mbuna. It would be easier to understand if patterns and polymorphisms were ancestral traits of the lineage (or lineages) that gave rise to the two big radiations. That a polymorphism could cause speciation and yet be retained through large radiations is paradoxical. However, it does not need to be contradictory. Studies on the genetics of haplochromine coloration revealed epistatic interactions between structural and regulatory genes, where the latter influence expression of the structural genes (fin coloration of haplochromines [KORNFIELD 1991]; «WB» and «OB» female colour pattern polymorphism [SEEHAUSEN, VAN ALPHEN & LANDE, unpubl.]). From ongoing experiments we have indications that similar epistatic gene interactions may also be involved in male nuptial colour pattern polymorphism.

We propose here a hypothesis for the genetics underlying male nuptial colour polymorphism that is suitable to explain the retention of the polymorphism through speciation events that are caused by its very presence. A similar model may be relevant also for female

(1) KONINGS (1995) gives distribution maps of *Labeotropheus* species. At eight localities he found two species living sympatrically. At all but one place the sympatric species display alternative hue patterns. At six places a yellow-ventral species coexists with a blue species, at one West coast locality a yellow-ventral species (*L. fuelleborni* Ahl, 1927) coexist with a yellow-dorsal species (*L. trewavasae* Fryer, 1956), and at another West coast locality two blue species were found together. (Yellow-ventral from the East coast was assigned to *L. trewavasae*, and blue from the East coast to *L. fuelleborni* while the reverse is true among the West coast fishes, except for the one blue population of *L. fuelleborni*). Numerous other examples can be found in Konings' book.

colour polymorphisms. If the three male colour patterns are coded for by non-allelic structural genes, that are switched on or off by cascades of regulatory genes, the retention of the polymorphism as a «trait of speciation» becomes plausible. Our model assumes two structural loci, one of which, when switched on, expresses a yellow-red ventrum, and the other one a yellow-red dorsum. When both are switched off, the body is entirely blue. These structural genes are switched on by a cascade of polymorphic regulatory genes as a consequence of recombination (Fig. 2). Expression of the structural genes for « red-ventrum » and « red-dorsum » has consequences for the probability of mating with females that differ in their preferences for red and blue mates. Such preference variation has been found (SEEHAUSEN, 1999a), and could be due to variation in spectral sensitivity, or « wiring » in the eye and the brain, causing variation in individual perception of the male colours. The selective mating between red males and red-sensitive females, and blue males and blue-sensitive females would lead to speciation. Species of Lake Victoria haplochromines differ in their relative sensitivities to blue and red light (SMIT & ANKER, 1997).

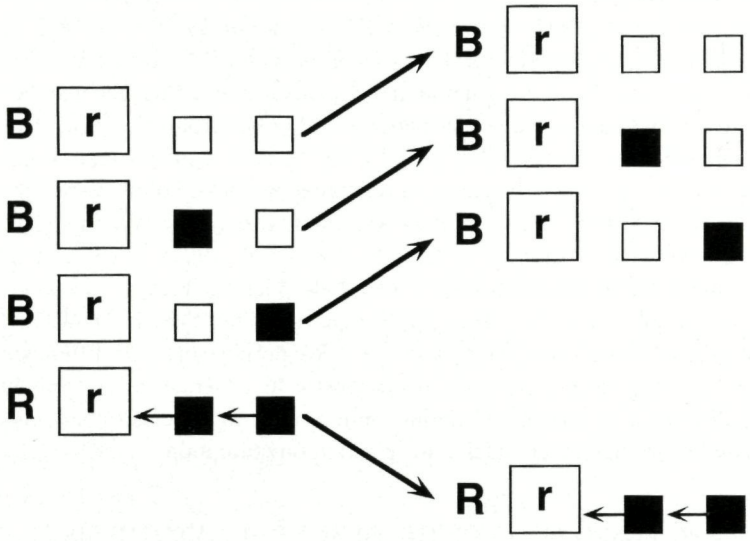


Fig. 2. – Schematic genetical model for persistance through speciation of the colour polymorphism that causes speciation. Boxes represent genes, lettres outside boxes represent planotypes Large boxes represent a structural gene complex for «red coloration» that is expressed (capital R) only when particular alleles at a number of polymorphic regulatory loci (small boxes) come together. If the structural gene is not expressed, the phenotype is blue (capital B). If females vary in their preference for blue and red, selective mating of blue males with blue-preferring females and red males with red-preferring females may lead to speciation. Both daughter species inherit the structural gene complex that causes colour variation when switched on in some and switched off in other individuals. While the red daughter species has lost the variation at the regulatory loci that can cause variation in expression of the structural gene, and mutational events would be required to restore it, the blue daughter species inherits the variation. New red morphs can be generated in the blue daughter species purely by recombination. The rate at which red morphs are produced will depend on the number of regulatory loci required to interact in order to express the structural gene, and on the number of alleles at these loci.

The structural genes for alternative colours that cause speciation when exposed, would be inherited by both daughter species. While the red daughter species has lost the variation at the regulatory loci that can cause this variation in expression of the structural genes, and mutational events would be required to restore it, the blue daughter species inherits the variation (Fig. 2). Selection would operate mainly upon the regulatory gene interaction while the structural gene complexes would be conserved (though variation in extension and hue of the yellow/red colour patches implies responsiveness to selection within the structural gene complexes). New red morphs, and therewith the variation in colour expression, that holds the potential to break up the sexual coherence of populations, can be generated in the blue daughter species purely by recombination.

Species of other lacustrine cichlid lineages can also be polymorphic for coloration. Several colour polymorphisms have been described by KOHDA *et al.* (1996) and are shown by KONINGS (1988), but none of the colour patterns involved resembles those associated with disruptive selection and sympatric speciation in Lake Victoria. Despite existence of intraspecific (SEEHAUSEN, 1996: 258 for *Astatoreochromis alluandi* Pellegrin, 1903; TWENTYMAN-JONES *et al.*, 1997 for *Pseudocrenilabrus philander* (Weber, 1897), and interspecific (GREENWOOD, 1979; 1989 for *Astatoreochromis* and *Pseudocrenilabrus*; SKELTON, 1993 for *Serranochromis*) colour variation in the haplochromine lineages that did not show any explosive diversification, the colour patterns and polymorphisms associated with disruptive sexual selection in Lake Victoria and Lake Malawi haplochromines appear to be also entirely absent from these lineages. In a detailed study of colour variation only one colour type of the genus *Pseudocrenilabrus* was found in any one lake in southern Africa despite a 16-30,000 years history (TWENTYMAN-JONES *et al.*, 1997). In contrast, even small lakes that are inhabited by haplochromines of the Lake Victoria lineage, such as crater lakes in Uganda, commonly have more than one sympatric colour type (L. and C. CHAPMAN, L.S. KAUFMAN & R. OAUTO-KWAYO, pers. comm.). We propose that this difference between species-poor and species-rich lineages in propensity to generate colour polymorphisms explains the difference in rates of speciation, and in resulting species richness under environmental conditions that are conducive to selection on coloration.

PROPOSED TESTS OF THE GENETICAL HYPOTHESIS

Our hypothesis ought to be tested by breeding experiments but also yields at least two explicit and testable predictions about phylogenetic patterns.

(1) The frequency of intraspecific colour polymorphism should differ between species with different predominant colour patterns. Polymorphisms should be more common in species that have the pattern with inherited epistatic variation at the regulatory gene level as their predominant pattern. Hence, species with « blue » as predominant male nuptial colour pattern should have red morphs more often than species with « red-ventrum » or « red-dorsum » as predominant colour pattern should have « blue » morphs.

(2) The male nuptial colour polymorphism should have been present in the common ancestor of various genera of Lake Victoria cichlids, and possibly in the common ancestor of the species flocks of Lake Victoria and Lake Malawi (or the Mbuna of Lake Malawi).

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BIODIVERSITY OF FORAMINIFERA AND OTHER PROTISTS IN THE DEEP SEA : SCALES AND PATTERNS

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Abstract. Ocean-floor sediments harbour a variety of protistan taxa, including ciliates, flagellates, naked amoebae, testate amoebae, foraminifera and xenophyophores. Only the foraminifera and xenophyohores, however, are reasonably well studied at the species level. Despite being an important component of deep-sea communities, these protists are frequently disregarded in biodiversity studies. This is unfortunate because « live » (rose Bengal stained) foraminifera are rich in species and morphologically very diverse. Individual samples from well-oxygenated bathyal and abyssal settings may contain up to 150 and sometimes more than 200 live species (>63-μm fraction). The local diversity of foraminifera seems broadly comparable to that of nematodes among the meiofauna and polychaetes among the macrofauna. Particularly at abyssal sites, many species are undescribed and belong to poorly-known, soft-shelled taxa. Extrapolating from local to global diversity (a popular activity in biodiversity research) is hampered by lack information about species distribution patterns, particularly for the soft-shelled taxa. However, many deep-sea foraminiferal species in « normal » well-oxygenated deep-sea settings appear to be widely distributed, implying relatively modest levels of global diversity.

Trends in foraminiferal diversity in response to regional gradients of increasing organic enrichment and decreasing oxygen concentrations are fairly well described ; species richness decreases, and dominance increases. Changes in foraminiferal diversity with increasing bathymetric depth down the continental slope have also been reported, but latitudinal diversity gradients remain largely undocumented among foraminifera in modern deep-sea settings. Because of their extensive fossil record, calcareous and other hard-shelled species can be used to address the influence of historical processes on large-scale diversity patterns. For example, the establishment of an Antarctic ice sheet 35 million years ago has been linked to the development of an ancient latitudinal diversity gradient among deep-sea foraminifera in the Southern Hemisphere.

Xenophyophores are much less speciose than foraminifera. It has been estimated by TENDAL (1996) that only about one hundred species, described and undescribed, exist in modern oceans. Where the two groups coexist at a single locality, there may be an order of magnitude fewer xenophyophore species than foraminiferal species. The much lower number of xenophyophore species probably reflects their larger size and narrower ecological tolerance compared to foraminifera.

Key words : Foraminifera, protist, diversity, xenophyophore, paleoceanography.

INTRODUCTION

Biodiversity embraces all aspects of biological variety, including variety at the genetic, morphological, species, higher taxon, and community levels (*e.g.* HARPER & HAWKSWORTH, 1994; MAY, 1994; WILLIAMSON, 1997). Ideas about biodiversity have been developed largely by terrestrial biologists and only fairly recently has attention been directed to marine systems (Committee on Biological Diversity in Marine Systems, 1995; Ormond *et al.*, 1997). Yet the seas are inhabited by more major animal groups than the land (MAY, 1994), and macrobenthic soft-bottom deep-sea communities are extremely rich in species, at least at local scales (*e.g.* HESSLER & SANDERS, 1967; GRASSLE & MACKIOLEK, 1992; GAGE, 1996; SMITH *et al.*, 1998). Recently, the diverse nature of the deep-sea meiobenthos, a size fraction which is difficult and laborious to study, has received emphasis (LAMBSHEAD, 1993; LAMBSHEAD *et al.*, 1995). The scale of deep-sea diversity is comparable to that found in such obviously heterogeneous environments as coral reefs and tropical rain forests. Given the relatively featureless appearance of much of the ocean floor, explaining this phenomenon has presented ecologists with a major challenge (GAGE, 1996; SMITH *et al.*, 1998). Small-scale stochastic processes, for example, species successions in response to patches of labile organic matter (GRASSLE & MORSE-PORTEOUS, 1987; GRASSLE & MACKIOLEK, 1992) or to hydrodynamic disturbance (GAGE, 1996, 1997), appear to be important in maintaining local diversity. However, these centimetre to metre-scale processes do not necessarily explain large-scale diversity patterns, for example in relation to bathymetric and latitudinal gradients (REX *et al.*, 1997). In the case of latitudinal diversity gradients, it seems likely that evolutionary or historical processes underlie the patterns observed.

Certain groups of organisms are particularly rich in species and therefore of special importance in biodiversity research (HAMMOND, 1994; MAY, 1994). Foraminifera may merit inclusion in this category. However, most foraminiferal workers are geologists, reflecting the fact that these protists are the most common deep-sea benthic organisms preserved in the fossil record. Much of the geologically-orientated research has concerned the search for proxies of parameters which are useful in paleoceanographic reconstructions (*e.g.* MURRAY, 1995), for example, bathymetry (PHLEGER, 1960), near-bottom water masses (SCHNITKER, 1980, 1994) and organic matter fluxes to the seafloor (HERGUERA & BERGER, 1991; CORLISS & EMERSON, 1990; JORISSEN *et al.*, 1995). With some exceptions (*e.g.* the work of BUZAS *et al.*, 1969, 1994; CULVER & BUZAS, 1998, DOUGLAS, 1981; DOUGLAS AND WOODRUFF, 1998), the species diversity of deep-sea foraminifera has received relatively little attention. Moreover, those studies that have been undertaken have focused on the hard-shelled component of the fauna, rather than the soft-shelled forms which constitute a major proportion of many assemblages (GOODAY, 1994, 1996; GOODAY *et al.*, 1998). This lack of attention contrasts with the efforts devoted by benthic ecologists to establishing patterns and scales of deep-sea metazoan diversity in relation to bathymetric, latitudinal and other environmental gradients (ETTER & GRASSLE, 1992; LEVIN & GAGE, 1998; REX *et al.*, 1997; SMITH *et al.*, 1998).

This paper starts with a brief survey of protistan taxa reported from the deep sea and then reviews the scale of species diversity in foraminifera and xenophyophores (a related

group), spatial patterns in foraminiferal diversity, and the importance of the fossil foraminifera in diversity research. Except where stated, data refer to « live » (*i.e.* Rose Bengal stained) faunas.

PROTISTS IN THE DEEP SEA

All the main free-living protozoan groups occur in marine environments and most have been reported from the deep sea. Their size range is enormous, spanning five orders of magnitude from tiny flagellates, a few microns in length (TURLEY *et al.*, 1988 ; PATTERSON, 1990 ; ATKINS *et al.*, 1998), to xenophyophores with tests reaching >20cm in size (TENDAL, 1972). Small naked protists in deep-sea sediments (the « nanobiota ») are, however, very difficult to study and therefore poorly documented (THIEL, 1983 ; BURNETT & THIEL, 1988).

SMALL & GROSS (1985) discovered a variety of ciliates (8 classes, 14 families, 15 genera, at least 20 species) in water and hard substrate samples from hydrothermal vents at 21°N on the East Pacific Rise. These included abundant folliculinid heterotrichs attached to artificial surfaces (VAN DOVER *et al.*, 1988). SMALL & GROSS (1985) also reported an amoeboid organism and a colonial flagellate, each possibly representing a new family, in water samples from the same area. In contrast to their abundance on hard substrates near vents, ciliates are generally rare on soft bottoms in the deep sea (BURNETT, 1977, 1979, 1981 ; ALONGI 1987), probably because they require interstitial space for ciliary movement (BURNETT, 1981). Instead, flagellates and amoebae dominated the eukaryotic nanobiota in the central North Pacific (5498-5800m) (BURNETT 1977 ; SNIDER *et al.*, 1984) and at depths around 1200m in the San Diego Trough (BURNETT, 1979, 1981) and Coral Sea (ALONGI, 1987). A barophilic bodonid flagellate was associated with phytodetritus in the abyssal NE Atlantic (LOCHTE & TURLEY, 1988 ; TURLEY *et al.*, 1988) and flagellates which grew faster under pressure than shallow-water strains of the same species have been reported from 2500m from a hydrothermal vent area on the East Pacific Rise (ATKINS *et al.*, 1998). Although testate amoebae are generally restricted to fresh water and nearshore habitats, a very large (up to 34mm diameter) spherical species of the genus *Gromia* (Order Filosea) has recently been discovered in the bathyal (1200-1650m) NW Arabian Sea (GOODAY *et al.*, in revision).

Apart from the observations of SMALL & GROSS (1985), there are virtually no data on the species diversity of deep-sea ciliates, flagellates and amoebae. Xenophyophores and foraminifera, the testate rhizopod taxa which form the main focus of this paper, are much better known. Xenophyophores have some distinctive « soft part » features which distinguish them from foraminifera (TENDAL, 1972). They are generally classified as a distinct higher taxon, usually a class (TENDAL, 1996). Like some foraminifera, xenophyophores have an agglutinated test. Unlike most foraminifera, all xenophyophores are either large (>500µm) or very large (>1cm, sometimes >10cm) and are confined to depths below about 500m. Foraminiferal assemblages are very speciose and often exhibit high densities in the deep sea. The gross taxonomic composition of NE Atlantic faunas has been described in a number of publications (GOODAY, 1986, 1994, 1996 ; GOODAY *et al.*, 1998). Soft-shelled monothalamous taxa (allogromiids, saccamminids, psammosphaerids), *Lagenammina*

species, and hormosinaceans (*Leptohalysis* spp., *Reophax* spp) are important, particularly at abyssal oligotrophic sites. Hyaline calcareous taxa (e.g. rotaliids) are most common in areas (*e.g.* continental margins) with a higher food input. They typically dominate faunas in organically-enriched, oxygen-depleted settings.

THE SCALE OF DEEP-SEA FORAMINIFERAL DIVERSITY

Morphological diversity

Deep-sea foraminifera exhibit an extraordinary morphological diversity (BRADY, 1884 ; CULVER & BUZAS, 1998). At individual abyssal sites they can range from a few tens of microns up to several centimetres in size, and include forms with organic, agglutinated and calcareous tests. Common morphotypes includes spheres, flasks, various types of tube (branched or unbranched, open-ended or with a bulb-shaped initial part) and chambered tests with chambers arranged planispirally, trochospirally, or in biserial, triserial or more complex arrangements. One exclusively deep-sea taxon, the Komokiacea, displays morphologies (elaborate systems of branching tubules) not traditionally associated with foraminifera (TENDAL & HESSLER, 1977 ; SHIRES *et al.*, 1994). If test morphology and function are closely linked, then the great range of foraminiferal sizes and morphologies in the deep sea implies a corresponding diversity of ecological strategies. For example, test morphotypes can be related to preferences for particular microhabitats within the sediment profile (CORLISS, 1985 ; CORLISS & EMERSON, 1990 ; GOODAY, 1994) or to trophic strategies (JONES & CHARNOCK, 1985).

Local species diversity

Most deep-sea foraminiferal populations consist of a few common species and numerous rare ones. DOUGLAS & WOODRUFF (1981) summarise foraminiferal species richness and diversity data from bathyal and abyssal environments. Most of these earlier data relate to « total » (live plus dead) assemblages.

GOODAY *et al.* (1998) presented a summary of foraminiferal diversity measures from five sites in the bathyal (1340m) and abyssal (4545-4942m) NE Atlantic and the NW Arabian Sea (3350m). In Table 1, I report the same data in condensed form together with similar data from a bathyal site located at 412m in the core of the Arabian Sea oxygen minimum zone (OMZ). Rarefaction curves for the five sites are given in Fig. 1. Foraminiferal species richness and diversity were high in the well-oxygenated bathyal and abyssal NE Atlantic, and at the deep Arabian Sea site where oxygen levels were around 3.0ml.l^{-1}. They were much lower at the 412-m Arabian Sea site where oxygen concentrations were around 0.13ml.l^{-1}. Dominance (R1D) showed the opposite trend. It was low (generally <10%) at the abyssal sites, slightly higher (11-15%) in the bathyal Porcupine Seabight, and higher again (27-44% depending on size fraction) in the core of the Arabian Sea OMZ.

Diversity data for the foraminiferal macrofauna (>300µm) from three sites on the North Carolina Slope are also reported in Table 1 and included in Fig. 1. These sites lie

along a gradient of increasing organic matter input but without corresponding oxygen depletion in the bottom water (SCHAFF *et al.*, 1992). Values for species richness and diversity measures were generally higher in both the 0-2cm and 0-15cm layers at Sites I and II compared to the organically-enriched Site III.

TABLE 1

Summary of foraminiferal diversity measures from well-oxygenated sites in the NE Atlantic (GOODAY et al., 1998), the NW Arabian Sea within (412m) and below (3350m) the oxygen minimum zone (GOODAY et al., in revision a), and the North Carolina slope (HUGHES & GOODAY unpublished). Asterisk (*) indicates that the data set consists of median values. Localities: PSB = Porcupine Seabight; PAP = Porcupine Abyssal Plain; MAP = Madeira Abyssal Plain; CVAP = Cape Verde Abyssal Plain. Other abbreviations: OMZ = oxygen minimum zone; TS = topmost sediment; N = number of specimens; S = number of species; RID = Rank 1 dominance; SW2 = Shanon-Wiener index (log_2); $E(S_{100})$ = Expected number of species in a sample of 100 specimens

Locality	*Size fraction (μm)*	*Depth (m)*	*Layer*	*N*	*% Calc*	*S*	*RID*	*SW2*	*Fishers α*	$E(S_{100})$
				Northwest Atlantic						
*PSB	>45	1340	0-5cm	640	20.8	118	11.1	5.7	43.2	46.8
*PSB	>45	1340	0-1cm	335	26.8	89	14.6	5.4	35.4	45.5
*PAP	>63	4850	0-1cm	586	10.1	141	9.5	6.2	58.9	55.0
*PAP	>63	4850	0-10cm	1168.5	9.9	210.5	6.0	6.6	75.0	59.6
*MAP	>63	4940	0-1cm	345	14.2	108	5.2	6.2	62.4	60.0
MAP	>63	4940	0-10cm	575	9.2	158	9.4	6.4	71.9	59.5
CVAP	>63	4545	0-1cm	341	1.75	117	10.0	6.2	62.9	59.1
				Northwest Arabian Sea						
Below OMZm	>63	3350	0-1cm	1282	6.2	208	5.3	2.02	67.1	61.4
Below OMZ	>125	3350	0-1cm	893	1.5	158	6.8	1.92	61.0	58.7
Core of OMZ	>63	412	TS	3647	84.9	64	27.0	0.98	8.24	18.6
Core of OMZ	>125	412	0–1cm	6188	70.7	49	44.1	1.23	9.28	23.9
				North Carolina Slope						
*Site I	>300	850	0-2cm	231	3.8	53	12.1	1.50	22.14	37.3
Site I	>300	850	0-15cm	301	2.3	59	22.9	1.39	21.93	34.8
Site II	>300	850	0-2cm	286	6.4	64	12.9	1.53	25.61	39.9
Site II	>300	850	0-15cm	486	4.3	71	16.7	1.47	22.90	36.0
*Site III	>300	850	0-2cm	323	68.5	25	65.3	0.65	6.33	15.5
*Site III	>300	850	0-15cm	966	73.8	33	71.7	1.08	6.62	14.3

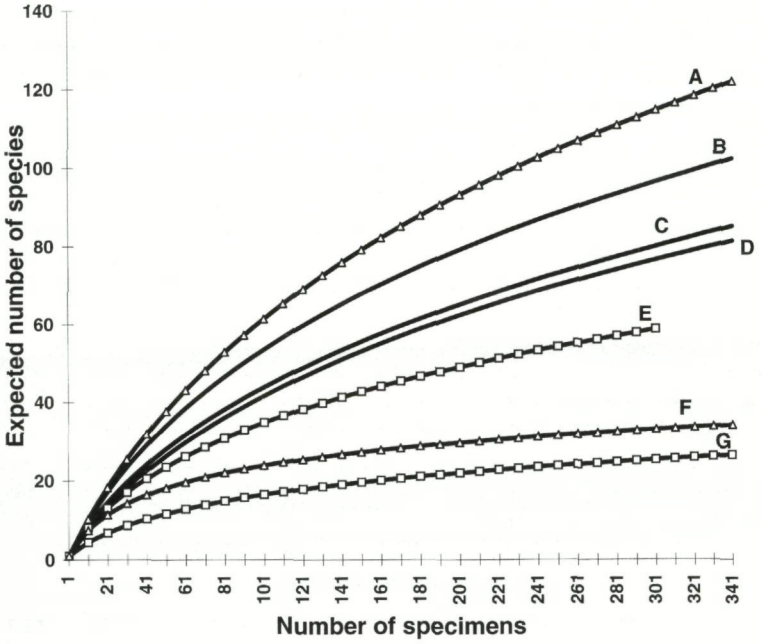

Fig. 1. – Rarefaction curves based on «live» (Rose Bengal stained) foraminifera from individual samples obtained at sites in the N. Atlantic and NW Arabian Sea. A, F are from the NW Arabian Sea, *Discovery* Stations 12687 (3350m depth) and 12692 (412m depth) respectively. B,C,D are from the Porcupine Abyssal Plain, 4840m depth, *Discovery* Station 11908#70, *Challenger* Station 54301#9 and *Discovery* Station 13077#21 respectively. E and G are from the North Carolina continental slope, 850m depth, Site I and Site III respectively of SCHAFF *et al.* (1992). Curves A and F are based on the >125µm fraction, 0–1cm layer; curves B–D on the >63µm fraction, 0–1cm layer; curves E,G on the >300µm fraction, 0–15cm layer.

The percentage of easily fossilizable calcareous foraminifera was inversely related to diversity. It was generally around 10% or less in the abyssal NE Atlantic, increased to 20-27% in the bathyal PSB, and reached 70-85% (depending on size fraction) in the core of the Arabian Sea OMZ. A similar trend was apparent among macrofaunal foraminifera on the North Carolina slope where the percentage of calcareous specimens was much lower at Sites I and II than at Site III.

Species distribution patterns

An extensive literature exists on deep-sea foraminifera and large-scale distribution patterns are well established for the better known hard-shelled species, many of which are calcareous. These sources suggest that common species typically have cosmopolitan distributions on the ocean floor (BRADY, 1884; SCHNITKER, 1980; DOUGLAS & WOODRUFF, 1981; MURRAY, 1991). As long as the environmental conditions are appropriate, the same

species tend to occur, irrespective of location. GOODAY *et al.* (1998) analysed the distribution of the more abundant species which could be consistently recognised in samples from three North Atlantic abyssal plains (the Cape Verde, Madeira and Porcupine) and the 35-m Arabian Sea site. Only 17 of the 78 species were restricted to a single site, 17 occurred at all four, 20 at three and 24 at two of the sites, suggesting that many of the species occurring at these well-oxygenated localities were fairly widely distributed.

XENOPHYOPHORE SPECIES DIVERSITY

Xenophyophores are the only deep-sea protistan taxon, apart from the foraminifera, for which reliable data are available at the species level (TENDAL, 1996). This group is much less speciose than the foraminifera. Where faunas are well studied, the number of species present at a particular site is always <20. Seventeen species were recognised among about 200 specimens collected at the DISCOL site in the eastern equatorial Pacific (GOODAY & MAYBURY unpublished). At the well-studied BENGAL site on the Porcupine Abyssal Plain, careful examination of box cores collected over several years has yielded only 8 species among 87 specimens (GOODAY, unpublished). LEVIN & THOMAS (1988) recognised between 2 and 5 distinct xenophyophore morphotypes on seamounts in different areas of the east Pacific (10°-31°N). Many xenophyophore species remain undescribed, for example most of those in the DISCOL area and in the Pacific material of Levin & Thomas (1988).

SPATIAL PATTERNS OF FORAMINIFERAL SPECIES DIVERSITY

Foraminiferal diversity patterns have been described over bathymetric and latitudinal gradients, and in relation to organic enrichment and oxygen depletion. As in the case of local (α) diversity, most studies are based either on total (live + dead) modern assemblages or on fossil faunas, and concern only the hard-shelled taxa. Little is known about patterns of xenophyophore diversity in relation to environmental gradients.

Large-scale patterns

Bathymetric patterns. In general, total foraminiferal diversity appears to decrease down the continental slope and then increase again on abyssal plains (DOUGLAS & WOODRUFF, 1981). At various sites along the eastern continental margin of North America (from the Canadian Arctic to the Gulf of Mexico) diversity in core top samples reached minimum values on the upper slope and then increased downslope in a non-linear fashion (BUZAS & GIBSON, 1969; GIBSON & BUZAS, 1973; CARTER *et al.,* 1979). In BUZAS & GIBSON's (1969) data from the western North Atlantic (29-5001m), species numbers peaked at 40-50 on the outer shelf (100-200m depth) and then declined on the continental slope before increasing to values of 60-80 or more at abyssal depths (4000-5000m). The

pattern was based on total (live + dead) faunas but was also evident in the live assemblage. DOUGLAS & WOODRUFF (1981) also reported that total foraminiferal diversity was higher in the abyssal (80-100 species) than in the bathyal (40-50 species) Pacific.

The opposite trend, an overall decrease in foraminiferal diversity down the continental slope, has also been described. In the NW Gulf of Mexico, species richness and diversity (Shannon-Wiener Index) declined with increasing depth below 100m (BUZAS & GIBSON, 1969), LAGOE (1976) found a depth-related decrease in species richness and Shannon-Wiener values between 1069m and 3709m in the Arctic Ocean, and CUTTER *et al.* (1994) reported no trend in values of the Shannon-Wiener Index (loge) down the continental slope off Cape Hatteras. On the bathyal California borderland, live foraminiferal diversity showed a general, slight decrease with bathymetric depth, but the pattern displayed many complexities related mainly to the distribution of oxygen-depleted basins (DOUGLAS, 1981). Walsh (in DOUGLAS & WOODRUFF, 1981) presented diversity data for live, dead, calcareous live and calcareous dead foraminiferal assemblages in replicated samples from 3200-4600m on the East Pacific Rise. The live component of the fauna showed a much stronger decrease in species richness and Shannon-Wiener Index values than the dead component, which was modified by down-slope transport of foraminiferal tests.

These observations suggest that foraminifera do not conform to the parabolic diversity pattern exhibited by metazoan macrofauna and megafauna (*e.g.*, gastropods, polychaetes and fish) on continental slopes (REX, 1981, 1983; REX *et al.*, 1997; ETTER & GRASSLE, 1992; PATERSON & LAMBSHEAD, 1995). Caution should be exercised, however, when comparing metazoan patterns with foraminiferal patterns based on the hard-shelled component of the fauna. Establishing bathymetric diversity trends among « complete » faunas (*i.e.* live, soft- and hard-shelled foraminifera) might be instructive, although the effort involved in such a study would be considerable.

Latitudinal patterns. The existence of latitudinal diversity patterns in the sea has been the subject of considerable debate (CLARKE, 1992). There does seem, however, to be a decline in diversity towards higher latitudes in the Northern Hemisphere, although perhaps not in the Southern Hemisphere (BREY *et al.*, 1996; CLARKE & CRAME, 1997; REX *et al.*, 1993; 1997).

On the basis of an extensive data set derived from literature and original sources, GIBSON & BUZAS (1973) concluded that modern foraminiferal sample (α) diversity shows a general increase from north (Canadian Arctic) to south (Gulf of Mexico) along the eastern margin of North America (0-100m and 100-1000m depth intervals). The trend, however, was not a smooth one. For example, an area to the south of Nova Scotia (~41-43°N), and sites located on deltas in the Gulf of Mexico were characterised by low diversity within the depth range 0-100m. CULVER & BUZAS (1998) examined the frequency of species occurrence (i.e. the number of localities at which a species occurs) within five regions (Pacific, Arctic, Atlantic, Gulf of Mexico, Caribbean) around North America. They found that this parameter, which is related very closely to species diversity, was higher at low latitudes (Caribbean) than at high latitudes (Arctic).

These trends were described on the continental shelf and slope. No attempt has yet been made to establish whether a latitudinal diversity gradient exists among living benthic foraminifera in truly deep-ocean settings, similar to that suggested by REX *et al.* (1993, 1997) for metazoan macrofauna. However, THOMAS & GOODAY (1996), studying Ocean

Drilling Project (ODP) cores, reported that a deep-water latitudinal gradient in foraminiferal species richness (increasing from high to low latitudes) was initiated about 36 million years ago in the Southern Hemisphere. They speculated that an increase in seasonality at high latitudes, coincident with the build-up of ice on the Antarctic continent, may have depressed foraminiferal diversity, perhaps by encouraging opportunistic species. The ODP cores yielded a few data points which suggested that the diversity gradient may persist in the modern ocean, although this question requires considerable further study. SMART & MURRAY (1995) also described diversity (Fisher α and Shannon-Wiener indices) in fossil (early to middle Miocene) foraminiferal faunas. They found that diversity was lower on Atlantic than Indian Ocean abyssal plains, but, unlike THOMAS & GOODAY (1996), they found no discernible trend in diversity with latitude.

Regional patterns in relation to organic enrichment and oxygen depletion

The interplay between food and oxygen availability is a major factor in both foraminiferal (JORISSEN *et al.,* 1995 ; DE STIGTER, 1996) and metazoan ecology (LEVIN & GAGE, 1998). Organic enrichment is typically associated with oxygen depletion in near-bottom water and sediment pore water (DIAZ & ROSENBERG, 1995). Oxygen depletion is persistent where oxygen minimum zones (OMZs) impinge on the continental slope and in some silled basins and fjords where bottom-water circulation is poor and the system is enriched with organic matter derived from natural sources or pollution (ALVE, 1995a, 1995b). Other basins are periodically flushed, sometimes seasonally, with oxygenated water, leading to cycles of oxygen depletion and renewal (BERNHARD & REIMERS, 1991). These conditions (oxygen depletion combined with organic enrichment) exert a strong influence on the species composition of foraminiferal faunas, leading to the development of distinctive assemblages dominated by taxa such as *Bolivina* d'Orbigny, 1839, *Brizalina* O.G. Costa, 1856, *Bulimina* d'Orbigny, 1826, *Cassidulina* d'Orbigny, 1826, *Epistominella* Husezima and Maruhosi, 1944, *Spiroplectammina* Cushman, 1927 and *Textularia* Defrance, 1824 (SEN GUPTA & MACHAIN-CASTILLO, 1993 ; BERNHARD *et al.,* 1997).

It is well known that these assemblages exhibit low species richness and high dominance (PHLEGER & SOUTAR, 1973 ; VAN DER ZWAAN & JORISSEN, 1991 ; SEN GUPTA & MACHAIN-CASTILLO, 1993). Few studies, however, have documented diversity parameters across gradients of organic enrichment and oxygen depletion in any detail. Data from the two Oman margin sites suggest that species richness ($E(S_{100})$) and diversity (Fisher α and Shannon-Wiener indices) are both much lower, while dominance is higher, in food-rich, low-oxygen settings (Table 1). Similar features characterise foraminiferal assemblages in the severely oxygen-depleted (O_2<0.1ml.l^{-1}) Santa Barbara Basin (BERNHARD *et al.,* 1997). However, dense, low-diversity, high-dominance assemblages are not invariably associated with oxygen-depleted bottom water, and no species is confined to these environments. Faunas from organically-enriched regions where the bottom water is well oxygenated may exhibit similar diversity characteristics and species compositions (SEN GUPTA *et al.,* 1981). This suggests that organic enrichment, rather than oxygen depletion, encourages population growth in opportunistic species, although oxygen depletion may deter less tolerant species and therefore have an impact on the number of species present in such areas (LEVIN & GAGE, 1998).

THE IMPORTANCE OF THE GEOLOGICAL RECORD

Because benthic foraminifera have a rich fossil record, particularly in the Cenozoic, they can be used to investigate historical aspects of diversity, including issues such as evolutionary diversification, ancient diversity patterns, trends in diversity over geological time scales, and changes in the geographical distribution of species. These matters are difficult or impossible to address on the basis of modern samples. This approach has been exploited to considerable effect by M.A Buzas and S.J. Culver. These authors compiled three very extensive data bases on the distribution of modern species around the North American continental margin, the world-wide stratigraphic distribution of a subset of these North American species, and a detailed study of the stratigraphic distribution and first and last occurrences of species in six Cenozoic rock formations located in a restricted area (the Salisbury-Albemarle Embayment) on the United States Atlantic coastal plain (reviewed by CULVER & BUZAS, 1998). Analyses of these data yield results which are highly relevant for studies of modern diversity. These including the following.

1) A number of the modern species which occur in all five regions around North America have no fossil record, implying that they evolved and dispersed around the continent very recently (CULVER & BUZAS, 1998). This suggests that some foraminifera attained cosmopolitan distributions very rapidly, a result with important implications for global diversity estimates (FENCHEL, 1993). Many species, however, are endemic to one particular region. These are much more likely to have no fossil record than the cosmopolitan species, implying that they also evolved very recently, but failed to disperse.

2) Detailed examination of fossil foraminiferal species present in successive Cenozoic formations indicates that local species diversity is maintained through immigration from a regional species pool located on the Atlantic and Gulf coastal plains. However, which species immigrate into an unoccupied neritic area, in this case following a marine transgression, seems to be largely unpredictable and to depend on chance dispersals of individual species from the regional pool rather than the reassembly of a unified community (BUZAS & CULVER, 1994). This geological perspective is relevant to the question of how local and regional species pools interact (CORNELL, 1995).

Buzas and Culver studied shallow-water (neretic) habitats. Their approach may be less applicable in ocean-floor settings where barriers to dispersion, particularly at abyssal depths, are fewer and endemism likely to be less pronounced (GRASSLE & MORSE-PORTEOUS, 1987; GRASSLE & MACKIOLEK, 1992). Studies of foraminiferal and other microfossils through geological time are, however, potentially important for understanding the historical and evolutionary processes that help to shape modern large-scale diversity patterns in the deep sea (REX *et al.*, 1997). For example, they might provide a test of the hypothesis that species tend to evolve in bathyal systems and then invade abyssal environments (ETTER & REX, 1990; REX & ETTER, 1998). In the above-mentioned study of Thomas & Gooday (1996), data from ODP cores revealed that the development of a latitudinal diversity gradient in the Southern Hemisphere was coincident with the onset of Antarctic glaciation. CRONIN & RAYMO (1997) demonstrated a link between regular fluctuations in deep-sea ostracod diversity (Shannon-Wiener Index) and Quaternary glacial-

interglacial cycles. These examples demonstrate that the geological record can yield information on deep-sea diversity trends in relation to changing environmental conditions over time scales of thousands of years.

DISCUSSION

Species concepts in foraminifera

Almost all foraminiferal species and genera are recognised on the basis of test morphology, structure and composition. Analysis of ribosomal DNA sequences has recently made it possible to separate some morphologically very similar species (PAWLOWSKI *et al.*, 1995), but this approach is not generally applicable, and particularly not to deep-sea foraminifera. Thus, most foraminiferal species are, in effect, morphospecies. Many of those recognised in deep-sea samples are undescribed «working» morphospecies. Foraminifera are able to reproduce asexually, and MURRAY (1991) has suggested that this mode of reproduction is particularly prevalent among deep-sea species. Thus, as in the case of many inbreeding or asexual, free-living ciliates (FINDLAY *et al.*, 1996a), it may be inappropriate (as well as practically impossible) to apply biological species concepts to deep-sea foraminifera.

Why are foraminifera much more diverse than xenophyophores?

Foraminiferal species are much more numerous than xenophyophore species at both local and global scales. On the Porcupine Abyssal Plain, only eight xenophyophore species have been recognised in numerous box cores compared to as many as 150 foraminiferal species present in a single sample. Since there are rarely more than ten xenophyophore species at any single locality (an area which may cover 10km^2 or more), this level of disparity in species numbers is probably fairly typical. TENDAL (1996) estimated the total number of xenophyophore species, described and undescribed, at around 100. It is difficult to even guess at the corresponding number of deep-sea foraminiferal species, but the figure is likely to be at least two orders of magnitude higher (note that >200 foraminiferal species >63μm were present in 25.5cm^3 of sediment from one of the sites studied by GOODAY *et al.*, 1998).

The relatively small number of xenophyophore species compared to foraminiferal species present at any particular locality must reflect the much greater test size of most xenophyophores. MAY (1988) showed that, within a particular taxonomic group, smaller size classes contain more species than larger size classes. As FENCHEL (1993) and FINDLAY *et al.* (1996b) argue, however, this is not necessarily true at global scales since small species (e.g. ciliates and other protozoa) are more likely to be cosmopolitan than large species. Although some xenophyophores such as *Syringammina fragilis* (which occurs in New Zealand and Scottish waters; TENDAL, 1972, 1981) are widely distributed, there does appear to be a tendency towards endemicity within the group. For example, the Atlantic and Pacific faunas are rather distinct (GOODAY & TENDAL, 1988). One xenophyophore order (Stannomida) is abundant in parts of the Pacific but virtually absent in the Atlantic

(TENDAL, 1972, 1980, 1996). Many deep-sea foraminifera, on the other hand, occur widely at abyssal depths (GOODAY *et al.*, 1998). There may therefore be some additional factors which account for the low number of xenophyophore species compared to the undoubtedly much larger, but unquantified, total for deep-sea foraminifera.

In addition to occupying a much larger size range, foraminifera possess a greater variety of wall types (organic, agglutinated, calcareous) than xenophyophores which, with a few exceptions, construct agglutinated tests. Xenophyophores also have a peculiar but very consistent internal organisation consisting of a branching protoplasmic strand, enclosed within an organic tube system and closely associated with branching, string-like stercomata masses. This may impose ecological constraints on, for example, trophic mechanisms. Xenophyophores are most common in relatively food-rich regions, although they do occur in more oligotrophic areas as well (TENDAL, 1972 ; LEVIN & GOODAY, 1992). They seem to have narrower ecological tolerances than foraminifera and have not been reported in highly energetic (GAGE, 1997) or low-oxygen (O_2<0.2ml/l) areas (LEVIN & GOODAY, 1992). Together with factors associated with size differences, these structural and ecological factors may explain why foraminifera are more diverse than xenophyophores at global as well as at local scales.

How does protistan diversity compare with metazoan diversity in the deep sea?

Megafauna. As indicated above, the number of xenophyophore species present at any one locality ranges from 1 to <20. This is similar to the numbers of species belonging to deep-sea megafaunal groups such as the holothurians. At the PAP site, 16 holothurian species have been recorded (BILLETT pers. comm.) compared to 8 xenophyophore species. According to HANSEN (1975), the total number of described holothurian species in the deep sea is 380, and the total number belonging to the Order Elasipoda is about 171, compared to TENDAL's (1972) estimate of 100 described and undescribed xenophyophore species.

Macrofauna. GOODAY *et al.* (1998, p. 192) suggested that macrofaunal foraminifera (>500-µm size fraction) « are as speciose as metazoan taxa such as polychaetes, bivalves and isopods, but that, as a result of their much greater abundance, they may be less diverse when numbers are normalised by rarefaction ». Recently, PATERSON *et al.* (1998) recognised 101 polychaete species among 427 specimens recovered from 5 box-core samples (>300-µm fraction, 0-5cm depth) obtained at the BENGAL Porcupine Abyssal Plain (PAP) site in the NE Atlantic. By comparison, 100 and 123 large foraminiferal species were found among, respectively, 1501 and 4061 complete specimens sorted from two box-core samples (>500-µm fraction, 0-1cm depth) taken at the same site (GOODAY *et al.*, 1998). Values of $E(S_{100})$ for the polychaetes are around 44 (from PATERSON *et al.*, 1998, fig. 5a) compared to 35.5 and 29.3 for the foraminifera. These data therefore tend to support the conclusions of Gooday *et al.* (1998), although the comparison is blurred by the lack of comparability between size fractions and depth horizons. Thus, partly as a result of their sheer numerical abundance, foraminifera make a substantial contribution to macrofaunal species richness at this site and probably elsewhere in the deep sea (TENDAL & HESSLER, 1977).

Meiofauna. Nematodes dominate the deep-sea metazoan meiofauna numerically (VINCX *et al.*, 1994) and are a highly diverse taxon (LAMBSHEAD, 1993). Single samples may contain

over 100 species with the most abundant species usually representing <10% of the population (TIETJEN, 1989). DINET & VIVIERS (1979) recognised 50, 115 and 84 species at 4216m, 4220-4225m and 4700-4725m in the Bay of Biscay; corresponding specimen numbers were 105, 190 and 190 respectively. At the PAP site, numbers of nematode species in multicore samples (25.5cm^2 area, 0-1cm depth; >45-µm fraction) varied from 35 (59 specimens) to 71 (338 specimens) per core compared to 131-153 (499-651 specimens) foraminiferal species per core (>63-µm fraction) (GOODAY *et al.,* 1998). On the Madeira Abyssal Plain, nematode species numbers varied from 25 (53 specimens) to 49 (187 specimens) compared to 100 (290 specimens) to 149 (405 specimens) foraminiferal species.

To summarise, the very limited available data (most of it from the PAP), suggest that foraminiferal species richness is comparable to that of polychaetes in the macrofaunal size range and comparable to or rather greater than that of nematode in the meiofaunal size range.

Is diversity related to phylogeny?

Recent molecular evidence suggests that foraminifera branch close to the plasmodial and cellular slime moulds near the middle of the eukaryotic tree (PAWLOWSKI *et al.,* 1994). Foraminifera and metazoans are therefore phylogenetically distant as well as being structurally very different (unicellular vs multicellular). Nevertheless, locally (for example, in samples taken at one site), foraminifera and metazoan taxa such as polychaetes and nematodes, exhibit similar levels of species richness and diversity in the deep sea. They also display similar population responses to organic-enrichment and oxygen-depletion gradients (albeit possibly not bathymetric gradients). As far as local (α) diversity is concerned, the answer to the question posed above appears to be « no ».

There are, however, some important differences between foraminifera and metazoans. As mentioned above, foraminifera, unlike most metazoans (but like other protistan groups such as ciliates), can reproduce asexually and this may allow rare species to persist. It may also explain the extraordinary geological longevity of some foraminiferal species; for example, >35 million years in the case of *Epistominella exigua* (Brady, 1884) and *Alabaminella weddellensis* (Earland, 1936) (THOMAS & GOODAY, 1996), compared to an estimated average lifespan of 5-10 million years for most marine invertebrates (MAY *et al.,* 1995). Another important consideration is that many deep-sea foraminifera are widely distributed (GOODAY *et al.*, 1998) and occur wherever conditions are suitable. In this respect, they again resemble ciliates and other protistan groups (FENCHEL, 1993; FINDLAY, 1998; FINDLAY *et al.*, 1996b; 1998). Thus, the total number of foraminiferal species in the deep sea is likely to be much lower than recent estimates of the global numbers of macroinfaunal ($1x10^7$) and nematode species ($1x10^8$) in ocean-floor sediments (GRASSLE & MACKIOLEK, 1992; LAMBSHEAD, 1993).

SOME QUESTIONS AND FUTURE DIRECTIONS

Foraminifera are commonly disregarded by biologists investigating deep-sea biodiversity (species diversity). At the same time, geologists, who are at the forefront of

research into deep-sea foraminiferal ecology, are (understandably, and with some notable exceptions) more concerned with developing proxies for use in palaeoceanographic reconstructions than in determining scales and patterns of foraminiferal diversity. Nevertheless, foraminifera clearly represent an important and highly diverse component of the deep-sea benthos and one which should be incorporated into assessments of biodiversity on the ocean floor.

The following problems and questions are among those that need to be addressed in order to improve understanding of the scale and pattern of foraminiferal diversity in ocean-floor sediments.

1) Deep-sea foraminiferal assemblages incorporate a substantial pool of undescribed species, many of them belonging to poorly-known, soft-walled taxa. A wider recognition of these organisms will only come about if effort is devoted to the description of new species.

2) In order to estimate the global scale of foraminiferal diversity, it is important to establish the distributional ranges of species. Are cosmopolitan distributions really more prevalent among deep-sea species than they are in shallow water? Calcareous foraminifera, which are better known taxonomically than other meiofaunal groups in the deep sea, are well suited to an analysis of these patterns.

3) Information is required about large-scale trends in foraminiferal diversity. Does a latitudinal gradient exist among modern deep-sea foraminifera, similar to that reported for fossil foraminifera and modern macrofauna? Do bathymetric trends in foraminiferal diversity down the continental slope differ fundamentally from the parabolic patterns exhibited by metazoan taxa, and if so why?

4) Are the patterns and scales of foraminiferal diversity (both local and global) similar to those reported for metazoans? Or are they more similar to those of other protists such as free-living ciliates?

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2

Paper & Poster Contributions

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A GENETIC COMPARISON OF ATLANTIC AND MEDITERRANEAN POPULATIONS OF A SALTMARSH BEETLE

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Abstract. Enzyme and dispersal polymorphism in the saltmarsh carabid beetle *Pogonus chalceus* Marsham were compared between 30 Atlantic and nine Mediterranean European populations. Allozyme results showed that Mediterranean beetles (France, Spain) are genetically distinct from Atlantic populations. All Mediterranean beetles screened showed complete fixation at one locus (IDH1), which in Atlantic populations nearly always varied, whereas some unique Mediterranean alleles were observed for another locus (MPI). Genetic differentiation (allozymes) between Mediterranean populations, although highly significant, appeared to be much lower (F_{ST} =0.098) than between Atlantic populations (F_{ST} =0.178). Beetles from the Mediterranean showed a remarkably high dispersal power in all populations studied, whereas Atlantic populations showed wing polymorphism and reduced dispersal power to much more varying degrees. These results, along with relatively lower levels of *Pogonus chalceus* abundance in many Mediterranean saltmarshes, strongly suggest increased levels of extinction/recolonisation in relation to a lower degree of habitat persistence in Mediterranean compared with most Atlantic saltmarshes. Conclusions are relevant to issues in both evolutionary and conservation biology.

Key words : population genetics, genetic differentiation, dispersal power, gene flow, habitat fragmentation, allozymes, wing polymorphism, saltmarshes, Atlantic, Mediterranean, ground beetles (Carabidae), *Pogonus chalceus*.

INTRODUCTION

Geographic genetic structure – the distribution and abundance of genotypes between and within populations – is a fundamental part of ecology and evolution, combining both demographic and genetic processes, such as extinction/recolonisation, gene flow, drift and natural selection (RODERICK, 1996). Insects, with their extreme diversity and abundance, in many cases have evolved special features, and are increasingly used as model species in population and conservation genetics. For example, many insect species display dispersal polymorphism (*e.g.* DENNO *et al.*, 1996), which can be used to evaluate directly the effect of migration on geographic structure. Such data can then be compared with those obtained indirectly from genetic studies (RODERICK, 1996).

Natural areas in Europe have been severely reduced and highly fragmented. Many species therefore persist only in small and isolated populations. Habitat fragmentation in general results

in a reduced biodiversity (e.g. ANDRÈN, 1994), but may also lead to an increased genetic differentiation (HASTINGS & HARRISON, 1994; YOUNG *et al.*, 1996) as a result of reduced gene flow (SLATKIN, 1994). In theory, the negative influence of fragmentation on biodiversity is supposed to be caused by lowered genetic diversity and adaptability of species, eventually leading more rapidly to their extinction (FRANKHAM, 1995). Recently, we started studying population genetic consequences of fragmentation on a variety of beetle species, with emphasis on saltmarsh and woodland species. The rationale which formed the basis of this study is outlined in more detail elsewhere (DESENDER & TURIN, 1989; DESENDER *et al.*, 1998).

West-European saltmarshes are relatively recent habitats, in many cases well documented historically. They can mostly be considered true habitat islands, a prerequisite for the study of population genetic effects of isolation and fragmentation. Recent estimates all over western Europe show a dramatic decrease of the surface area of saltmarshes (DIJKEMA *et al.*, 1984). This is expected to enhance isolation and its negative effects on the genetic diversity of the highly specialised terrestrial arthropod fauna, including many halobiontic species, i.e. many ground beetles (Coleoptera, Carabidae). Mediterranean saltmarshes, although in general studied less intensively than Atlantic marshes, also are inhabited by an array of highly specialised terrestrial invertebrates, with again beetles as one of the most prominent groups (BIGOT, 1965). Also in this area, human impact on coastal habitats (especially estuaries) has been very pronounced during the twentieth century (e.g. GUILLEN & PANALQUES, 1997).

Ground beetles show large variation in morphological traits related to dispersal power, and many species even display dispersal polymorphism or dimorphism (DEN BOER *et al.*, 1980; DESENDER, 1989). Gene flow of such species can be quantified more or less directly by means of morphological or biometrical knowledge on the hind wings and flight muscles. In earlier work, we have documented the life cycle and population densities of such a wing polymorphic and halobiontic carabid beetle *Pogonus chalceus* (Marsham, 1802) from an Atlantic population (DESENDER, 1985). *P. chalceus* occurs exclusively in saltmarshes (THIELE, 1977), at densities generally between about 1-20 ind/m^2 (DESENDER & SEGERS, 1985; GAUTIER, 1979). The geographical distribution extends along the Atlantic coasts from Denmark to and including the major part of the Mediterranean (TURIN *et al.*, 1977). The species can be found on inland saline habitats (EVERSHAM *et al.*, 1996; DESENDER & MAELFAIT, in press), especially in the Iberian Peninsula (ZABALLOS & JEANNE, 1994).

Wing development in *P. chalceus* appears to be a polygenic trait with high heritability (DESENDER, 1985, 1989). An earlier population genetic study on the same species (based on allozymes as well as wing polymorphism) compared ten Atlantic populations (varying in size and isolation in space and time), and showed significant genetic differentiation (DESENDER *et al.*, 1998). The dispersal power in small populations was larger than in large populations, suggesting that the former are unstable and/or young. Dispersal power declined with increasing age of the saltmarsh, probably due to a continuous emigration of winged individuals. Age and size of saltmarshes, although difficult to study independently, both appeared to be important in determining the genetic structure of saltmarsh beetles. Population genetic data of *P. chalceus* are not yet available for the Mediterranean area.

In the present study, we therefore compared the geographic genetic structure in the halobiontic *P. chalceus* from 30 Atlantic and nine Mediterranean European populations. In particular, we investigated the following questions:

(1) Can Mediterranean *P. chalceus* populations be distinguished genetically from Atlantic populations?

(2) Is genetic differentiation between populations within each region comparable between the two regions?

(3) Are there general differences in dispersal power between populations in the two regions and, if so, can these be understood and related to allozyme genetic structure?

Results related to genetic diversity will be presented in a forthcoming paper.

MATERIAL AND METHODS

Study sites and sampling

Since 1992 we have collected *P. chalceus* beetles from 30 Atlantic and nine Mediterranean saltmarshes varying in size and isolation. Atlantic populations were sampled from several areas in the UK, France, The Netherlands and Belgium, including four small and relatively young populations on inland sites from Flanders and the southern part of the Netherlands (cf. DESENDER *et al.*, 1998). Nine Mediterranean populations were sampled from southern France (three coastal populations from lagoons) and Spain (including three coastal populations and three populations from inland high elevation salt ponds (elevation ca 600-800 m) near Albacete). The study areas are indicated on Fig. 1. In several areas, different populations were studied (Table 1).

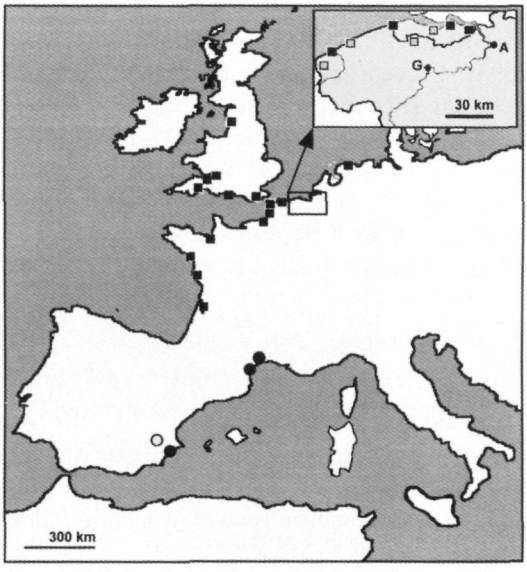

Fig. 1. – Geographic location of sampled saltmarsh areas (in some areas, more than one population was sampled; for names, see Table 1); square symbols: Atlantic areas, grey-filled: small/young inland marshes; circular symbols: Mediterranean areas, grey-filled: high elevation inland salt ponds near Albacete.

Beetles were collected by standardised handcatches (unit of effort), mostly by using an aspirator, or by flotation whenever brackish water was available in the immediate surroundings of a sampling site. Beetles were transported alive, counted and identified with a binocular dissecting microscope, and mostly kept at low temperature for some days without food to allow the gut to empty. They were then frozen in liquid nitrogen until subjected to electrophoresis.

Allozyme electrophoresis

Individuals were prepared for electrophoresis by homogenising part of the body in 50 µl of distilled water, and kept in eppendorf tubes on ice. Only the head and thorax of beetles were used for electrophoresis, while the abdomen was retained for morphological study and verification of identification. Variation at enzyme loci was analysed using cellulose acetate electrophoresis (HEBERT & BEATON, 1989), permitting the examination of each individual for variation at multiple enzyme loci. If available, about 30 to 50 individuals from each population were analysed. Overall, more than 2000 beetles were processed for this study. Results from six polymorphic enzyme loci (95%-criterion) are used in this paper: Aldehyde Oxidase (AO, E.C. 1.2.3.1), Glucose-6-phosphate Isomerase (GPI, E.C. 5.3.1.9), Isocitrate Dehydrogenase 1 and 2 (IDH1, IDH2, E.C. 1.1.1.42), Mannose Phosphate Isomerase (MPI, E.C. 5.3.1.8) and Phosphoglucomutase (PGM, E.C. 2.7.5.1). For more details on other enzymes tested, protocols of electrophoresis and buffer systems used, we refer to DESENDER *et al.* (1998).

Analyses of electrophoretic data were carried out using BIOSYS-1 (SWOFFORD & SELANDER, 1981) and GENEPOP (version 3; update from version 1.2: RAYMOND & ROUSSET, 1995). Genotype frequencies were tested against Hardy-Weinberg expectations using an exact test procedure (ROUSSET & RAYMOND, 1995). The significance of genetic differentiation was tested per region by contingency X^2-analyses (BIOSYS-1) and evaluated quantitatively by F_{ST}-statistics (NEI, 1977) (weighted) averaged over the six polymorphic (95%-criterion) enzymes. F_{ST}-values describe the proportion of diversity found in populations. Hierarchical F_{ST}-values were also calculated to look at partitioning of the genetic variance within and between the two regions studied. Overall genetic similarity between the populations was visualised in a PCA based on a variance-covariance matrix of allelic frequencies.

Dispersal polymorphism

Dispersal polymorphism was studied by investigation of wing and flight muscle development, on the same specimens used for electrophoresis as well as on additional alcohol-fixed samples. In carabid beetles in general, wing size follows an allometric relationship to body size, and the index « % MAX ALL » (DESENDER *et al.*, 1986) corrects for this allometry. Wing length and width, as well as elytral length and width are needed to calculate these values. Wing length x width is then expressed as a percentage of the maximal wing size for a beetle of a given (elytral) size. Population frequency distributions of « % MAX ALL » values were plotted for ten Atlantic populations (showing the range of variation in all studied Atlantic populations, see also DESENDER *et al.*, 1998) and the nine

Mediterranean populations. The plots were ordered according to decreasing mean population values, and the frequencies of individuals with functional flight muscles were separately indicated.

RESULTS AND DISCUSSION

Genotype frequencies were first tested against Hardy-Weinberg expectations and showed only very few significant deviations, which could be expected by chance alone. These results suggest that the studied populations were all in Hardy-Weinberg equilibrium.

Allelic frequencies of the polymorphic loci are given in Table 1. The PCA-plot (based on allelic frequencies) along the first and second axis is given in Fig. 2. Dispersal power, expressed as % MAX ALL, is shown in frequency distributions for ten Atlantic populations (Fig. 3) and for the nine Mediterranean populations (Fig. 4.).

The allozyme results showed that the beetles from the Mediterranean (France, Spain) were genetically distinct from those in the Atlantic populations. All Mediterranean beetles screened showed complete fixation in one enzyme (IDH1) – which in Atlantic populations nearly always varied (except for one population on an inland site, see further) – whereas some unique Mediterranean alleles were observed for another locus (MPI). Moreover, allelic frequencies regularly differed between the two regions for AO (AO A allele most frequent in coastal Mediterranean populations only), and for GPI (GPI A allele most frequent for coastal Mediterranean sites, and GPI B allele most frequent in Mediterranean populations). There were thus also a number of differences within the Mediterranean area, where all coastal populations (France and Spain) could be differentiated from the inland salt ponds near Albacete (Spain) due to the presence of high frequencies of the AO A and GPI A allele. Whether the similarity between coastal populations holds true for other parts of the Mediterranean remains to be investigated, but is not immediately expected because of the more or less discontinuous distribution of the species in other parts of the area (TURIN *et al.*, 1977). The populations from the inland marshes of Belgium and the southern part of the Netherlands (small and/or young populations of *P. chalceus*) showed near-fixation in the IDH1 B allele (in the MOK population even complete fixation, but relatively low sample size) and in this way mimic the Mediterranean populations. This explains why these Atlantic populations are situated closer to the Mediterranean ones along the first and second axis of the PCA-plot based on allelic frequencies (Fig. 2). The table with allelic frequencies, however, shows that they still can be discriminated from Mediterranean populations by the complete absence of the MPI A, MPI D, GPI A and GPI B alleles.

Genetic differentiation (allozymes) between Mediterranean populations, although highly significant for at least two enzymes (Table 2), appeared to be much lower (F_{ST}=0.098) than between Atlantic populations (F_{ST}=0.178). This difference cannot be explained by the difference in the number of populations screened so far for both regions. Indeed, recalculating F_{ST}-values for several sets of randomly chosen groups of only ten populations of the Atlantic region yielded only slightly lower mean values than those obtained from 30 populations. Hierarchical F-statistics showed an overall F_{ST}=0.244 (all 39 populations), F_{ST}= 0.150 within regions and F_{ST}= 0.094 between regions.

TABLE 1

Allele frequencies (zero-values not printed) and number of individuals scored (n) for polymorphic enzymes (95%-criterion) in Pogonus chalceus; populations (from left to right) arranged in four groups: (1) Atlantic, coastal, n=26; (2) Atlantic, inland, n=4; (3) Mediterranean, inland, n=3; (4) Mediterranean, coastal, n=6; population codes: France, Atlantic: AFM: Authie estuary, Fort Mahon; APM: Authie, Port Madelon; CAN= Canche estuary; FOR: le Fort Vert; GAC: la Gachère; GFP: Grand Fort Philippe; GIR: Gironde estuary; GUA-GUD: la Guérande: MSM: Mont St Michel; SOC: Somme estuary, le Crotoy; SOH: Somme, Cap Hornu; UK: EXE: Exe estuary; MOR: Morecambe Bay; RYE: Rye estuary; SEA-SEB: Severn estuary; THO: Thorney Island; The Netherlands: FRI: Friesland, Ferwerd-Holwerd; OSS: Ossenisse; SAE: Saeftinghe; BRA: the Braakman; Belgium: NIE: Nieuwpoort, IJzer estuary; ZWR- ZWC: the 'Zwin'; MOE: De Moeren; MOK: Molenkreek; OOS: Oostende; Spain: COR: Cordovilla; ELS: El Saladar; PET: Petrola; MAT: La Mata; PED: La Pedrera; POL: Sta Pola; France, Mediterranean: MAR: la Marende; LAP: Lapalme; SAL: Salses

	ATLANTIC COASTAL																								ATLANTIC INLAND				MEDITERRANEA INLAND			
POPUL.	AFM	APM	CAN	EXE	FRI	FOR	GAC	GFP	GIR	GUA	GUB	GUC	GUD	MOR	MSM	NIE	OSS	RYE	SAE	SEA	SEB	SOC	SOH	THO	ZWR	ZWC	BRA	MOE	MOK	OOS	COR	ELS	PET	MAT	PED
allele:																																			
AOA	.038	.087	.039	.083	.119	.089	.007	.125	.051	.022					.125	.088	.143	.020	.219	.077	.178	.094	.130	.085	.107	.185	.054	.009	.019	.130	.048	.015	.061	.438	.250
AOB	.363	.338	.304	.250	.357	.389	.089	.328	.205	.090	.122	.076	.250		.444	.262	.452	.122	.300	.231	.305	.271	.349	.162	.375	.303	.446	.284	.259	.185	.323	.294	.333	.250	.063
AOC	.300	.300	.373	.472	.292	.256	.521	.344	.282	.455	.541	.758	.750	.100	.297	.371	.262	.592	.210	.269	.229	.396	.336	.338	.250	.265	.161	.440	.333	.442	.274	.265	.288	.188	.188
AOD	.250	.262	.235	.194	.202	.267	.384	.203	.462	.388	.338	.167		.900	.122	.228	.095	.153	.214	.413	.288	.229	.171	.415	.250	.210	.298	.155	.370	.116	.177	.250	.121	.063	
AOE	.050	.013	.049		.030					.045					.013	.051	.048	.112	.057	.010		.010	.014		.018	.038	.042	.112	.019	.127	.177	.176	.197	.063	
MPIA																															.031	.100	.029		
MPIB		.071			.012	.056		.045							.019	.008			.042			.025	.017	.045	.022	.037	.125			.094	.125	.143	.100	.125	.063
MPIC	1.000	.929	1.000	1.000	.988	.944	1.000	.955	1.000	1.000	1.000	1.000	1.000	1.000	.981	.992	1.000	1.000	.958	1.000	1.000	.975	.983	.955	.978	.963	.875	1.000	1.000	.906	.828	.743	.829	.875	.938
MPID																															.016	.014	.043		
IDH1A									.012			.172																							
IDH1B	.587	.350	.280	.066	.546	.807	.153	.710	.049	.615	.029	.359	.625	.000	.623	.611	.214	.076	.337	.323	.170	.202	.143	.367	.268	.438	.853	.856	1.000	.921	1.000	1.000	1.000	1.000	1.000
IDH1C	.412	.650	.720	.934	.446	.193	.847	.290	.841	.385	.971	.469	.375	1.000	.377	.389	.786	.924	.663	.677	.830	.798	.857	.633	.732	.562	.147	.144		.079					
IDH1D					.008				.098																										
IDH2A		.013				.011				.007			.063			.009			.010			.010				.008					.016	.043	.014		
IDH2B	1.000	.988	.980	1.000	.994	.989	1.000	1.000	1.000	.993	1.000	1.000	.938	1.000	.997	.991	1.000	1.000	.976	1.000	.992	.990	.987	1.000	1.000	.988	1.000	1.000	1.000	1.000	.969	.957	.971	1.000	1.000
IDH2C			.020		.006										.003				.014		.008		.013								.016		.014		
IDH2D																										.004									
GPIA	.028	.013			.012		.013		.012	.043			.063		.009			.010	.005	.038	.025	.010	.023			.018								.063	.063
GPIB																															.014	.014			.188
GPIC		.013					.006								.009							.031						.148				.043	.029	.063	.063
GPID	.972	.975	1.000	1.000	.988	1.000	.981	.970	.988	.957	1.000	1.000	.938	1.000	.978	1.000	1.000	.970	.995	.962	.975	.959	.977	1.000	1.000	.982	1.000	1.000	.852	1.000	.953	.914	.943	.875	.688
GPIE								.030							.003			.020													.031	.029	.029		
PGMA			.028																			.013									.016		.014		
PGMB	1.000	1.000	.972	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.938	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.988	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.984	1.000	.971	1.000	1.000
PGMC													.063																				.014		
n =	40	40	51	38	88	45	77	33	43	69	37	33	8	5	163	160	23	52	105	53	61	49	75	65	30	128	51	58	27	146	32	35	35	8	8

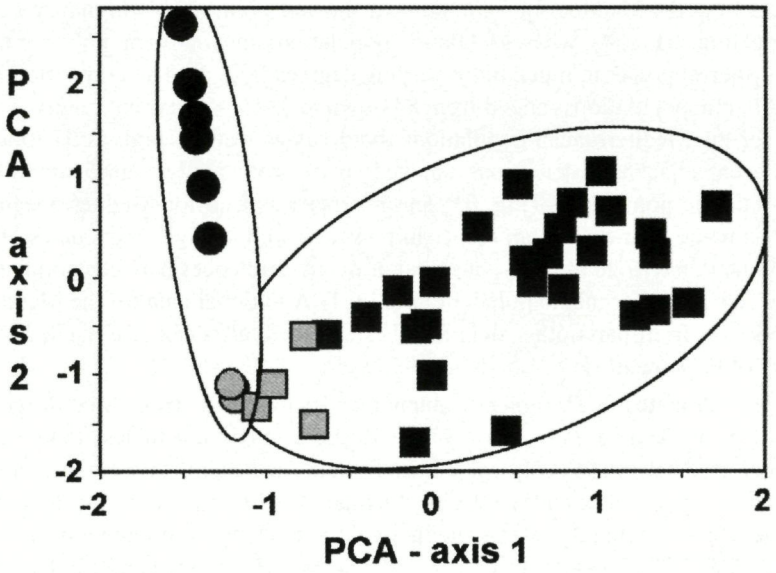

Fig. 2 . – PCA-plot along first and second axis based on allelic frequencies of 39 populations of *Pogonus chalceus*; square symbols: Atlantic populations, grey-filled: small/young inland marshes; circular symbols: Mediterranean populations, grey-filled: high elevation inland salt ponds; ellipses regroup Mediterranean and Atlantic populations, respectively.

TABLE 2

Contingency X^2-analyses at all polymorphic enzyme loci for (A) 30 Atlantic and (B) nine Mediterranean populations of Pogonus chalceus*; highly significant values ($p<0.01$) in bold italic*

Locus	No. of alleles	X^2-value	d.f.	p
(A) AO	5	742.28	116	***0.0000***
(A) MPI	2	46.17	29	0.0225
(A) IDH1	4	1758.88	87	***0.0000***
(A) IDH2	4	91.76	87	0.3429
(A) GPI	4	385.27	87	***0.0000***
(A) PGM	3	149.15	58	***0.0000***
(A) totals		3173.51	464	***0.0000***
(B) AO	5	147.55	32	***0.0000***
(B) MPI	4	34.09	24	0.0831
(B) IDH2	3	11.0	16	0.8046
(B) GPI	5	97.52	32	***0.0000***
(B) PGM	3	20.72	16	0.1893
(B) totals		310.97	120	***0.0000***

Beetles from the Mediterranean, moreover, showed a remarkably high dispersal power in all populations (Fig. 4), whereas Atlantic populations showed wing polymorphism and reduced dispersal power to much more varying degrees (Fig. 3). The population mean values for Atlantic populations ranged from 84 down to 32 % of maximal relative wing size, whereas for the Mediterranean populations these values were as high as 91 to 93 %. X^2-tests between adjacent distributions showed many statistically significant differences between Atlantic populations (Fig. 3). The reverse was true for Mediterranean populations, all showing near-maximum dispersal power as well as high frequencies of individuals with functional flight muscles, but no significant differences between populations, not even between coastal or inland populations (Fig. 4). Additional data for the Mediterranean area, especially from parts other than the Western Mediterranean, are needed to test the generality of these results.

The few estimates of *P. chalceus* abundance known to us from Mediterranean saltmarshes (Camargue area, GAUTIER, 1979) indicate a maximum of less than 1.5 ind/m^2, even in preferred habitats. Ecological studies from the same and other areas in the Western Mediterranean invariably mention strong fluctuations of numbers during the course of a year in these salty habitats, possibly mediated through changes in temperature and humidity (GAUTIER, 1979; VERDIER & QUÉZEL, 1951). Severe circumstances indeed regularly occur due to temporal desiccation of many lagoons from July onwards. Populations of *P. chalceus* in parts of the Mediterranean therefore are probably to be viewed as metapopulations made up of ephemeral local populations (cf. OLIVIERI & GOUYON, 1997). Along with the observed relatively low abundance levels of *P. chalceus* in many Mediterranean saltmarshes, our dispersal power data thus strongly suggest increased levels of extinction/recolonisation in Mediterranean compared with most Atlantic saltmarshes. Mediterranean populations suffer much more from temporarily dry and hot conditions during a large part of the year. The retention of a high dispersal power can then be interpreted as an adaptation for survival in temporarily more unstable marshes compared with many Atlantic saltmarshes, which would be closer to the species' optimal habitat, with less need for regular recolonisation. As a consequence of increased gene flow, one would then expect the genetic differentiation between Mediterranean populations to be lower. Our allozyme data confirm this hypothesis. Future field work, especially in other areas of the Mediterranean area, will gather more data to further test this hypothesis.

Models and (few) empirical work have shown that patterns of extinction and recolonisation indeed can influence the genetic differentiation between local populations (MCCAULEY, 1991), but that effects can be complex. The hypothesis that levels of gene flow among populations are correlated with dispersal power (« the dispersal-gene flow hypothesis ») has only recently been rigorously tested. This was done by comparing intraspecific geographic variation in dispersal strategies with levels of gene flow, as derived from genetic structure data (PETERSEN & DENNO, 1997): increased genetic structuring between populations of wing-dimorphic planthopper species was found in the region where they showed a lower dispersal power. Our results on *P. chalceus*, although still somewhat limited for the Mediterranean, present comparable evidence for the dispersal-gene flow hypothesis. PETERSEN & DENNO (1997) also investigated coastal saltmarsh-inhabiting species, in their case from the Atlantic and Gulf coasts of North America.

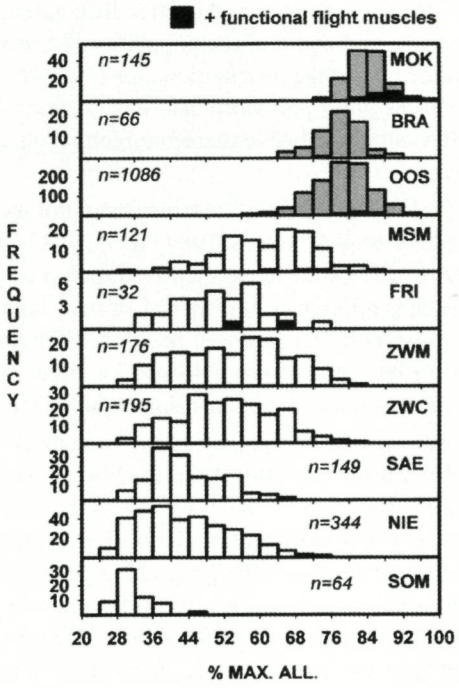

Fig. 3. – Wing polymorphism in Atlantic populations of *Pogonus chalceus*: percentage frequency distributions of % MAX ALL, arranged according to decreasing mean value of dispersal power; grey-filled histograms correspond to highly significantly differing samples from inland marshes; X^2-test results between adjacent distributions: MOK ≅ BRA ≅ OOS ≠ MSM ≠ FRI ≅ ZWM ≅ ZWC ≠ SAE ≅ NIE ≠ SOM, $p<0.01$; black columns: individuals with functional flight musculature; for population codes, see Table 1.

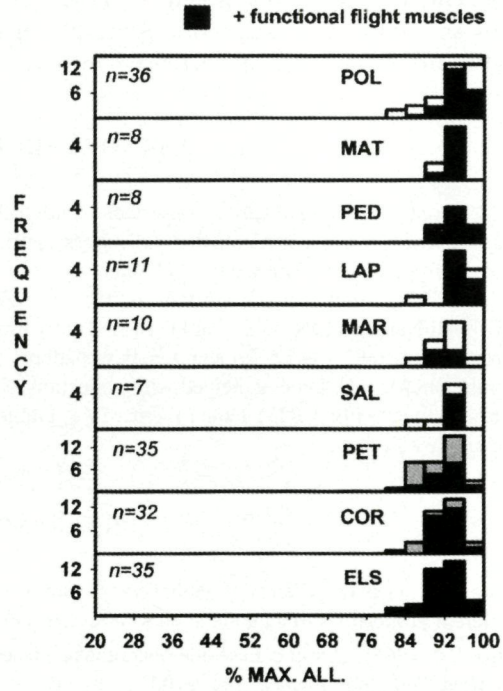

Fig. 4. – Wing polymorphism in Mediterranean populations of *Pogonus chalceus*: percentage frequency distributions of % MAX ALL; grey-filled histograms correspond to inland salt ponds near Albacete (Spain); no significant differences (X^2-test) between distributions; black columns: individuals with functional flight musculature; for population codes, see Table 1.

Among-populational genetic differentiation in *P. chalceus* was highly significant (Table 2), and many of the populations studied were genetically distinct, especially in the Atlantic part of the distribution area (DESENDER *et al.*, 1998). This was shown by allozyme as well as wing polymorphism data. Conserving a maximal overall genetic diversity for such a saltmarsh beetle therefore requires the protection of as much of the few remaining sites as possible.

Significant genetic substructuring (allozymes) has been reported for many insects including beetles (HSIAO, 1989; KING, 1987). Apart from the study of PETERSON & DENNO (1997), to our knowledge such substructuring has never been mentioned to be accompanied by such a difference in dispersal strategy. Geographic genetic structure in wing polymorphism nevertheless has been mentioned for many insect species (RODERICK, 1996). DENNO *et al.* (1996) gave strong evidence for an intraspecific inverse relationship between the dispersal capability of saltmarsh-inhabiting planthoppers and the persistence of their habitats.

Few genetic comparisons have been made for terrestrial coastal organisms between the Mediterranean and the Atlantic. MADEC *et al.* (1996) reported significant geographic genetic structure in the mollusc *Helix aspersa*, but some Mediterranean populations showed a stronger genetic relationship to all Atlantic ones, probably as a result of human transport in the past. The geographic genetic structure of a tephritid fly from 16 European regions (EBER & BRANDL, 1997) showed only low levels of differentiation, mainly resulting from a south-north decrease in allelic diversity. A genetic study on *Zannichellia* water plants (TRIEST & VANHECKE, 1991) showed a near-uniform intraspecific genetic structure between Mediterranean and Atlantic populations, linked to predominant inbreeding. Many more empirical population genetic studies are needed to evaluate more generally the patterns and processes linked to the geographic genetic structure of organisms, distributed both in the Atlantic and Mediterranean region.

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HIERARCHICAL POPULATION GENETIC ANALYSIS REVEALS METAPOPULATION STRUCTURE IN A PHYTOPHAGOUS GALÁPAGOS BEETLE

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Abstract. The Galápagos Archipelago has long been considered a living laboratory for the study of evolution. Due to geographic isolation and speciation many endemic animal and plant groups have radiated on the islands. Although the vertebrate fauna of these islands (*e.g.* giant tortoises, Darwin's finches) has been studied in great detail, little is known about invertebrates and especially insects. Results are given of a population genetic study on the phytophagous beetle *Nesaecrepida darwini*. This small alticine beetle is present on all major islands but shows a discontinuous population distribution. To obtain population genetic information we used cellulose acetate gel electrophoresis to study allozyme variation in 6 populations from 3 islands. Twelve presumptive loci, including 9 polymorphic ones, were analysed. The results show low heterozygosity values, with the lowest genetic diversity on the youngest island. F-statistics (mean F_{ST} = 0.431) indicate a very large amount of genetic differentiation between populations. Hierarchical analysis indicates little inter-island gene flow but also considerable genetic variation between populations occurring on the same island. These results strongly suggest a metapopulation structure with recurrent extinctions and recolonisations of populations within each island. Recent field observations support these findings.

Key words: Chrysomelidae, Galápagos, evolution, population genetics, genetic differentiation, phytophagy, metapopulation structure, gene flow, genetic drift.

INTRODUCTION

The Galápagos Archipelago is situated in the Pacific Ocean about 1000 km from the South American coast, straddling the Equator. It is a group of 13 large islands, 6 smaller ones and 107 islets and rocks, with a total land area of about 8000 square kilometres. The islands are volcanic in origin and several volcanoes in the west of the archipelago are still active (*e.g.* recent volcanic activity on Cerro Azul, Isla Isabela in September-October 1998).

The archipelago is home to many radiated groups of endemic species, the most famous examples being the Darwin finches, the giant tortoises, the mockingbirds, the *Opuntia* cacti, the *Scalesia* trees and the lava lizards. Although the invertebrate communities also have a considerable portion of endemics and include striking examples of radiated groups (COPPOIS, 1984; PECK, 1996; FINSTON & PECK, 1997), until now evolutionary research on these islands has mainly focused on vertebrate species (*e.g.* GRANT, 1981; FRITTS, 1984; SNELL *et al.*, 1984; STERN & GRANT, 1996; RASSMANN *et al.*, 1997). Since 1982, entomologists from the Royal

Belgian Institute of Natural Sciences have been conducting systematic and ecological work on insects and spiders of Galápagos (*e.g.* BAERT & MAELFAIT, 1986a, 1986b; DESENDER *et al.*, 1989, 1990) collecting material during expeditions in 1982, 1986, 1988 and 1991. In spring 1996, another expedition to three of the major Galápagos islands was organised in order to collect material in liquid nitrogen for studying population genetic aspects of several model spider and insect species, now also including phytophagous beetles. The aim of this study is to obtain information about genetic variability within and genetic exchange between the islands for several invertebrates, and to gain insight into the genetic structure of their populations.

In this paper we present the first results of these studies on a specialised herbivorous leaf beetle from Galápagos: *Nesaecrepida darwini* (Mutchler, 1925). This species probably occurs on all islands and is monophagous on saltbush (*Cryptocarpus pyriformis*), a plant that often is very abundant along the coastline in the littoral zone. Although the plant can cover vast areas of land, the beetles are only found on isolated patches, constituting geographically well-defined populations of hundreds to several thousands of individuals (pers. obs.). Large areas of host plant are left unoccupied by the beetles. It is unknown why these animals do not occur as continuous populations over larger areas.

Until now population genetic aspects of only one group of Galápagos insects have been published: FINSTON & PECK (1995, 1997) provided data on population structure and gene flow in the Galápagos beetles of the genus *Stomion,* a species swarm (13 species) of flightless beetles that are generalised litter feeders. In this study we focus on a genus with only one described endemic species that is able to fly, is present on all major islands and is a highly specialised feeder (monophagous). Population genetic results on other beetle species will be given in a future contribution (DESENDER & VERDYCK, unpublished data).

MATERIAL AND METHODS

During our 1996 expedition to Galápagos we collected at least 40 individuals from six populations of *Nesaecrepida darwini* on three islands (Isla San Cristóbal [estimated age 3 million years], Isla Santa Cruz [estimated age between 0.7 and 1.5 million years], and Volcan Sierra Negra on Isla Isabela [estimated age less than 0.7 million years]). For electrophoresis the abdomen of each individual was removed and homogenised in 35 µl of distilled water. After a pilot study on some 30 enzyme loci, a selection of 12 presumptive loci, showing clearly interpretable banding patterns, was used for analysis. The allozyme loci studied were arginine phosphokinase (*APK*, E.C. 2.7.3.3, 2 loci), aspartate aminotransferase (*AAT,* E.C. 2.6.1.1, 2 loci), isocitrate dehydrogenase (*IDH*, E.C. 1.1.1.42, 2 loci), malic enzyme (*ME*, E.C. 1.1.1.40), malate dehydrogenase (*MDH*, E.C. 1.1.1.37, 2 loci), peptidase-A (dipeptide substrate: leucyl glycine, *PEP-A*, E.C. 3.4.-.-), peptidase-D (dipeptide substrate: phenylalanine proline, *PEP-D*, E.C. 3.4.-.-), 6-phosphogluconate dehydrogenase (*6PGDH*, E.C.1.1.1.44). Staining recipes were as in HEBERT & BEATON (1989). Alleles were designated alphabetically according to decreasing mobility, the slowest allele being A. Nine loci (*APK1, AAT1, AAT2, IDH1, IDH2, ME, MDH1, PEP-A* and *PEP-D*) were polymorphic (five loci at the 95% level).

After interpretation, data were further analysed using BIOSYS-1 (SWOFFORD & SELANDER, 1989) and GENEPOP (RAYMOND & ROUSSET, 1995). Four genetic variability measures (mean number of alleles per locus, percentage of loci polymorphic, direct count heterozygosity and

heterozygosity values expected under Hardy-Weinberg conditions) were calculated at twelve loci in all populations (see Table 1). We tested for deviations of Hardy Weinberg equilibrium using exact probabilities (SWOFFORD & SELANDER, 1989), correcting for errors resulting from multiple tests by means of the tablewide sequential Bonferroni procedure (HOLM, 1979; RICE 1989).

TABLE 1

Allele frequencies in all populations of Nesaecrepida darwini

		Bahia Tortuga (Isla Santa Cruz)	*CDRS* (Isla Santa Cruz)	*Caleta Sapho* (Isla San Cristóbal)	*La Loberia* (Isla San Cristóbal)	*El Estero* (Isla Isabela)	*Villamil* (Isla Isabela)
APK1	N	45	50	43	43	45	42
	A	0.000	0.000	0.012	0.000	0.000	0.000
	B	1.000	1.000	0.988	1.000	1.000	1.000
APK2	N	45	50	43	43	45	42
	A	1.000	1.000	1.000	1.000	1.000	1.000
AAT1	N	45	50	43	43	46	43
	A	0.267	0.580	0.651	0.115	0.537	0.035
	B	0.733	0.420	0.349	0.885	0.463	0.965
AAT2	n	45	50	43	43	46	43
	A	0.978	0.990	1.000	1.000	1.000	1.000
	B	0.022	0.010	0.000	0.000	0.000	0.000
IDH1	n	44	49	43	43	45	43
	A	0.114	0.347	0.000	0.000	0.000	0.000
	B	0.886	0.653	1.000	1.000	1.000	1.000
IDH2	n	45	50	43	43	46	43
	A	0.000	0.000	0.000	0.012	0.000	0.000
	B	1.000	1.000	0.988	0.988	1.000	1.000
	C	0.000	0.000	0.012	0.000	0.000	0.000
ME	n	45	50	43	43	46	43
	A	0.000	0.070	0.000	0.000	0.000	0.000
	B	1.000	0.930	0.977	1.000	1.000	1.000
	C	0.000	0.000	0.023	0.000	0.000	0.000
MDH1	n	45	50	43	42	46	43
	A	1.000	0.990	0.605	0.571	1.000	1.000
	B	0.000	0.000	0.081	0.012	0.000	0.000
	C	0.000	0.010	0.314	0.417	0.000	0.000
MDH2	n	45	50	43	43	46	43
	A	1.000	1.000	1.000	1.000	1.000	1.000
PEPA	n	45	40	43	38	41	43
	A	0.011	0.000	0.047	0.000	1.000	0.721
	B	0.989	1.000	0.953	1.000	0.000	0.279
PEPD	n	45	38	43	36	39	43
	A	0.000	0.000	0.012	0.000	0.000	0.000
	B	1.000	1.000	0.988	0.986	1.000	1.000
	C	0.000	0.000	0.000	0.014	0.000	0.000
6PGDH	n	45	49	43	43	46	43
	A	1.000	1.000	1.000	1.000	1.000	1.000

Rogers' Genetic distances were calculated between all populations and both UPGMA and distance Wagner dendrograms were constructed.

Population structure was analysed using Wright's F-statistics (WRIGHT, 1978) (F_{ST} measures the amount of differentiation between subpopulations relative to the limiting amount under complete fixation). A hierarchical analysis of population differentiation was performed at two levels: localities within islands, and between islands.

RESULTS

Allele frequencies for all populations studied are shown in Table 1. The different genetic variability measures for all populations are shown in Table 2. Only the locus *AAT1* in the population of Caleta Sapho (one out of more than 20 tests only) shows significant deviation from Hardy-Weinberg equilibrium, due to a heterozygote excess.

TABLE 2

Genetic variability measures for all populations (standard errors in parentheses)

Population	*Mean Sample Size per Locus*	*Mean no. of alleles per locus*	*% of loci polymorphic*	*Mean Heterozygosity*	
				Direct count	*HdyWbg expected*
Bahia Tortuga (Santa Cruz)	44.9 (0.1)	1.3 (0.1)	33.3	0.054 (0.033)	0.055 (0.035)
CDRS (Santa Cruz)	48.0 (1.2)	1.4 (0.1)	41.7	0.082 (0.045)	0.093 (0.053)
La Loberia (San Cristobal)	41.6 (0.7)	1.4 (0.2)	33.3	0.063 (0.042)	0.064 (0.044)
Caleta Sapho (San Cristobal)	43.0 (0.0)	1.7 (0.2)	58.3	0.079 (0.050)	0.100 (0.054)
Villamil (Isabela)	42.8 (0.1)	1.2 (0.1)	16.7	0.045 (0.039)	0.040 (0.034)
El Estero (Isabela)	44.3 (0.7)	1.1 (0.1)	8.3	0.028 (0.028)	0.042 (0.042)

Rogers' genetic distances between all populations are presented in Table 3. Distances between populations vary from 0.054 to 0.156, distances between populations of one island generally being smaller than distances between populations of different islands. The UPGMA and distance Wagner dendrograms are shown in Figs 1 and 2. In both dendrograms, three main groups can be distinguished, each corresponding to the populations of one island. Isla Isabela is separated first, whereas Santa Cruz and San Cristóbal are clustered more closely. These last two islands are considerably older than Isla Isabela.

Table 4 provides a summary of the F-statistics at all loci (FIT is the overall inbreeding coefficient of an individual, which includes a contribution due to actual nonrandom mating within subpopulations [FIS] and another contribution due to the subdivision itself [F_{ST}]). F_{ST} values vary between 0.010 and 0.795. The mean F_{ST} value over all loci is 0.431. Variance components and F-statistics combined across loci for the hierarchical analysis are shown in Table 5. About half of the total variance is attributable to variance between islands. $F_{ISLAND\text{-}TOTAL}$ is somewhat smaller than $F_{LOCALITY\text{-}ISLAND}$, hence genetic differentiation is considerably important also between populations within an island.

TABLE 3

Matrix of Rogers (1972) genetic distances between populations

Population	*Bahia Tortuga*	*CDRS*	*Villamil*	*El Estero*	*La Loberia*
Bahia Tortuga (Santa Cruz)	-				
CDRS (Santa Cruz)	0.054	-			
Villamil (Isabela)	0.090	0.142	-		
El Estero (Isabela)	0.116	0.123	0.065	-	
La Loberia (San Cristobal)	0.062	0.111	0.104	0.156	-
Caleta Sapho (San Cristobal)	0.081	0.077	0.143	0.124	0.061

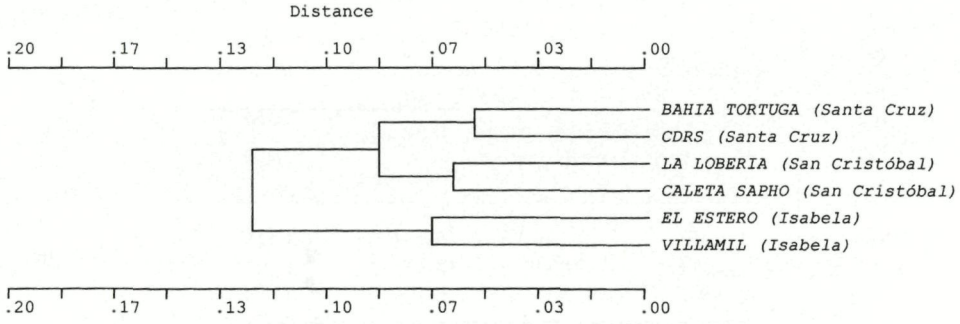

Fig. 1. – UPGMA dendrogram based on Rogers' genetic distance

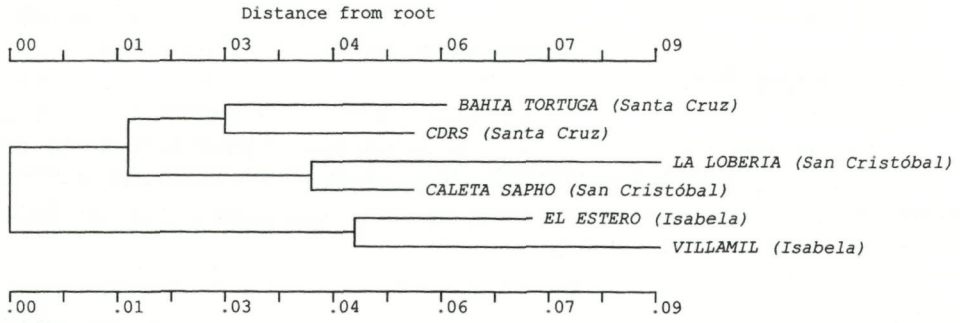

Fig. 2. – Distance Wagner dendrogram based on Roger's genetic distance

TABLE 4

Summary of F-statistics at all loci

Locus	*F(IS)*	*F(IT)*	*F(ST)*
APK1	-0.012	-0.002	0.010
AAT1	0.256	0.444	0.244
AAT2	-0.019	-0.005	0.013
IDH1	-0.033	0.205	0.230
IDH2	-0.012	-0.003	0.009
ME	-0.062	-0.013	0.046
MDH1	-0.050	0.246	0.281
PEPA	-0.041	0.787	0.795
PEPD	-0.013	-0.003	0.010
Mean	0.096	0.469	0.413

TABLE 5

Variance components and F-statistics combined across loci

Comparison		*Variance*	F_{xy}
X	*Y*	*Component*	
Locality	Island	0.25405	0.244
Locality	Total	0.53930	0.407
Island	Total	0.28525	0.215

DISCUSSION

Heterozygosity values (Table 2) in all populations are relatively low compared to those found in studies on continental Chrysomelidae (see overview in VERDYCK, in press) and other beetles (see overview in HSIAO, 1989). Observed heterozygosity is also considerably higher in the flightless Galápagos species of the genus *Stomion* (average 0.085) (FINSTON & PECK, 1997).

Populations from Isla Isabela (Villamil and El Estero), the youngest island investigated in our study, show the lowest genetic diversity for all measures. This can be explained if the origin of these populations is to be found in a more recent colonisation of this island by a limited number of individuals (founder effects), originating from nearby older islands (*e.g.* Floreana, Santa Cruz).

The observed clustering pattern of both dendrograms may be explained by the fact that populations of the two oldest islands (Santa Cruz and San Cristóbal) probably have had some genetic exchange in their recent history, whereas Isabela is genetically much more separated.

The mean F_{ST} value of 0.431 is one of the highest ever recorded between populations of a chrysomelid species (an overview of genetic population structuring in chrysomelids at different micro- and macrogeographical scales is provided by KNOLL *et al.* (1996)). VERDYCK *et al.* (1998) studied continental populations of the alticine beetle *Phyllotreta tetrastigma*. This species has (apart from belonging to the same subfamily of beetles) several other characteristics in common with *N. darwini*. It is a monophagous species, capable of flying, with a discontinuous distribution (because its host plant *Cardamine amara* is restricted to wet woodlands), which can sometimes be very locally abundant. However *P. tetrastigma* shows remarkably little genetic differentiation over a relatively large geographic scale (Western Europe). For the only other Galápagos insects studied, FINSTON & PECK (1995) found a mean F_{ST} of 0.30 across taxa in the flightless *Stomion* beetles.

The results obtained for *N. darwini* are very remarkable and in contrast with those found for other good flying and even for flightless species. The high amount of genetic differentiation between the islands indicates that, although the species is macropterous, inter-island gene flow is not sufficient to counteract effects of differentiation between islands. Moreover, we observed a relatively high differentiation between populations from the same island, sometimes even at relatively small distances. The main reason for this might be a metapopulation structure (patchy distribution) in combination with a pattern of recurrent extinctions and recolonisations giving rise to many founder events. Recent field observations in spring 1998 showed that the population on Villamil had probably become extinct whereas the populations on Santa Cruz (CDRS and Bahia Tortuga) still existed (the other populations were not visited in 1998). This confirms the hypothesis of a metapopulation model for *N. darwini*. A more general concluding hypothesis is that the population genetics and thus the evolution of this species in Galápagos have been profoundly shaped by genetic drift during recurrent founder events within the archipelago. This hypothesis will be further tested in the future, by means of genetic data on additional populations and by using other biochemical markers, such as mtDNA-data.

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ISOLATION AND PARTIAL SEQUENCING OF A NOVEL MYOTROPIN FROM THE BRAIN OF THE DESERT LOCUST *SCHISTOCERCA GREGARIA* FORSK

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Abstract. A neuropeptide that stimulates the motility of the cockroach hindgut has been isolated from an extract of 7000 brain – corpus cardiacum – corpora allata – suboesophageal ganglion complexes of the desert locust *Schistocerca gregaria*. During HPLC purification, the myotropic activity of column fractions was monitored on the isolated hindgut of *Leucophaea maderae*. Due to the low amount of material, this myotropic peptide - designated as schistomyostimulin or Scg-MST – could only be partially sequenced : DSRW?GPK(?). Scg-MST shows no relevant sequence similarities with other peptides from vertebrate or invertebrate sources. It is the fifteenth myotropic peptide chromatographically isolated from *S. gregaria*.

Key words : insect, peptide, myotropin, grasshopper.

INTRODUCTION

Regulatory peptides mediate many physiological processes. In insects alone the number of different neuropeptides isolated and identified to date exceeds 200 (GÄDE, 1997). It is assumed that the number of different neuropeptides in each species may also exceed 200. In locusts the total number of neuropeptides identified to the present time is 58 (SCHOOFS *et al.*, 1997 ; VEELAERT *et al.*, 1998). Most of the locust peptides have been isolated on the basis of their stimulatory effect on insect visceral muscles and were designated myotropins. Some of the myotropins appear to be unique to insects or arthropods ; others seem to be members of peptide families spanning across phyla. Members of the myotropin peptide families have been associated with a variety of physiological activities such as myotropic activities, pheromonotropic activities, diuresis, diapause induction, and stimulation of cuticular melanization (for review see SCHOOFS *et al.*, 1993). Some of the members may be important neurotransmitters present in nerve endings innervating the locust oviduct, the salivary glands, the male accessory glands and the heart (SCHOOFS *et al.*, 1992) whereas others are stored in neurohaemal organs until release into the haemolymph (SCHOOFS *et al.*, 1992).

Two adipokinetic hormones (Scg-AKH-1 and -2) have been purified from *Schistocerca gregaria* (Table 1). Recently two schistomyotropins (Scg-MT-1 and -2), belonging to the -FXPRLamide family were purified from an extract of 7000 brains. Scg-MT-2 has a modified -FXXPRLamide C-terminus. The crustacean cardioactive peptide was also purified from brains of the desert locust (Scg-CCAP). This peptide has a corpus cardiacum-activating effect, being a releasing factor of adipokinetic hormone in locusts. SchistoFLRFamide (Scg-FLRFamide) and the Schistostatins (Scg-ASTs) are inhibitors of visceral muscle contractions.

TABLE 1

Myotropic and myoinhibiting peptides isolated from the desert locust Schistocerca gregaria *(*VEELAERT *et al.*, 1998)

Scg-AST-1	LCDFGVa	VANDEN BROECK *et al*, 1996
Scg-AST-2	AYTYVSEYKRLPVYNFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-211-18	LPVYNFGLa	VEELAERT *et al.*, 1996b; VANDEN BROECK *et al*, 1996
Scg-AST-3	ATGAASLYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-4	GPRTYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-5	GRLYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-6	ARPYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-7	AGPAPSRLYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-8	EGRMYSFGLa	VEELAERT *et al.*, 1996a; VANDEN BROECK *et al*, 1996
Scg-AST-9	PLYGGDRRFSFGLa	VANDEN BROECK *et al*, 1996
Scg-AST-10	APAEHRFSFGLa	VEELAERT *et al.*, 1996; VANDEN BROECK *et al*, 1996
Scg-AKH-1	pELNFTPNWGTa	STONE *et al.*, 1976
Scg-AKH-2	pELNFSTGWa	SIEGERT *et al.*, 1985
Scg-MT-1	GAAPAAQFSPRLa	VEELAERT *et al.*, 1997b
Scg-MT-2	TSSLFPHPRLa	VEELAERT *et al.*, 1997b
Scg-CCAP	PFCNAFTGCa	VEELAERT *et al.*, 1997a
Scg-FLRFamide	PDVDHVFLRFa	ROBB *et al.*, 1989

In this paper we describe the isolation and partial identification from *S. gregaria* of a novel myotropin that shows no relevant sequence similarity with any known vertebrate or invertebrate neuropeptide.

MATERIAL AND METHODS

Animals, tissue extraction, and purification

S. gregaria was raised under laboratory conditions (ASHBY, 1972). Brains and adjacent retrocerebral complexes (7000) from 12-14-day adults were dissected and immediately placed in an ice-cold methanol/water/acetic acid (90:9:1) solution. The tissues were sonicated and centrifuged for 30 min (10,000 *g*; 4°C). Methanol was evaporated and the remaining aqueous residue was re-extracted with ethylacetate and n-hexane to remove the bulk of lipids. The organic solvent layer was decanted and the aqueous solution was dried in siliconized round-bottom flasks. Subsequently, it was dissolved in aqueous trifluoroacetic acid

(TFA) (0.1 %) and pre-purified on Megabond Elute C18 cartridges (10g/cartridge) (Varian, Harbor City, CA) that had been activated with CH_3CN/H_2O/TFA (80 :19.9 :0.1) and then rinsed with 0.1 % TFA. The cartridges were eluted with 25 ml of 50 % and 80 % CH_3CN in 0.1 % TFA. Columns and operating conditions for high performance liquid chromatography (HPLC) on a Gilson HPLC system with variable wavelength detector (214 nm) were: (1.) Deltapak C18 column (25 x 100 mm) (Waters Associates, Milford, MA), solvent A: 0.1 % TFA in water; solvent B: 50 % CH_3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 8 min, followed by a linear gradient to 100 % B in 150 min; flow rate: 6 ml/min; detector range: 1 absorption unit full scale (Aufs); (2.) Protein C4 column (4.6 x 250 mm) (Vydac, Hesperia, CA), solvent A: 0.1 % TFA in water; solvent B: 50 % CH^3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 20 min, followed by a linear gradient to 100 % B in 50 min; flow rate: 1 ml/min; detector range: 0.5 Aufs; (3.) Phenyl spheri-5 column (4.6 x 250 mm) (Brownlee, Applied Biosystems, Foster City, CA), solvent A: 15 % CH_3CN in 0.1 % aqueous TFA; solvent B: 65 % CH_3CN in 0.1 % aqueous TFA. Column conditions : 100 % A for 20 min, followed by a linear gradient to 100 % B in 50 min; flow rate: 1.5 ml/min; detector range: 0.5 Aufs; (4.) Protein and Peptide C18 (4.6 x 250 mm) (Vydac, Hesperia, CA), solvent A: 10 % CH_3CN in 0.1 % aqueous TFA; solvent B: 40 % CH_3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 10 min, followed by a linear gradient to 100 % B in 60 min; flow rate: 1.5 ml/min; detector range: 0.5 Aufs; (5.) Microsorb-MV C18 (4.6 x 250 mm) (Rainin Instruments Co., Woburn, MA), solvent A: 15 % CH_3CN in 0.1 % aqueous TFA; solvent B: 50 % CH3CN in 0.1 % aqueous TFA. Column conditions: 100 % A for 20 min, followed by alinear gradient to 100 % B in 60 min, flow rate; 1ml/min; detector range: 0.2 Aufs.

Mass spectrometry and peptide sequencing

A sample containing 0.5-1 pmol/µl of the active peak was subjected to Maldi-TOF analysis (HILLENKAMP *et al.*, 1991). One µl was mixed with 1 µl of a 50 mM solution of α-cyano-4-hydroxycinnamic acid in CH_3CN :EtOH (50 :50) containing 0.1 % TFA and applied on the multi-sample target. This mixture was air-dried and the target was then introduced in the instrument, a Micromass Tofspec (Micromass, Wythenshawe, UK) equipped with a N_2-laser (337 nm). The samples were measured either in the linear (acceleration voltage 24 kV) or in the reflectron mode (acceleration voltage 24 kV, reflectron voltage 28.6 kV). In either case, the laser energy was reduced until an optimal resolution and signal/noise ratio was obtained. The results of 20 to 50 shots were averaged to obtain the final spectrum. Automated amino acid sequencing was performed on a Beckman LF3600TC gas-phase protein sequencer (Beckman, Fullerton, CA) according to the Edman degradation principle.

Myotropic bioassay

The myotropic bioassay was performed as described by SCHOOFS *et al.* (1993). Hindguts of *Leucophaea maderae* were dissected under Ringer solution. Each hindgut was placed in an assay chamber filled with Ringer solution and attached to a transducer device, which allows recording of the frequency and of the amplitude of muscle contractions.

Ringer solution containing a purified material was applied to the bioassay chamber with the isolated hindgut of *L. maderae*.

RESULTS

Passage through two Megabond Elute columns was used to pre-purify the brain extract containing 7000 equivalents. The material eluting with 50 % CH_3CN showed myotropic activity. During the HPLC purification process all fractions were tested in the myotropic assay. The fraction eluting at 66-72 min on the first HPLC column showed myotropic activity (Fig. 1A.). This fraction was further purified and myotropic activity eluted subsequently at 43 min on the second column (Fig. 1B), 38 min on the third column (Fig. 1C), 46 min on the fourth column (Fig. 1D) and at 45 min on the fifth column (Fig. 1E). After the fifth column purification step, the peptide showed apparent homogeneity. The mass spectrum is shown in Fig. 2. Only one molecular ion is present with mass of 968.3 Da.

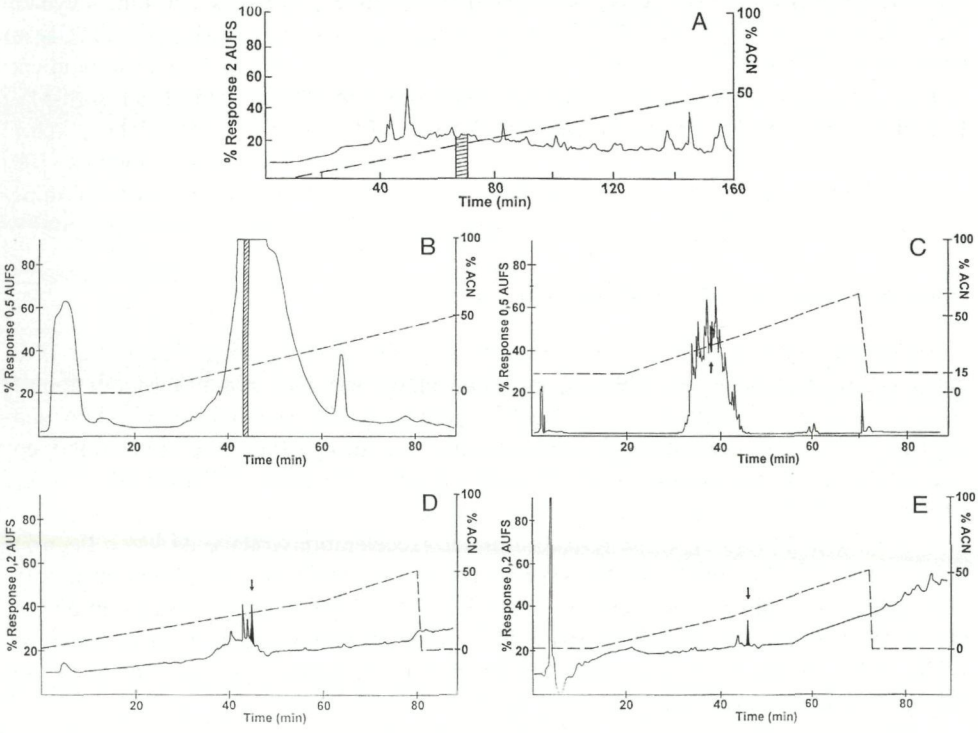

Fig. 1. – Purification of brain extract of *Schistocerca gregaria*. **A :** Fractionation on Deltapak C18 column. Fraction eluting at 66-72 min (shaded part) shows myotropic activity. **B :** Second fractionation on a Protein C4 column. The fraction eluting at 43 min contained the myotropic activity (shaded part). **C :** Third fractionation on a Phenyl spheri-5 column. Myotropic activity eluted at 38 min (Black peak and arrow). **D :** Fourth fractionation on a Protein and Peptide C18 column. Myotropic activity eluted at 46 min (Black peak and arrow). **E :** Final purification on a Microsorb-MV C18 column. Myotropic activity eluted at 45 min (black peak and arrow).

This may represent (a) a protonated peptide ion $(M+H)^+$. In that case the presence of a minor peak at 984.6 may represent an oxidized form of the peptide. Alternatively (b) the 984.6 peak may be indicative of a potassium adduct, in which case the 968.3 Da compound may represent the sodium adduct $(M+Na)^+$. This will also mean that the $(M+H)^+$ ion is not seen in the MALDI spectrum. This phenomenon, however, has previously been observed with insect neuropeptides (VERHAERT & DE LOOF, 1998; VERHAERT *et al.*, 1998). The molecular mass of the peptide will be 967.3 in case a and 945.3 in case b.

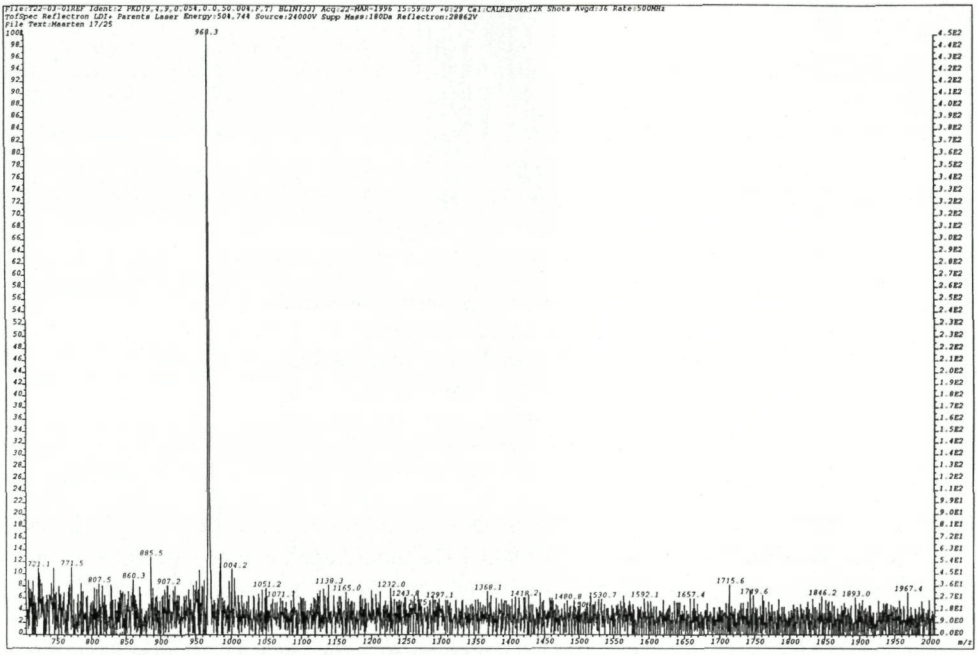

Fig. 2. – MALDI-TOF mass spec spectrum of Scg-MST showing a single ion species of m/z 968.3 Da.

Fig. 3 shows the effect of 20 brain equivalents of the purified peptide. Its activity is comparable to the effect of the schistomyotropins and the locustamyotropins on the hindgut of *L. maderae*. The peptide was sequenced by Edman degradation and a partial sequence was obtained: DRSW?GPK?. The peptide is called schistomyostimulin (Scg-MST).

DISCUSSION

Five HPLC runs were needed to isolate a myotropic peptide from the nervous system of the desert locust, *S. gregaria*. After final purification, 20 brain equivalents still showed myotropic activity on the hindgut of *L. maderae*. Maldi-TOF spectrometry revealed an ionic mass of 968.3 Da presumptive $(M+H)^+$ if not $(M+Na)^+$. Due to the low amount of purified material only a partial sequence was obtained: DSRW?GPK(?) (? : missing amino

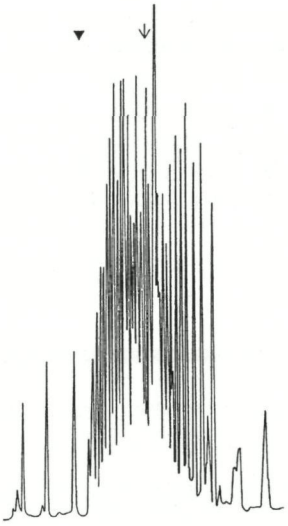

Fig. 3. – Myotropic assay on the hindgut of *Leucophaea maderae*. Response of the hindgut to 20 equivalents of purified Scg-MST. Arrow-head, application of Scg-MST; arrow, rinse with saline; 1cm horizontal axis is 1min.

acids). This partial sequence has to our knowledge no relevant similarities with any known amino acid sequence. The mass 967.3 or 945.3 Da should enable us to calculate the missing amino acid(s) of myostimulin. Unfortunately no obvious amino acid combination fits this mass. This could be explained if (one of) the missing amino acid(s) were modified which would agree with the observation that no relevant signal was observed at the respective blank cycle(s) during Edman degradation reactions.

Of all known insect regulatory peptides, myotropins are by far the largest group. In *S. gregaria*, two myotropic peptides, the schistomyotropins (Scg-MT-1 and -2) have been identified previously. They are closely related to the locustamyotropins (Lom-MT-1 till -4) and the locustapyrokinins (Lom-PK-1 and -2) (SCHOOFS *et al.*, 1997). All those peptides belong to the -FXPRLamide peptide family (X being Ser, Val, Thr, Gly), which occurs widely among insects. This pentameric carboxyterminal sequence is also found in the pheromone-biosynthesis-activating peptide of *Heliothis zea* (Hez-PBAN: RAINA *et al.*, 1989) and *Bombyx mori* (Bom-PBAN I and II: KITAMURA *et al.*, 1989,1990) as well as in the melanization and reddish-coloration hormone of *Pseudaletia separata* (Pss-MRCH: MATSUMOTO *et al.*, 1992). The same C-terminal -FXPRLamide motif is also found in the diapause hormone of *B. mori* (Bom-DH: IMAI *et al.*, 1991). Two adipokinetic hormones, Scg-AKH-1 and Scg-AKH-2 were isolated from *S. gregaria* (STONE *et al.*, 1976). These hormones induce the mobilization of lipids from the fat body (ORCHARD & LANGE, 1983) and induce the synthesis of flight-specific lipophorins (VAN DER HORST *et al.*, 1979). Lom-AKH-$1^{14\text{-}10}$, most probably a degradation product of Lom-AKH-1, is a stimulator of visceral muscle contractions (SCHOOFS *et al.*, 1993).

By screening chromatographically-purified fractions in a bioassay to measure adipokinetic hormone release, a peptidergic, adipokinetic, hormone-releasing factor (Scg-CCAP) was isolated from 7000 brains of the dessert locust *S. gregaria*. Scg-CCAP stimulates the release of adipokinetic hormone in a dose-dependent manner in *S. gregaria* (VEELAERT *et al.*, 1997a). The peptide was first isolated from the shore crab, *Carcinus maenas*, as a cardioactive peptide, CCAP (STANGIER *et al.*, 1987). Later, CCAP was isolated from *Locusta migratoria* by affinity chromatography (STANGIER *et al.*, 1989).

The locusts *L. migratoria* and *S. gregaria* are the insect species from which the largest number of myotropins (including Scg-MST) have been isolated and sequenced (SCHOOFS *et al.*, 1997; VEELAERT *et al.*, 1998).

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ESTIMATING ISOLATION AND GENETIC DIFFERENTIATION IN TWO BELGIAN POPULATIONS OF MOORHENS *GALLINULA CHLOROPUS* BY USING MINISATELLITE AND MICROSATELLITE DNA MARKERS

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Abstract. Isolation of a population can result in decreased genetic variability as a consequence of inbreeding, random genetic drift and reduced gene flow. This effect is reinforced when it concerns a small population. We used two molecular techniques, multilocus minisatellite DNA fingerprinting and microsatellite analysis, to compare population genetic parameters between a small, possibly isolated natural population of moorhens and a large, presumably non-isolated population at a distance of approximately 30 km. Although sample sizes were still relatively low, both minisatellite and microsatellite analysis indicated that the small population is not genetically impoverished, despite being located at the centre of a large city. Nevertheless, we found significant between-populational genetic differentiation, which suggests that there is little gene flow between the two populations studied.

Key words : *Gallinula chloropus*, DNA-fingerprinting, minisatellites, microsatellites, isolation, inbreeding, population differentiation.

INTRODUCTION

Individuals in small populations are more likely to be inbred because there is a high probability that random pair formation will occur between related individuals (AVISE, 1994; NEIGEL, 1996). Genetically, inbred populations are expected to show increased homozygosity due to the increased probability that individuals carry alleles that are « identical by descent » (AVISE, 1994). Small populations are also more susceptible to loss in genetic polymorphism due to random genetic drift. The main process that counteracts this decrease in genetic variability in small populations is gene flow, largely achieved by migrating individuals (NEIGEL, 1996). Therefore, looking at the genetic variability or the level of inbreeding in a small population can provide information about the degree of isolation, in terms of gene flow, of that population.

Genetic drift, selection and mutation may all result in the divergence of allele and genotype frequencies between isolated populations, such that populations become geneti-

cally different, a process known as population differentiation. This genetic divergence is also reduced by exchange of genetic material (NEIGEL, 1996). As such, the amount of genetic differentiation provides information on the amount of gene flow between populations.

During the course of the last decade, a diverse array of new molecular genetic tools has become available for high-resolution genetic studies of population-level processes (PARKER *et al.*, 1998). In recent years, minisatellite and microsatellite DNA markers have proven their usefulness in numerous applications, including studies on population genetics and relatedness (AVISE, 1994; BRUFORD *et al.*, 1996; BURKE *et al.*, 1996). Minisatellite and microsatellite loci belong to the classes of loci known as variable number tandem repeat (VNTR) loci. Both are usually highly polymorphic, owing to variation in the number of tandem repeats. As a consequence, they can provide information on the genetic structure of individuals (heterozygosity) or populations (allelic diversity) where less sensitive methods such as enzyme electrophoresis might fail (BURKE *et al.*, 1996). Contrary to minisatellites, microsatellite loci can be assayed using the polymerase chain reaction (PCR) combined with electrophoresis (BURKE *et al.*, 1996). This approach allows determination of the exact length in basepairs of alleles, which can be unambiguously assigned to certain loci (QUELLER *et al.*, 1993). As a result, microsatellites are highly suitable to assess genetic variation in terms of heterozygosity levels, genetic polymorphism, and allele frequencies, within and between populations, which makes them powerful tools for estimating inbreeding levels and population differentiation (BRUFORD *et al.*, 1996). Although multilocus minisatellite analysis has also been successfully used in population genetic studies (*e.g.* WAUTERS *et al.*, 1994), this method has more drawbacks than the previous one (QUELLER *et al.*, 1993; AVISE, 1994; BURKE *et al.*, 1996). One of the problems is that multilocus minisatellite analysis reveals complex multiple banding patterns, or «DNA fingerprints», in which it is usually not possible to distinguish every allele and in which alleles generally cannot be assigned to a particular locus (JEFFREYS *et al.*, 1985a,b; BRUFORD *et al.*, 1992; AVISE, 1994; BURKE *et al.*, 1996). Minisatellite fingerprinting is therefore less suitable for studies requiring information on allele frequency distributions, such as needed to estimate the degree of gene flow between populations (BURKE *et al.*, 1996). However, « DNA fingerprints » can be used to elucidate inbreeding because they make it possible to estimate the mean relatedness within populations (AVISE, 1994). BELLAMY *et al.* (1991) have shown in humans that even a moderately inbred population can be recognised by a higher mean band sharing than a known outbred population.

The Antwerp Zoo, which is located at the centre of the large Belgian city of Antwerp, contains a small population of free-living moorhens. The main goal of our study was to find out whether the surrounding city functions as a barrier that decreases migration in such a way that the moorhens at the Antwerp Zoo form an isolated population. This was tested by estimating the level of inbreeding in the Zoo population compared with that in a large, presumably non-isolated moorhen population at Planckendael, using both multilocus minisatellite and microsatellite DNA markers. The microsatellite analysis also allowed us to determine genetic differentiation between the two populations and to estimate gene flow.

MATERIAL AND METHODS

Study areas and blood sampling

In Belgium, moorhens are common in all kinds of habitats as long as fresh water and low cover are present (WOOD, 1974; STEVENS, 1989). Therefore, moorhens can be abundant in city parks even though they do not inhabit the rest of the city. At the time of its foundation in 1843, the Antwerp Zoo was located at the edge of the town (BAETENS, 1993). Due to increasing urbanisation ever since, the surrounding area of the Zoo has been built over within a radius of five kilometres. The Zoo, with an area of about ten hectares, is now located at the centre of the city and forms, together with a public park nearby the Zoo, an isolated patch of suitable habitat for moorhens in the crowded city (BAETENS, 1993). No records were kept, but moorhens have inhabited the Zoo for as long as can be remembered by long-term employees, which means at least 40 years. Based on thorough capturing and personal observations, the population size was estimated to be about 40 adult moorhens during the sampling period.

The animal park Planckendael, founded in 1956, is situated in a semi-rural setting, near Mechelen, Belgium, at about 30 kilometres distance from the Antwerp Zoo, and has an area of roughly 36 hectares (BAETENS, 1993). Moorhens at Planckendael have been ringed and studied at irregular intervals since 1992, and the turnover population size was estimated to be approximately 300 adult individuals (MERCKX, unpubl.).
At Planckendael, animals were captured between 1993 and 1996; at the Antwerp Zoo all animals were caught in 1996. A wing vein was punctured with a sterile needle (Terumo, 0.55*25 mm) and blood was collected with Na-heparinized haematocrit-capillaries. Within three hours after capture, the blood was stored at -70°C until analyses were done.

Multilocus minisatellite DNA analysis

After a normal phenol-chloroform extraction, DNA was fragmented using the restriction enzyme MboI. DNA fingerprints were generated by «Southern Blotting» following BRUFORD *et al.* (1992). The radio-active (α32dCTP) probe 33.15 was used for hybridisation (JEFFREYS *et al.*, 1985a,b). The mean probability of two individuals sharing a band of apparently similar mobility on the gel, was estimated using the band-sharing coefficient or similarity index $2n_{ab}/(n_a+n_b)$, where n_a and n_b are the numbers of bands present in individuals a and b and n_{ab} is the number of bands shared by a and b (WETTON *et al.*, 1987; WESTNEAT, 1990; LYNCH, 1990; BRUFORD *et al.* 1992; PINXTEN *et al.*, 1994). A band was considered identical in two individuals if it had migrated no more than 0.5 mm from a band in another individual (WESTNEAT, 1990) and if the bands were of approximately the same intensity. A weakly hybridised band in individual «a» was excluded if its possible presence in individual «b» could not be verified because there was a stronger band on the same mobility level or because the entire lane of individual «b» was lighter (WESTNEAT, 1990; BURKE & BRUFORD, 1987). The band-sharing coefficient was calculated for 10 adult pairs from the Antwerp Zoo and 13 from Planckendael. Pairs were ordered in adjacent lanes on the same gel to assure precise comparison of band mobility. The higher the band-sharing coefficient, the more related are the members of the pair (LYNCH, 1990; BRUFORD *et al.*, 1992). A mean

band-sharing coefficient was calculated for each population. These coefficients were arcsine square-root transformed to be normalised, and compared by means of a t-test using the statistical package SPSS/PC (SPSS, 1986). Values given are mean ± SE.

Microsatellite DNA analysis

Twenty-six and thirty adult moorhens were sampled at the Zoo and at Planckendael, respectively. DNA was obtained from blood by a normal phenol-chloroform extraction. Microsatellite polymorphism was analysed at 9 loci. The primer sets used were originally developed for the Tasmanian native hen *Gallinula mortierii* (unpubl. data). For the exact defining of the primer sets we refer to Jason Buchan (see acknowledgements) by whom they were kindly provided. We adjusted reaction conditions to moorhens by « trial and error ». Amplification was achieved in a 25 µl reaction volume containing 0.2 to 0.4 µM of each primer (one of which was fluorescently labelled with CY5, Pharmacia), 1.5 to 2 mM $MgCl_2$, 200µM dNTP's, about 200 ng of genomic DNA, 1 x buffer (1.5 mM $MgCl_2$ included for the DynaZyme buffer) and 0.5 to 1 U of Pro-HA DNA polymerase (Eurogentec) or DynaZyme polymerase (Life Sciences Int.). The optimal thermal profile for PCR amplification is : 94°C, 3 min. ; 30 cycles of : 94°C, 50 sec. ; locus specific annealing temperature (Table 1), 55 sec. ; 72°C, 45 sec. and a final extension step at 72°C for 5 min. in a Techne Genius. Successful PCR reactions were diluted 1 :10 and exact fragment size was calculated by Allelinks (Pharmacia), after electrophoresis and fluorescent detection on a 6% acrylamide gel in an automated sequencer (A.L.F. express, Pharmacia).

TABLE 1

Volumes (µl) of reagents and annealing temperatures used in the PCR amplification of 9 microsatellite loci in Gallinula chloropus. *The total PCR volume was 25 µl. The length range of the PCR products is given in base pairs (bp). Multiplexing of PCR reactions was feasible for Tm19–Tm20 and Tm18–Tm38, while PCR products were run together for loci Tm31B–Tm105 and Tm36–Tm101.*

	Tm18–38	Tm19–20	Tm27	Tm31B	Tm36	Tm101	Tm105
DNA (200ng/µl)	1	1	1	1	1	1	1
H_2O	14.85	15.85	17.9	19.85	18.85	18.4	18.35
10 X buffer	2.5	2.5	2.5	2.5	2.5	2.5	2.5
$MgCl_2$ (25 mM)	2	2	2	/	0.5	1.5	1.5
DNTPs (10mM)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Forward primer (10 µM)	1–1	1–0.5	0.5	0.5	0.75	0.5	0.5
Reverse primer (10µM)	1–1	1–0.5	0.5	0.5	0.75	0.5	0.5
Taq (5U/µl)	0.15 PRO	0.15 PRO	0.1 PRO	0.15 DYN	0.15 DYN	0.15 PRO	0.15 PRO
Locus specific annealing temperature (°C)	57	57	55	52	57	57	57
Length range (bp)	145–147/ 112–126	149–155/ 132–136	143–173	180–252	146–153	132–133	111–151

For each population, microsatellite loci were tested for deviations from the Hardy-Weinberg equilibrium and for linkage disequilibrium (following the method of BLACK & KRAFSUR, 1985), using exact probabilities generated in Genepop 3.1b (Markov chain parameters: dememorization :10000; batches :200; iterations per batch (or permutations) :10000; RAYMOND & ROUSSET, 1995, 1998). To correct for multiple tests, p values were adjusted (p_a) for comparison to alpha (=0.05) based on the number of tests, Ntest, carried out: the most significant value was multiplied with Ntest, the second most with Ntest-1, etc. (modification of the sequential Bonferroni method of RICE, 1989; see ROS *et al.*, 1997).

To estimate within population genetic variability, we looked at the level of polymorphism (P), the mean number of alleles per locus (MNA) and heterozygosity levels (H). WRIGHT's F_{IS} (1951) measures the deviation from Hardy-Weinberg proportions within populations and was calculated as a metric of the level of inbreeding for each population. Significance of F_{IS} values was estimated by resampling over alleles (1000 permutations). F_{ST} represents the amount of genetic differentiation between two populations (WRIGHT, 1951, 1969) and was estimated by means of θ (WEIR & COCKERHAM, 1984). We estimated the overall F_{ST} and the F_{ST} for each locus separately. We also checked whether linkage or deviation of H-W of loci had an effect on the overall F_{ST}. Significance of θ values was estimated by permuting individual alleles across the two areas (Markov chain parameters: dememorization: 10000; batches: 200; iterations per batch (or permutations): 10000). Using θ and assuming an island model, it is possible to estimate Nm or the mean number of individuals that migrate between the studied populations each generation to maintain the population differentiation (WEIR & COCKERHAM, 1984). Genetic diversity (H) was compared between the two populations by means of permutation following VAN DONGEN *et al.* (1998). Parameters H, P, MNA, θ-values and significance of θ-values were calculated with Genepop3.1b (RAYMOND & ROUSSET, 1995b, 1998). Nm, F_{IS} and significance of F_{IS} were calculated using Genetix version 3.0 (BELKHIR *et al.*, 1996). The probability level for significant differences (α) was set at 0.05.

RESULTS

Multilocus minisatellite DNA analysis

In each lane we could score a mean of 21.3 ± 5.5 bands. The difference between the mean band-sharing coefficient of ten adult pairs at the Zoo (0.257 ± 0.038) and that of 13 pairs at Planckendael (0.254 ± 0.036) was not significant (t-test, t=0.15, df=21, p=0.88).

Microsatellite DNA analysis

At Planckendael, we found a significant linkage between loci Tm18-TM19 and loci Tm36-Tm38 (permutation test, sequential Bonferroni correction, p_a=0.042 and p_a=0.0036 respectively), while there was a significant linkage between locus Tm18 and Tm38 at the Zoo (permutation test, sequential Bonferroni correction, p_a=0.027). We found a significant

deviation from Hardy-Weinberg equilibrium at locus Tm36 at the Zoo and at locus Tm38 at Planckendael (after Bonferroni correction respectively: p_a=0.023 and p_a=0.0072); the other loci showed no such deviation (after Bonferroni correction: p_a>0.05)

TABLE 2

Allele frequencies at nine microsatellite loci for 26 adult moorhens at the Zoo and 30 at Planckendael. Names of alleles represent the size in base pairs (or fragment length) of the alleles

Locus	*Allele*	*Antwerp Zoo*	*Planckendael*	*Locus*	*Allele*	*Antwerp Zoo*	*Planckendael*
Tm20	132	0.6346	0.6	Tm36	146	0.1346	0.2
	134	0.2115	0.3833		151	0.6538	0.5833
	136	0.1538	0.0167		153	0.2115	0.2167
Tm27	137	0.0192	0	Tm38	112	0.1346	0.0333
	143	0.0577	0		114	0.0192	0.1667
	144	0	0.0333		116	0.2885	0.1
	145	0.0385	0.1		118	0.2692	0.0667
	147	0.2115	0.1167		120	0.1154	0.1
	149	0	0.0167		122	0.1731	0.5167
	151	0.3462	0.1667		126	0	0.0167
	153	0.0769	0.1	Tm101	132	0.9615	0.9464
	155	0.1538	0.0167		133	0.0385	0.0536
	157	0	0.2333	Tm105	111	0.0192	0.0167
	159	0.0385	0.1833		115	0.0192	0.1
	163	0	0.0333		117	0.0192	0
	173	0.0577	0		119	0.1538	0
Tm31B	180	0	0.0833		131	0.1154	0.0167
	194	0.1154	0.1333		133	0.0192	0
	200	0.0192	0		135	0.0192	0.0667
	202	0.0962	0.0667		137	0.0192	0.05
	206	0.0385	0		139	0.0962	0
	210	0.0192	0.0333		143	0.2692	0.0333
	216	0.0385	0.2		145	0.0769	0.4167
	218	0.0577	0		147	0.0192	0.25
	220	0.0385	0		149	0.0192	0
	224	0.0385	0.05		151	0.1346	0
	228	0.2885	0		153	0	0.05
	234	0	0.1833	Tm18	145	0.4423	0.2333
	236	0.1538	0		147	0.5577	0.7333
	238	0.0385	0		149	0	0.0167
	240	0	0.0167		151	0	0.0167
	246	0.0577	0.2	Tm19	149	0	0.2
	252	0	0.0333		155	1	0.8

Table 2 shows the allele frequencies that were determined at the nine microsatellite loci for 26 adult moorhens at the Zoo and 30 at Planckendael. Some loci are highly variable (e.g. Tm31B) while locus Tm101 is uniform. Locus Tm19 seems to be fixed in the Zoo population. Table 3 presents the genetic diversity in both populations. No significant

differences in genetic variability between the two populations were found (permutation tests, all $p>0.05$). The inbreeding coefficient F_{IS} equalled 0.061 for the Zoo population and 0.033 for the Planckendael population; both values were not significantly different from 0 (permutation tests, $p=0.1$ and $p=0.18$ respectively). As shown in table 4, there was a significant overall genetic population differentiation with an estimated θ value of 0.082 (permutation test, $p<0.05$). This value corresponds to 2.79 migrants per generation (Nm). Table 4 also lists F_{ST}-estimates for separate loci. Omitting linked loci or the loci that deviated from Hardy-Weinberg equilibrium did not alter the magnitude order nor significance of the overall F_{ST}-estimate (values not given).

TABLE 3

Microsatellite variability for two populations of moorhens: expected heterozygosity (Hexp), observed heterozygosity (Hobs), proportion polymorphic loci (P) and mean number of alleles per locus (MNA). SD = Standard Deviation

	Hexp (SD)	*Hobs (SD)*	*P*	*MNA*
Antwerp Zoo	0.54 (0.32)	0.52 (0.34)	0.9	5.89
Planckendael	0.56 (0.25)	0.55 (0.27)	1.0	5.56

TABLE 4

Estimates of genetic differentiation between the Antwerp Zoo and Planckendael moorhen population for each locus separately and for all loci combined. Significance levels are defined as follows: $p<0.05$:; $p<0.01$:**; $p<0.001$:****

Microsatellite locus	*F_{ST}–estimate*	*significance*
Tm18	0.062	*
Tm19	0.178	***
Tm20	0.032	**
Tm27	0.066	***
Tm31B	0.091	***
Tm36	–0.016	NS
Tm38	0.118	***
Tm101	–0.032	NS
Tm105	0.142	***
Total	0.082	***

DISCUSSION

Although sample sizes were rather low, both genetic analyses provided indications that the small moorhen population inhabiting the Antwerp Zoo is not genetically impoverished compared to the ten times larger population at Planckendael. Firstly, the study of microsatellite variability at nine loci revealed that the Zoo population does not suffer a decreased genetic variability. There were no significant differences in heterozygosity

levels nor in the proportion of polymorphic loci nor in mean number of different alleles between the two populations studied. For both populations, inbreeding coefficients (F_{IS}) were too small to regard the populations as being inbred. This also suggests that there is no unusually high level of relatedness among moorhens in either of the populations. In addition, mean band-sharing coefficients, calculated from the DNA-fingerprints, did not differ significantly between the Zoo and Planckendael populations. This indicates that moorhens at the Zoo are not móre related to each other than at Planckendael. We found similar mean band-sharing coefficients to those reported for unrelated animals in other studies (European starling *Sturnus vulgaris*: 0.21 (PINXTEN *et al.*, 1994); indigo bunting *Passerina cyanea*: 0.23 (WESTNEAT 1990); blue tit *Parus caeruleus*: ± 0.20 (KEMPENAERS *et al.*, 1992); moorhen: 0.29 (MCRAE & BURKE, 1996)), whereas close relatives have mean coefficients of around 0.60 in the indigo bunting (WESTNEAT 1990) and the blue tit (KEMPENAERS *et al.*, 1992). Thus our data indicate that the small moorhen population inhabiting the Antwerp Zoo is probably not isolated from other moorhen populations, as this would have resulted in a higher band-sharing and inbreeding coefficient.

We found a significant overall genetic population differentiation, which suggests that there is little gene flow between the Zoo and Planckendael populations. When considering the loci separately, the population differentiation was significant for seven of the nine loci; only Tm36 and Tm101 would have concealed differentiation. Nm equalled 2.71, which indicates that the observed genetic differentiation can be maintained when two to three individuals migrate between the study areas each generation. However, one should be aware of the fact that Nm is only an indirect assessment of the degree of gene flow, and should assume that an equilibrium exists, that the alleles considered are neutral in terms of selection, and that the migration is equal in both directions (NEIGEL, 1996). Because DNA was collected at Planckendael up to three years earlier than at the Zoo, we cannot exclude completely that the differentiation we found is in fact a temporal differentiation and that there was no genetic differentiation at the time we sampled moorhens at the Zoo. This would imply that the genetic composition of both populations altered during the time interval of our study, for instance as a consequence of a bottle-neck due to bad weather conditions. This is an unlikely scenario, however, because moorhen density at Planckendael remained high at all times. Moreover, we only used adults in our analysis, and at least some of the adults ringed in 1992 were still alive in 1997.

The fact that we found no signs of isolation even though the populations were genetically differentiated is probably due to an « isolation by distance » effect where the Zoo and Planckendael are reproductively isolated from each other due to geographical distance (WRIGHT, 1943), while migration of moorhens occurs between the Antwerp Zoo and the immediate surroundings. The distance between the Zoo and Planckendael is about 30 kilometres, which is rather large, given that the moorhens in both populations are considered to be mainly resident (MERCKX, 1993; VAN DUYSE, 1997).

A disadvantage of microsatellites is that identifying appropriate regions from a genomic library for a new species can be time-consuming (PARKER *et al.*, 1998). Known primers are not expected to amplify the same locus across related taxa unless the microsatellite region is flanked by highly conserved sequences where priming sites are located (ELLEGREN, 1992). Recent work, however, suggests that this may occur more often

than originally thought (PRIMMER *et al.*, 1996; PARKER *et al.*, 1998). Therefore, we began our study on moorhens by trying primers that had been developed for a closely related *Gallinula* species, namely the Tasmanian native hen. In agreement with several recent studies (DEKA *et al.*, 1994; PRIMMER *et al.*, 1996), the primers originally developed for Tasmanian native hens also worked for moorhens. As far as we know, our study reports the first application of microsatellite analysis in moorhens.

It has been recommended that one should be careful in interpreting data obtained from molecular markers (AVISE, 1994). Sometimes the results depend on the markers used, for example when different parts of the genome experience different selection pressures. Nevertheless, we believe that our results are reliable. Firstly, we combined two different molecular techniques, both revealing the same main result, i.e. that the Zoo population does not seem to be genetically impoverished. HAIG (1998) recently emphasised that studies that use several molecular techniques are likely to provide a more definitive assessment than those that use only one technique. Secondly, because minisatellite and microsatellite regions are non-coding regions of DNA, they are generally considered to be good, neutral mendelian markers (JEFFREYS *et al.*, 1985a,b; JARNE & LAGODA, 1996). Thirdly, our microsatellite analysis revealed significant linkage in only one pair of loci at the Zoo and in two pairwise combinations at Planckendael. These three combinations represent only 4.2% of the total of 72 combinations. Therefore, it is likely that we looked at loci that are representative of the entire genome. However, we cannot exclude that the Zoo population of moorhens is isolated to some extent. It could for instance be that the reference population at Planckendael is isolated and that the levels of genetic diversity we found may be typical of « isolated » moorhen populations in both populations. Though we have no proof, we presume, however, that the Planckendael population is not isolated because of its situation in a semi-rural setting, surrounded with plenty of suitable habitat for moorhens. It is also possible that the Zoo has become isolated only recently and that the effects are not yet noticeable. Nevertheless, this seems unlikely because urbanisation has been going on for over a century. Although more data are needed, we tentatively conclude that the surrounding city probably does not serve as a significant barrier for moorhens.

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POSSIBILITIES AND LIMITATIONS OF THE USE OF ARCHAEOZOOLOGICAL DATA IN BIOGEOGRAPHICAL ANALYSIS : A REVIEW WITH EXAMPLES FROM THE BENELUX REGION

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Abstract. Archaeozoology is the study of interactions between man and other animals through time, by the analysis of animal remains and traces found during archaeological excavations. Gradually, it has also been recognised by the « mainstream » zoological world that archaeozoological data can provide insight into the evolution or regional variation of the Late Pleistocene and Holocene faunas of the Low Countries, just as palaeontological research does for older periods. The wide scope of recent archaeozoological research is, however, often not fully recognised, not only in terms of taxonomic diversity, but also with regard to the detail of information gathered. This review highlights these interpretative possibilities, but also outlines the possible limitations of archaeozoological datasets. These limitations are linked with the characteristics of the sites investigated, *i.e.* the conditions affecting preservation, the formation processes, the cultural framework, or the origin of the deposits. The dataset is also biased by archaeological methodology, particularly in relation to the sampling and recovery of organic remains. Furthermore, the information gathered will differ strongly between taxa, according to their taphonomic status, their place within different human cultures, the chances of fossilisation of their remains, and eventual identification problems. When the limiting factors described are not known or not well understood, the use of archaeozoological data in faunal reconstructions may lead to false conclusions.

Key words : archaeozoology, biogeography, biodiversity, introduction, extinction.

INTRODUCTION : THE BIODIVERSITY OF THE PAST

The past is an important dimension to an holistic approach to the environment (MACINNES & WICKHAM-JONES, 1992). It is indeed impossible to understand and discuss the composition of the fauna in a certain geographical area without any data on its evolution. A diachronic dimension is even more important when faunas are managed, *e.g.*, when the reintroduction of wild species is considered or when management strategies for natural reserves are discussed, involving the herding of domestic grazers in semi-confinement. When, for example, a discussion arose in the Netherlands about the introduction of « wild sheep » into the wild fauna of some natural reserves, supporters of this idea put forward the argument that this would only be a reintroduction, because « wild sheep » had occurred

in Europe during the Pleistocene and some populations were still surviving on Corsica and Sardinia. The subsequent proposal was to introduce animals from this Mediterranean stock into the Low Countries. Thanks to research on the evolution of wild and domestic sheep, critics of the idea could, however, explain that the Pleistocene sheep of Europe was in fact a different species (*Ovis savinii*) than the one still surviving on Corsica and Sardinia (*Ovis ammon*). Moreover, the Mediterranean « wild sheep » represent no more than feral populations of domestic sheep (*Ovis ammon* f. aries) that were introduced to the islands, from the Near East, in Neolithic times (VAN WIJNGAARDEN-BAKKER, 1991).

Data on the composition of former faunas can be gathered in many ways. Most well known is the contribution of palaeontology, generally involving the study of fossil remains and traces of former organisms. In principle, palaeontology studies material from all time periods, from the Precambrian to recent times. However, in the mind of the general public (and of scientists not familiar with the field), palaeontology often deals only with « old » material, dating from the Pleistocene or before, and is therefore considered to be of little importance for the study of recent faunal evolution. This common misunderstanding mostly originates because « younger » (*i.e.*, Holocene) sites are generally excavated by archaeologists and not by palaeontologists. Consequently, the study of organic remains from these sites is not labeled « palaeontology » but « archaeozoology », or « archaeobotany ». Both archaeozoology and archaeobotany are, however, no more than palaeontological disciplines with a specific character, because humans were the conscious or unconscious taphonomic agents responsible for the accumulation of the organic remains found at excavated sites.

During the last decades, it has gradually been recognised by the « mainstream » zoological world that archaeozoology – here defined as the investigation, through the analysis of animal remains and traces found at archaeological excavations, of the interaction between man and animal through time – can provide insight into the evolution or regional variation of our recent faunas (see BENECKE, in press, for a recent review for European faunas). This paper presents a survey of the difficulties that can arise when archaeozoological data are used without caution. A student of biogeography cannot safely incorporate archaeozoology into any considerations without knowing the possibilities and limitations of the field.

POSSIBILITIES OF ARCHAEOZOOLOGICAL ANALYSIS

In its pioneering years, archaeozoology mainly focused upon the history of large mammals. Most of the material recovered from archaeological excavations came indeed from large domestic animals (cattle, sheep, pigs) in the case of Neolithic or younger sites, or from large game in the case of Palaeolithic or Mesolithic sites. Gradually, however, the remains of smaller animals, including invertebrates, were incorporated into archaeozoological analyses. This evolution became possible by the use of refined sampling and recovery methods on sites, and by the gradual accumulation of experience with the treatment, identification and interpretation of small archaeological organic remains. Nowadays, archaeozoological research covers a wide scope of organisms, of

which the presence or absence at archaeological sites can be evaluated. Remains of invertebrates from the famous early medieval Coppergate site at York (UK) included specimens belonging to sponges, nematodes, annelids, molluscs, echinoderms, crustaceans, chilopods, diplopods, and a wide variety of insect orders (KENWARD & HALL, 1995). During the last decade, mites (Acari) have also become increasingly popular as ecological indicators for archaeological sites (SCHELVIS, 1992) (Fig. 1). Recently, it has been demonstrated that even the remains of testate amoebae can be found in archaeological contexts (BOBROV, 1998).

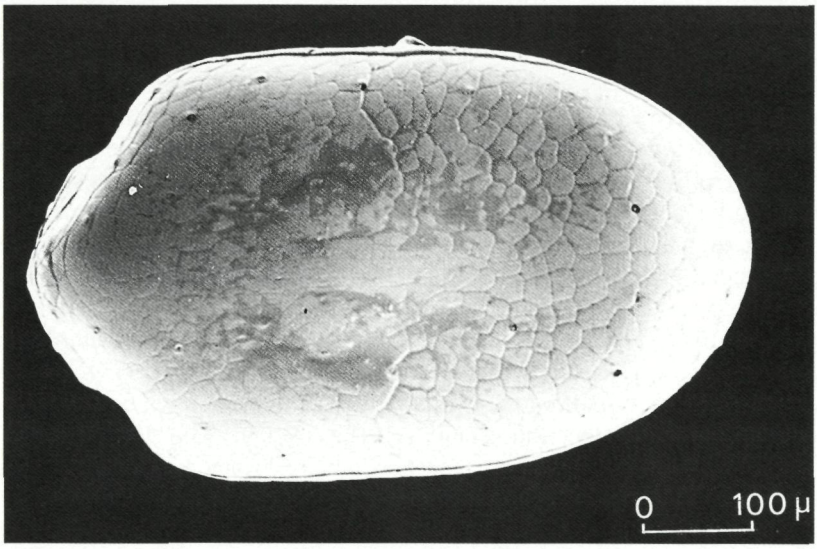

Fig. 1. – Exoskeleton of a mite belonging to the Gamasida, recovered from a medieval site at Oudenburg (B.) (from SCHELVIS & ERVYNCK, 1993).

The species lists from archaeozoological contexts give information about the composition of former faunas. The assemblages studied are dated by the analysis of archaeological artefacts found in the same contexts, their stratigraphic provenance, historical information, and, occasionally, by physico-chemical methods. The use of the latter technology is often not needed, especially when, as is the case for the Roman period, it is often possible to date faunal collections within a time interval of 20 to 25 years, on the basis of their archaeological context alone (*e.g.*, ERVYNCK & VANDERHOEVEN, 1997). In most cases, archaeozoological collections can also be placed into a taphonomic context, meaning that it is often clear why remains ended up at a human dwelling place, and what agents were responsible for their accumulation and preservation. From the associated archaeological and historical data, archaeozoological finds can be placed in a socio-economic context, revealing, for example, whether the material was deposited in a rich site with many long-distance contacts, or in a poor, rural household depending upon a subsistence economy. Finally, pedological, geomorphological and climatological information, together with associated plant remains, allow situating the animal remains in their former environment.

It would be a mistake to assume that archaeozoological information about past faunas is only needed for periods or cultures without written archives. Generally, historians (and biologists working with historical data) underestimate the potential of information that can be extracted from archaeological research and how fruitful can be the critical confrontation between written and excavated sources. As to the consumption of animal food products, for example, it has been amply demonstrated that, even for a period with a rich historical documentation such as the Late Middle Ages, written records alone are not sufficient to allow a more or less reliable reconstruction (ERVYNCK *et al.*, 1996; ERVYNCK & VAN NEER, 1998). When reviewing the historical records for faunal biogeographical information, it becomes clear that archaeozoology is the only reliable source of information about past faunas for most of the period that we call « history ». For his history of birds in Belgium, the oldest reliable scientific source that DESMET (1987) could use was the 19th century « Faune belge » by DE SELYS-LONGCHAMPS (1842), whilst archaeological records of birds cover the whole of the Holocene. The danger exists that, when only 19th and 20th century scientific data are used in historical biogeography, the impression arises that our 19th century fauna represents the original or 'natural' one, except for some extinctions of larger species, which are usually situated in early prehistory. It is therefore often surprising for students of the fauna of the Low Countries to learn from archaeozoological analyses that Dalmatian pelican (*Pelecanus crispus*) was still present around 2400 BC (CLASON *et al.*, 1979), that aurochs (*Bos primigenius*), black vulture (*Aegypius monachus*) and great auk (*Pinguinus impennis*) survived into Roman times (DE GRAEVE, pers. comm.; VERHAGEN, 1991; VAN WIJNGAARDEN-BAKKER, 1978), and that brown bear (*Ursus arctos*) only became extinct after the 12th century AD (ERVYNCK, 1993a). In contrast, archaeozoology has proven that animal groups such as our freshwater fish fauna, of which the decline (and extinction) is commonly thought to have begun during the Industrial Period, already suffered significant diminishing population numbers during the Middle Ages (VAN NEER & ERVYNCK, 1993; 1994; in press). Finally, even for the biogeography of the most recent periods, for which real scientific data is supposedly available, archaeozoological data can be extremely meaningful. For example, the often quoted theory that the post-medieval decline of the black rat (*Rattus rattus*) in Northwestern Europe is the result of competition with the brown rat (*Rattus norvegicus*), which was introduced in the 18th century, is contradicted by the archaeological finds of late 19th to 20th century black rat nests in the center of Gent (ERVYNCK, 1990; ERVYNCK & BASTIAENS, in press).

On a global scale, archaeozoological analysis has already accumulated a vast *corpus* of information, since its beginnings in the 19th century. One major advantage lies in the virtually infinite amount of new material that is still hidden on archaeological sites and which may be available for study in the future. Moreover, previously studied collections always yield new information through methodological developments, and represent an archive of which the potential has not yet been fully explored. Archaeozoology has not only constantly made progress in terms of the taxonomic diversity of the material studied, but has also revealed ever-more-detailed information, gathered from excavated remains. Archaeozoological research yields more than mere species lists. It is now possible, from the skeletal elements of vertebrates, to deduce morphological characteristics such as body size or the domestication status of former animals, their growth rate, the distribution of age at death of past populations, their sex ratio, prevailing pathologies, aspects of feeding con-

ditions, etc. (see DAVIS, 1987 or CHAIX & MENIEL, 1996 for a general review). One of the most important recent innovations is the extraction of biomolecular information from ancient bone, but methodological problems still occur (GÖTHERSTRÖM & LIDEN, 1998).

LIMITATIONS IN THE USE OF ARCHAEOZOOLOGICAL DATA

When archaeozoological data are used in biogeographical studies, the simplest question asked is often whether the species list from a site provides information about the presence or absence of a certain animal species in a certain period, in the area around the site. When dealing with this question, many of the limitations of the archaeozoological dataset become apparent. They can be discussed in a logical order, following the chain of events from the presence of an animal at a former human dwelling place to the analysis of its remains by an archaeozoologist (Fig. 2).

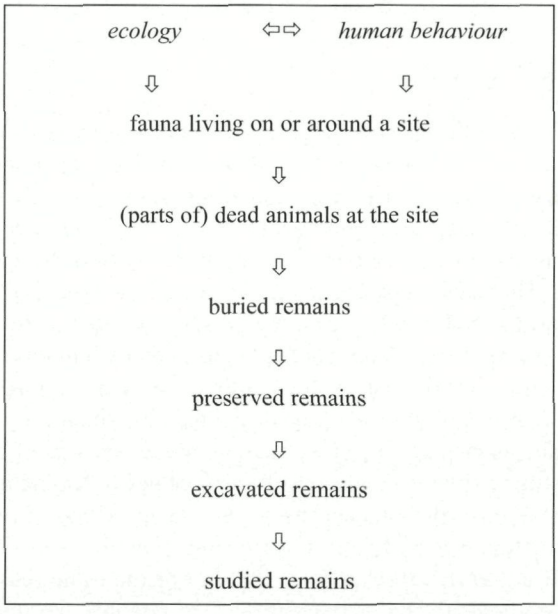

Fig. 2. – Schematic diagram illustrating the events from the death of an animal and the archaeozoological analysis of its remains (modified after DAVIS, 1987).

Nature of the sites investigated

The composition of the wild fauna that lived in the natural and cultural environment of a former site and its surroundings is the result of ecological conditions and human behaviour (land use, creation of artificial environments, hunting pressure, introduction of domesticates, etc.). Through the analysis of the archaeozoological finds from a site, it is

possible to reconstruct the past fauna, but what is often not fully recognised is the extent to which an archaeozoological study collection represents but a heavily biased sample from the former fauna. First of all, human behaviour has dictated what animals were originally brought to the site, whilst the structure of the site itself influenced which commensal species or parasites could live in or around it. Therefore, even contemporaneous sites located within the same landscape can sometimes yield very different faunas. The commensal fauna of a food storage site will be different from that of a ceremonial place. Within a cultural period characterised by socio-economic differentiation, certain animal species are more likely to be found at high status sites compared to low status ones. The privileges of the feudal nobility on game species such as red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*) and brown bear (*Ursus arctos*) (SMIT, 1911) are responsible for the fact that medieval remains of these species are only found at castles and not at rural sites (ERVYNCK, 1992). Since, in late medieval abbeys, a larger quantity and a wider variety of fish were consumed compared to contemporaneous urban households (ERVYNCK, 1997), the investigation of the first group of sites is more appropriate in revealing changes in the freshwater fauna.

Taphonomic groups

One further reason that archaeozoological collections are not a random sample of a former fauna relates to why animals were killed and why their carcasses (or parts of them) were brought to a site. Animals could be killed because they served as food, because a primary material suitable for artefact production (antler, horn, fur, *etc.*) could be gained from them, because of ritual motives, or because they were considered a nuisance and were therefore destroyed. These different reasons for killing define different taphonomic groups (GAUTIER, 1987) and inevitably influenced the presence of animal remains on a site and their preservation. The remains of animals that were eaten by humans usually ended up in the consumption refuse contexts that are frequently excavated at archaeological sites. The remains of species that were only killed, or of animals of which only certain body parts were brought to a site as primary material, have a reduced chance of being found during excavation. This pattern explains the dearth of archaeological data hampering a documentation of the population histories of such species as beaver (*Castor fiber*) and wolf (*Canis lupus*), species which disappeared from the Belgian fauna only in post-medieval times (FRECHKOP, 1958; TACK *et al.*, 1993; CRIEL, 1994). For the same reasons, there are also insufficient archaeozoological data to describe the demographic evolution of, *e.g.*, wild cat (*Felis silvestris*), badger (*Meles meles*), fox (*Vulpes vulpes*), and the smaller carnivores, which still survive in Belgium. In medieval times, these species were exterminated, or killed for their fur, but their carcasses were not often brought to a site for consumption. This explains the scarcity of their remains from archaeological sites of that period. In contrast, brown bear (*Ursus arctos*) was eaten after being hunted (ERVYNCK, 1993a), and therefore the history of this mammal is much better documented.

Animals of which the remains can be found at archaeological sites, but that ended up there without the knowledge or intent of man, are termed « intrusives » (GAUTIER, 1987). Mostly, this taphonomic group consists of small species belonging to the commensal or parasitic fauna present at a site, or to the wild fauna living close to it. These animals (gas-

tropods, insects, arthropods, amphibians, reptiles, small mammals) are mostly found in special contexts that acted as pitfalls, such as wells and cesspits, or at places where prey remains have accumulated through the actions of non-human taphonomic agents such as owls. Such contexts, however, are not present at all sites, have not yet been excavated in sufficient numbers and have often been inadequately sampled (see further). For these reasons, few archaeozoological data have yet been gathered on the natural history of the intrusive fauna.

Preservation

After animal remains arrive at a site, it is necessary for their preservation that they rapidly become incorporated into an archaeological context. Animal skeletal elements that are deposited on the surface are easily destroyed by activities of scavengers, trampling, weathering, etc. The structures present at a site, used for the deposition of consumption refuse, thus partly determine the chances of preservation for animal remains. This explains why in Belgium, animal bones are rare at Iron Age sites (ERVYNCK, 1994) but are very frequent at Roman sites. The latter group is generally characterised by complex buildings and large infrastructures (sewers, cesspits, etc.), whilst the Iron Age sites are less complex and more rural in nature. Without doubt, the systems of garbage disposal will have been totally different between both types of habitation.

The taphonomic characteristics of groups of archaeozoological material are also of major importance for their preservation. Consumption refuse will generally consist of isolated skeletal elements that are often severely fragmented. Conversely, animals that have not been eaten may leave complete skeletons to be found, with individual elements still in anatomical position. The same is sometimes true for intrusives that have been caught in a pitfall, although the small intrusive animals that have been consumed by a predator living in or close to a human dwelling place are often only represented by disarticulated, fragmented and poorly preserved skeletal elements.

Besides the taphonomic characteristics of the contexts and material investigated, other, general factors also influence the preservation of animal remains. Certain soil types are disadvantageous for the preservation of chitin, shell and bone. The sandy soils of parts of Flanders and of the Campine area are well-drained, causing an alternation of wet and dry conditions that is destructive for buried animal remains. Local factors damaging for potential archaeozoological finds are bioturbation, soil erosion, and a wide range of human interventions. Lowering of the ground water table, for example, has been proven to be harmful for the preservation of the buried organic archaeological heritage.

Of course, the characteristics of the animal remains themselves are largely responsible for their preservation or destruction. As a general rule, the larger skeletal elements (of large species) always have a better chance to survive destruction than the smaller skeletal elements (of small species) (see LYMAN, 1994 for a review). Regardless of size, however, the structure of the animal remains is also determinative. Within the fishes, for example, remains of species with cartilaginous skeletons have significantly less chance of being found at archaeological sites than species with bony skeletons. Even within the latter group, however, some species such as mackerel (*Scomber scombrus*) or salmonids (*Salmo*

sp.) store lipids in cavities within their skeletal elements (Fig. 3), a factor that determines the poor survival of remains of these fish at archaeological sites. It is believed that in the soil the lipids turn into lipid acids, which dissolve the mineral fraction of the surrounding bone and influence the denaturation of collagen (MÉZES & BARTOSIEWICZ, 1994).

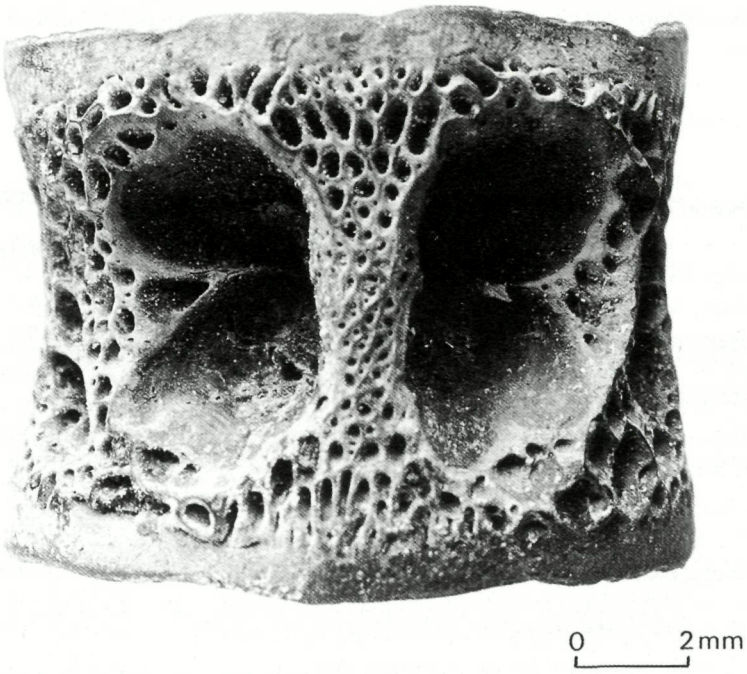

Fig. 3. – Vertebra of a salmon (*Salmo salar*), showing the porous structure where the storage of lipids is located (from VAN NEER & ERVYNCK, 1993).

Finally, it is sometimes hard to distinguish whether certain groups of animal remains are rare because of preservation conditions or of other causes. In medieval sites from the Low Countries, *e.g.*, bird remains are always rather scarce but it is often not clear whether this pattern is the result of the poor preservation chances of friable bird bones, or because birds were expensive food items during the period considered (ERVYNCK, 1993b).

Sampling and recovery

An archaeozoological dataset is always biased by archaeological methodology, especially when considering the sampling and recovery of organic remains. During excavations, most animal remains are simply handcollected, although it has long been proven that this method is an inadequate way to recover the remains of many small species (most invertebrates, smaller fish, amphibians, reptiles). Moreover, within the group of species recovered, handcollecting favours the recovery of the largest elements. More refined sampling methods are therefore needed to

adjust for this bias. During excavations by the Institute for the Archaeological Heritage of the Flemish Community, suitable contexts are sampled (at least 10 l of sediment) and sieved on a 0.5 mm mesh, which is sufficient to recover most molluscs, carabid beetles, fish and other small vertebrates. Moreover, the contents of deposits containing interesting archaeozoological assemblages are sometimes completely sieved. Additionally, smaller samples are taken that can be flotated in order to recover microscopic remains such as parasite eggs or exoskeletons of mites (see SCHELVIS & ERVYNCK, 1992). When reviewing older excavation reports, however, one must bear in mind that the refined sampling and recovery of animal remains only became a standard procedure at Flemish sites after 1985.

Analysis of the remains

Once animal remains are recovered from an archaeological site, they must be analysed, a process where identification is the first step. However, the identification of archaeological animal remains differs significantly from the identification of recent animals. Most identification keys for recent specimens are not applicable to archaeozoological material, and it is therefore not surprising that identification to species level is not always possible. In some cases, identification problems hamper the study of a whole animal group (*e.g.*, land slugs, small passerines). In other cases only the discrimination between a small number of species poses problems (*e.g.*, sheep and goat, flatfish species within the Pleuronectidae). Moreover, these identification problems differ between skeletal elements (for the vertebrates) and depend upon the preservation condition or the taphonomic status of the collection.

Identification possibilities of course depend upon specialist experience and the availability of reference collections. Moreover, there exists a marked discrepancy between the number of species within each animal phylum and the number of archaeozoologists working on these groups (Fig. 4) (SCHELVIS, 1993). Therefore, in many countries, several cate-

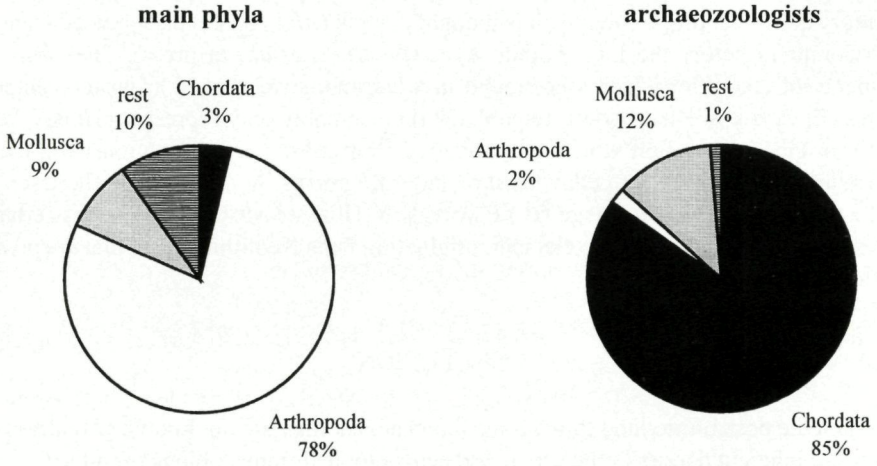

Fig. 4. – Discrepancy between the number of species within each phylum of the animal kingdom and the number of archaeozoologists working on these groups (after SCHELVIS, 1993).

gories of archaeozoological remains are simply not studied because of a lack of expertise. Moreover, the impetus to work on certain animal groups is influenced by the cultural questions asked. Groups important for human food economy have consequently gained more attention than groups than can only be used as ecological indicators. Furthermore, even within the group of ecological indicators, the characteristics of some groups (*e.g.*, carabid beetles, see ERVYNCK *et al.*, 1994) make them more reliable for interpretative purposes, which explains why they are preferentially studied.

Absence and presence

The arguments listed demonstrate that the absence of a particular species from the archaeozoological inventory of a site must always be evaluated against information on the nature of that site, the cultural period studied, the taphonomic status of the material, the preservation conditions at the site and of the species considered, the sampling and recovery methodology applied, and the specialist expertise available. All this explains why the absence of a particular species from an archaeozoological inventory does not necessarily imply that the species was absent from a former fauna.

When the presence of a species' remains is established by archaeozoological analysis, this is also not always straightforward proof of the former occurrence of the organism around the site studied. Animal remains can belong to the taphonomic groups of the so-called « reworked » and « late » intrusives, meaning that they originate from deposits that are older than the context in which they eventually ended up, or that they only became part of an archaeological context some time after that context was deposited. An example of the latter group are the remains of burrowing animals, that, after their death, became incorporated in the older archaeological layers or deposits which they were disturbing.

An additional problem is presented when animals or parts of animals are transported over long distances, in which case the presence of their remains in an archaeozoological inventory is misleading. Generally, it is thought that elk (*Alces alces*) disappeared from the Low Countries before the Late Middle Ages (ERVYNCK *et al.*, in press). Therefore, the fragments of elk antler which were found in a late medieval context from the center of Bruges (ERVYNCK & HILLEWAERT, unpubl. data) presumably only represent primary material for artefact production which was imported from more northern regions. In fact, all Roman and medieval finds of elk consist of antler fragments. A long bone of elk described from a medieval context in Liège (B.) (GAUTIER & HOFFSUMMER, 1988) almost certainly represents a reworked, intrusive element, originating from Neolithic layers that are present beneath the medieval deposits.

CONCLUSION

When the possibilities and limitations of archaeozoology are not known or understood, there is an inherent danger in the use of archaeozoological finds in biogeographical reconstructions. The possibility to follow a species' history through archaeozoological research differs markedly according to its status in former times (*e.g.*, protected game versus species on the extermination list), the taphonomic context in which the remains can be

found (*e.g.*, consumption refuse versus intrusive remains), and the socio-economic context of the sites investigated (*e.g.*, high status versus low status sites).

The previous discussion has illustrated what conditions must be met before archaeozoological data on the presence or absence of species can be safely interpreted. However, the archaeological context in which animal remains are found is always the most critical parameter for the significance of a faunal assemblage. Archaeozoology studies the interaction between man and animals, and not only the presence or absence of species, and is therefore a truly interdisciplinary field of science. When biogeography uses archaeozoological data, it should therefore take the same approach. The history of our fauna can only be understood when, more than extinction or introduction, processes such as pollution, destruction of habitats, synanthropisation of animals, domestication or scientific manipulation of organisms are studied (SIMMONS, 1979). Such analyses, however, are only possible when data from history, archaeology and ecology are fully integrated.

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BEETLE DIVERSITY AND HISTORICAL ECOLOGY OF WOODLANDS IN FLANDERS

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Abstract. Extensive data on the present-day carabid beetle fauna occurring in woods of Flanders were compared with a unique data-set of archaeological carabid remains from a Late and Post-Roman forest at Velzeke (Eastern Flanders), within the framework of a study on the historical ecology of woodlands in Flanders. Integration of these data on 14 woods revealed that most have been significantly impoverished with respect to their stenotopic woodland beetle fauna. The carabid species' diversity is higher in several small and relatively recent woodlands compared to that in larger ancient forests, regardless of whether this diversity is evaluated by rarefaction or by mean species richness per standardised year sample data. This pattern is primarily caused by the presence, in forest fragments, of many species from surrounding open habitats. Typical woodland beetles show a reduced dispersal power (constant brachyptery) and appear to be strongly linked to larger ancient woods. Knowledge of historical ecological factors, other than actual size of the forests, further aids the explanation of the observed ground beetle assemblages in the specific forests or sites. Preliminary results of population genetics, for the eurytopic forest carabid beetle *Abax ater*, showed significant genetic differentiation between populations (due to reduced gene flow) at a relatively large spatial scale, although genetic erosion cannot (yet?) be observed for this species.

Key words : woodland, biodiversity, historical ecology, deforestation events, woodland area, wood exploitation, archaeology, habitat fragmentation, ground beetles (Carabidae), ancient woodland indicators, dispersal power, population genetics, *Abax ater*, region of Flanders

INTRODUCTION

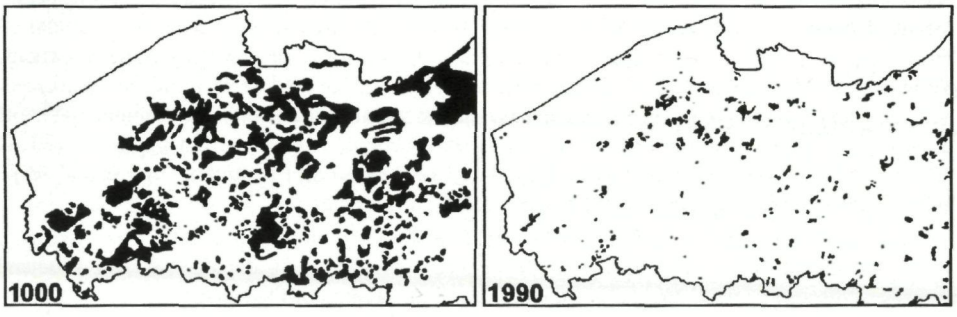
A short history of woodland in Flanders

It is a safe assumption that, at the beginning of the Holocene, there was more woodland in Flanders than there is now. However, the history of woodlands in Flanders cannot be described by a simple model of linear decline, but is characterised by periods of regression and expansion (BLOEMERS & VAN DORP, 1991 ; TACK *et al.*, 1993 ; TACK & HERMY, 1998). A first regression period started with the introduction of Neolithic cultures into our regions. At that moment, two processes initiated the decline of the original forest, *i.e.*,

deforestation due to woodland being turned into fields, and degradation due to areas being used as grazing grounds for large herds of domestic animals. The former process had a particularly severe impact during that period, and this is demonstrated by the finds of Neolithic Michelsberg sites (BLOEMERS & VAN DORP, 1991) in areas that are now under forest and which, as is suggested by the absence of younger sites, were never inhabited again.

How woodland evolved during the Bronze and Iron Ages is still largely unknown, but it can be assumed that the processes of forest degradation and disappearance continued, reaching a peak during the Roman period, as a result of large scale agricultural exploitation. After the fall of the Roman Empire, political instability caused a reduction of the human population and a decline of agricultural exploitation. Therefore, during the Early Medieval period, woodland recovered to a certain extent. During the High Middle Ages, however, renewed deforestation took place (VERHULST, 1990), in many cases precisely in those areas that had become woodland again during Early Medieval times.

From the end of the 13th century onwards, gradual deforestation was occasionally interrupted by renewed cultivation of woodland, mostly for economic purposes, such as the demand for firewood. During the 19th century, a final large scale deforestation took place in Flanders. As a result, woodlands in Flanders nowadays are extremely fragmented and/or reduced in size (compare Fig. 1 around 1000 AD, before the extensive Late Medieval deforestation, and Fig. 2 based on a recent map for Western and Eastern Flanders).



Figs 1-2. – Fragmentation in woodlands of Western and Eastern Flanders: Fig.1: situation around 1000 AD, before extensive Medieval deforestation (after TACK *et al.*, 1993, modified); Fig. 2: based on a recent map.

Carabid beetles from Flemish woodlands

Within the framework of several projects, including a long-term insect monitoring study, quantitative data on the carabid beetle fauna from many Flemish woodlands have recently been accumulated. When ecological data about carabids are gathered through actual sampling and collecting in the field, or through the study of museum collections,

the time-span covered is generally restricted to the last two centuries. Through archaeological analysis, however, it is possible to gain zoogeographic and ecological information about insect communities from much older periods. At some archaeological sites, specific man-made structures, such as wells, ditches or cesspits, have acted as pitfalls in the past, and have given rise to an accumulation of insect remains. These assemblages can be dated by the association of artefacts that are found in stratigraphic connection with them, or by physico-chemical dating techniques. The importance of the archaeological samples lies in the fact that they can originate from biotopes or environments that have disappeared today. Abundant archaeological carabid remains of a Late and Post-Roman forest at Velzeke (Eastern Flanders) have revealed a unique example of a woodland faunal composition around 500 AD (DESENDER *et al.*, unpubl.). Besides the investigation of recent and archaeological woodland carabid faunas, an independent study has been performed on the historical ecology of woodlands in Flanders (see TACK *et al.*, 1993). This study included aspects of fragmentation and site history, such as changes in area, de- or reforestation and forest exploitation.

In this paper, an attempt is made to integrate data from 14 Flemish woods, in order to analyse the current ground beetle diversity and faunal quality (values for conservation), within the framework of the historical ecology of these woods, and to compare them with faunal assemblages of Late and Post-Roman date from the same region.

Recently, population genetic studies on selected species of woodland ground beetles were undertaken, in order to evaluate the role of historical and present-day ecology and population characteristics in the observed genetic differentiation and diversity. Such studies may throw light on the mechanisms (apart from habitat quality decline or the loss of suitable habitat *per se*) responsible for the loss of typical species during woodland fragmentation, *e.g.*, by genetic erosion or reduced gene flow. Eventually, this may lead to an increased understanding of the actual conservation values of Flemish woodlands and suggest remedies for future woodland rehabilitation. In the present report, only preliminary population genetic results for the eurytopic forest carabid species *Abax ater* are summarised.

MATERIAL AND METHODS

Study sites

Fig. 3 shows the geographical locations of the 14 Flemish woods, from which faunal data have been used in the present paper. These locations have been superimposed on a recent map locating the woodlands in the region (see also Table 1). Two of the woods investigated are situated at the border of the Flemish region and continue into adjacent regions, i.e., the « Zoniënwoud » (located on the territories of Flanders, Brussels and Wallonia), and the Flemish « Bos Ter Rijst » at Edingen (which continues on Wallonian territory where it is called « Bois du Strihoux »). Obviously, most of the studied woods are situated relatively close to each other (except for the two woods at Wijnendale, cf. nrs 1 and 8 on Fig. 3). The forests are predominantly located on loamy soils, a higher sandy soil component being present only in most parts of the « Meerdaalwoud » (Fig. 3, nr 12), and at Wijnendale (Fig. 3, nrs 1 and 8). The archaeological site (Fig. 3, nr 14) is situated near

the centre of the entire study area and very close to about half of the studied woods. The central location of the archaeological study site ensures a more straightforward comparison and evaluation of historical ecological influences (see further). At present, woodland no longer exists on the exact location of this site, and there is some discussion as to how large the wood might have been at the time of the accumulation of the beetle remains (see further and DESENDER *et al.*, unpubl.).

Although, by now, data have been accumulated on the occurrence of ground beetles in many of the other woodlands of Flanders, i.e., from the coastal region (mostly relatively recent plantations on sandy soil) and from the Campine region (mostly pine woods on poor sandy soil), these have been deliberately excluded from the present analysis. Indeed, because of the different ecological conditions in these woods, comparison with the archaeological woodland samples and other (deciduous) forests situated on loam or sandy loam soil is difficult.

TABLE 1

Characterisation of study areas, according to deforestation events (A, B, C: see text), size class (S: small, M: medium-sized, L: large and XL: «extra large» (see text)) and exploitation history (DS: disturbed soil, US: mostly undisturbed soil); added numbers as used in Fig. 3

Woodland study areas	*nr*	*deforestation*	*size class*	*exploitation*
Wijnendale (satellite forest patch)	1	A	S	DS
Parikebos	2	A	S	DS
Zegelsem - Burreken	3	B(C)	S	DS
Schorisse (Bos Ter Rijst)	4	B	M	DS
Bos t'Ename	5	B(A)(C)	M	DS
Neigembos	6	A	M	DS
Brakelbos	7	A	L	DS
Wijnendalebos	8	A(B)	L	DS
Edingen (Bos Ter Rijst-Bois du Strihoux)	9	A	L	DS
Kluisbos	10	A	L	DS
Walenbos	11	C(A)	S-L*	DS
Meerdaalwoud	12	A	L	DS
Zoniënwoud	13	A	XL	US
Velzeke (archaeological samples)	14			

* Walenbos was formerly small but has recently been expanded to a large woodland.

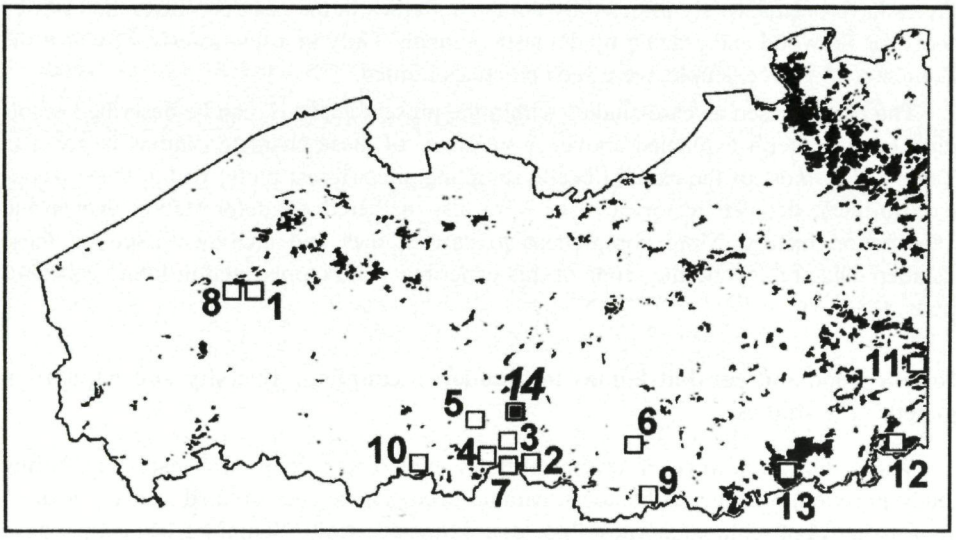


Fig. 3. – Location of the studied woods in Flanders; archaeological site at Velzeke (nr 14) labelled differently; numbers refer to the woods as mentioned in Table 1.

A historical approach to recent woods in Flanders

Considering their history, woodlands in Flanders can be described according to three basic factors: deforestation events, changes in dimensions resulting in actual size, and exploitation history. Considering the historical data that allow reconstruction of deforestation history in some detail, the maps by de Ferraris, drawn around 1775, are the oldest, more or less reliable source. Starting from these maps, present day woodlands have been subdivided into three categories: (A) woods that are drawn by de Ferraris, are still present today, and have known a continuous existence (« ancient forests »), (B) woods that are present on de Ferraris' maps, are extant today, but have not known a continuous existence in the intermediate period (« exploitation forests »), and (C) areas that show no woodland cover on de Ferraris' maps but are woodland today (« recent forests »).

Considering surface cover, four groups can be discriminated (see TACK *et al.*, 1993). The category of « small woods » comprises forests that at present cover less than 20 hectares (ha) and that covered less than 50 ha around 1775 AD, when the maps of de Ferraris were drawn. « Medium sized woods » cover at present 20 to 200 ha, and covered 50 to 500 ha around 1775. The group of « large woods » unifies forests that today cover more than 200 ha, and covered more than 500 ha at the end of the 18th century. Within the latter group, an exception must be made for the Zoniënwoud, which nowadays still covers an area of more than 4000 ha. Because of its extremely large size (according to Flemish standards), this forest must be placed in a fourth group.

The exploitation history of Flemish woodlands has been very diverse. Within the context of carabid (« ground beetle ») ecology, however, woodlands must mainly be subdivided according to former disturbances of the soil. In most of the Flemish forests, the soil

layers have been severely affected by human activities in the past, *e.g.* extraction of tree roots for firewood and grazing by domestic animals. Only in a few forests, of which the Zoniënwoud is an example, were such practices limited.

The forests, used as case-studies within the present analysis, can be described according to the criteria explained above. A summary of these characterisations is given in Table 1. For most of the carabid beetle sampling sites (forest plots) within these woods (see further), detailed historical data were also gathered on deforestation events and exploitation history. More comprehensive case studies and analyses based on these detailed data are beyond the scope of this paper but will be presented in future contributions.

Recent woodland carabid faunas in Flanders: sampling, diversity and population genetic case studies

Within the framework of several projects, including a long-term insect monitoring study in several habitats of Flanders, carabid beetles have been studied at many natural, semi-natural and cultivated sites since approximately 1980. Sampling campaigns have mostly been undertaken by means of at least one year cycle of pitfall trapping. Sampling involved at least 3 traps (glass jam jars, partly filled with a fixative, and with a diameter of approx. 10 cm) per site or micro-habitat (data from occasional year samplings with more than 3 traps per site were standardised by rarefaction to 3 sampling units). The traps were continuously in operation during a complete year cycle and emptied at fortnightly or three-weekly intervals.

Several of the larger or medium-sized woods, mentioned in Table 1 (*e.g.*, Zoniënwoud, Walenbos, Wijnendalebos and Bos t'Ename) have by now been sampled at some 10 to 20 different plots, sometimes during multiple year cycles (*e.g.*, DESENDER *et al.*, 1987; DESENDER & VANDEN BUSSCHE, in press). The data-set from most of the smaller woods by now includes replicate complete year cycle samples from at least 2 to 3 different sites, except for the extremely small satellite forest patch at Wijnendale (Fig. 3, nr 1), which could only be sampled as a single sampling station. Most of the year cycle sampling campaigns have been performed since 1985. Several of the woods were also recently sampled or resampled. As a result, today, the total data-set on the 13 woods from Table 1, has grown to around 100 site-year-samples and includes more than 60,000 ground beetles belonging to around 120 species.

All carabid beetles from the samples were identified to species level, counted and checked for their dispersal power (hind wing development and flight muscle development). Whether species are constantly brachypterous, macropterous or showing wing dimorphism or polymorphism, and to what degree they are able to disperse by flight, has been well documented in earlier papers (*e.g.*, DESENDER, 1989). Moreover, data from neighbouring regions and countries allow most species, recorded in Flemish woodlands, to be classified independently according to habitat preference (*e.g.*, ASSMANN, in press; BAGUETTE, 1993; LINDROTH, 1945; LUFF, 1998; THIELE, 1977; TURIN *et al.*, 1991). Detailed knowledge of geographical distribution and recent expansion or regression of individual species is available for Belgium (DESENDER, 1986a-d) and a documented Red

Data Book has recently been published for the region of Flanders (DESENDER *et al.*, 1995). For the purposes of the analysis described in this paper, a distinction was made between (1) stenotopic and (2) eurytopic woodland species, (3) ubiquists (also occurring in forest as in open landscape habitats), and (4) species from different types of open landscape habitats, mainly marshland, humid grasslands or cultivated fields.

Carabid diversity was assessed in three different ways: (1) total species richness per wood (obviously a biased diversity estimator due to an inevitably lower mean number of individual plot-year cycles in small to very small woods), (2) mean species diversity per sampling site (plot-year cycle) for a given wood and (3) rarefaction: calculation of the mean number of species for 100 individuals per sampling site, based on the actual number of individuals per species, for a given wood (cf. HECK *et al.*, 1975; HURLBERT, 1971; JAMES & RATHBUN, 1981). Habitat preference coding (see above) was then used for a more detailed comparison of the diversity observed and the data from historical ecology.

Preliminary population genetic data were gathered by cellulose acetate electrophoresis for the eurytopic woodland carabid beetle *Abax ater*, sampled from all 13 woods in this study (except for the small satellite forest patch at Wijnendale due to insufficient sample size). For each population, at least 40 beetles were analysed for 5 allozymes. More details on the technique and the statistical software used are given by HEBERT & BEATON (1989) and by DESENDER *et al.* (1998). Analysis of the preliminary data on *Abax ater* was restricted to a simple assessment of genetic diversity (mean number of alleles/locus, cf. BERG & HAMRICK, 1997) and of genetic differentiation between the study woods in relation to geographic location (isolation by distance?, reduced gene flow?). More extensive analyses based on these and additional data, and on other species, will be given in future publications.

Archaeological woodland fauna in Flanders: unique ground beetle data from a Roman well at Velzeke

In 1988, the Provincial Archaeological Museum of south-east Flanders excavated a stone well of Roman type at Velzeke (Eastern Flanders, Belgium), at the edge of a Roman site that flourished from the first to the third century AD (Van der Plaetsen, pers. comm.). The lower 3.5 m of the well's fill consisted of a deposit of organic debris that was subdivided in 11 sampling units and sieved on 0.5 mm meshes. The residues proved to be rich in zoological remains, i.e., bone, mollusc shells and the chitinous remains of insects. From the latter group, only the carabid remains were used in an attempt to reconstruct the former landscape around the well. Justification for this selection can be found elsewhere (ERVYNCK *et al.*, 1994), as well as a detailed account of the ground beetle results from the study (DESENDER *et al.*, unpubl.).

From each subunit, the remains of at least 100 carabids could be identified, yielding a total sample of more than 1100 ground beetles, belonging to 58 species. Most of the subunit samples (especially subunit 2-9) yielded a carabid faunal assemblage indicative of woodland habitat. They were dominated by stenotopic and eurytopic woodland species, implying that woodland surrounded the well at the time of deposition. Radiocarbon dating indicates the existence of this fauna around 500 AD, covering a time span of around 150

years (VAN STRYDONCK, pers. comm.). These carabid faunal data were compared to present-day data from the 13 woods previously described. Diversity was assessed in similar ways to that outlined above, but here, each subunit sample was considered a replicate sample for this wood in order to estimate mean values and associated standard errors.

RESULTS AND DISCUSSION

Carabid beetle diversity and historical ecology

Fig. 4 summarises the total carabid diversity from the 14 woods investigated, arranged in 4 size groups according to historical ecological characteristics. The archaeological data are also shown for comparison. Total species richness varies widely between circa 20 to nearly 70 carabid species per wood. Somewhat surprisingly, many large forests as well as the very large « Zoniënwoud » do not show a higher number of species compared to most of the small and medium-sized forests. A regression analysis of species richness on log(area) does indeed show that area is not a significant predictor of total diversity (r^2= 0.029, n.s.). An increased species richness nevertheless would be expected in these larger forests for two reasons. Firstly, large to very large woodlands have received a much higher sampling effort (number of separate sampling sites within one wood). The very large « Zoniënwoud » has, for example, by now been sampled already at more than 25 different sites, all included in this dataset. Secondly, larger woodlands would be expected to include a larger variety of micro-habitats, possibly increasing the total species richness of beetles. More recent woodlands, as well as those exploited relatively recently (*cf.* Fig. 4, asterisk-labelled bars), do not appear to show consistently lower or higher diversity compared to genuinely ancient forests.

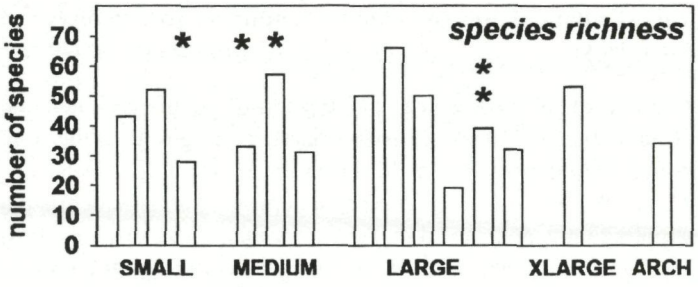


Fig. 4. – Total ground beetle species richness for the investigated woods (*= exploitation forests; **= recent forest; woods are ordered as in Table 1).

Values of much more straightforward and unbiased estimators for the comparison of beetle diversity between the woods are plotted in Fig. 5. These include: (A) mean total number of species per sampling series per wood and (B) mean expected number of species for 100 individuals calculated by rarefaction. Paradoxically, both estimators on average suggest higher diversity values in smaller sized forests. Weighted regressions of diversity on log(area) are highly significant and show a negative relationship based on both diversity estimators (*cf.* Fig. 7, A and B).

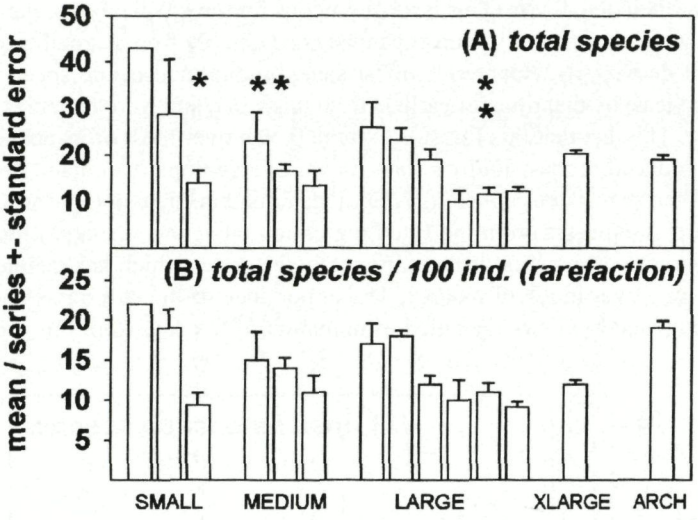


Fig. 5. – Carabid beetle diversity (measured per wood, woods ordered as in Table 1) as (A) mean total number of species per sampling series and (B) species per 100 ind., calculated by rarefaction ; ARCH= data from the archaeological samples, added for comparison ; *= exploitation forests ; **= recent forest.

Disentangling carabid diversity, according to habitat preference of the species involved, clearly shows that increased mean diversity in smaller-sized forests is caused by a pronouncedly higher number of open landscape carabid species (Fig. 6, A). This suggests severe edge effects from forest-surrounding open habitat types, increasing with decreasing forest patch size. A regression of open landscape species diversity on log(area) is indeed highly significant and again negative (Fig. 7, C). Other authors have described how the invertebrate fauna of fragmented woodland is more influenced by surrounding habitats than is the case for more contiguous forest (HALME & NIEMELÄ, 1993 ; MAELFAIT *et al.,* 1992). In some recent reviews (EHRLICH,1996 ; ZUIDEMA *et al.,* 1996), it has been suggested that small forest fragments are dominated by edge effects. Much more research is required on this topic, however, especially for invertebrates, since most research has been focused towards birds (EHRLICH,1996).

Whereas eurytopic woodland species or ubiquists (Fig. 6, B and C) do not show an obvious trend (nor any significant species-area relationships), an entirely opposite trend is shown for stenotopic woodland species (Fig. 6, D). Such species appear to be powerful indicators of larger ancient woods. Only the « Zoniënwoud » samples approached the high mean value of stenotopic woodland beetles found in the archaeological assemblages. A regression analysis of these diversity data on log(area) shows a significantly positive relationship (Fig. 7, D). A closer look at somewhat deviating points reveals a number of interesting and suggestive patterns. Without exception, woods, that have been temporarily heavily or partly exploited during the last 200 years, or that can be more or less classified as recent forest (differently-labelled on Fig. 6, D), are situated in the lower part of the plot,

irrespective of their actual size. This is most obvious for the « Walenbos », the single wood in this series which has recently been expanded considerably from a small forest fragment at the time of de Ferraris. Apparently, most stenotopic ground beetle species must have disappeared at least by that time (or earlier) from this wood and were not able to recolonise the area since. The fact that this forest is extremely wet over most of its actual area could have further reduced the possibilities for survival of stenotopic woodland beetles. In the upper part of the regression plot (Fig. 7, D), mean number of stenotopic woodland carabids is higher (as expected from the fitted regression on actual size area) for one wood : the « Zoniënwoud ». This wood is the only one in the series which has retained relatively undisturbed soils over much of its area. The importance of the soil disturbance factor is further substantiated by a more detailed examination of the data from this forest. Indeed,

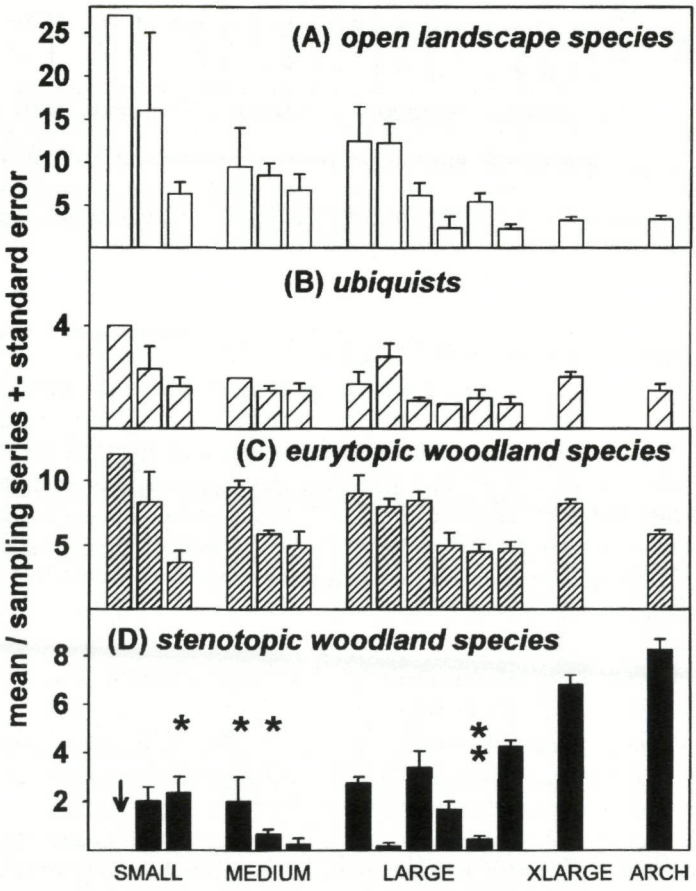


Fig. 6. – Carabid beetle diversity (measured per wood, woods ordered as in Table 1) as mean number of (A) species from open landscape habitats, (B) ubiquists, (C) eurytopic woodland species and (D) stenotopic woodland species ; ARCH= data from the archaeological samples, added for comparison ; *= exploitation forests ; **= recent forest.

some sample series from sites in the « Zoniënwoud », documented to be situated on soil, cultivated from the 14th until the 18th century, have yielded 3.00 ± 1.29 (95% c.i.) stenotopic woodland carabids compared to a significantly higher value of 7.78 ± 0.52 (95% c.i.) obtained for sites on more or less undisturbed soils. In a recent review of invertebrates and boreal forest management, NIEMELÄ (1997) similarly concluded that undisturbed old-growth forest must be set aside to sustain specialist species and to serve as sources for recolonisation.

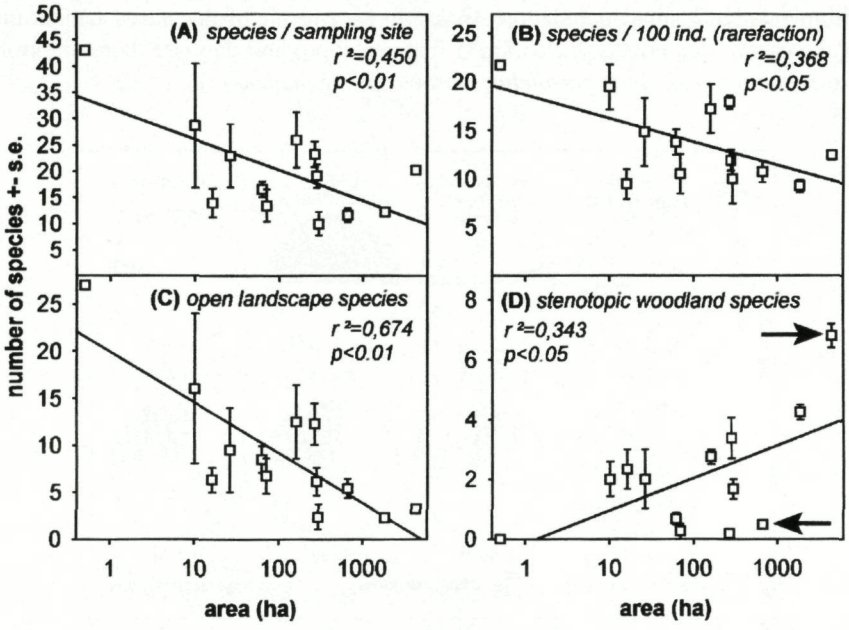


Fig. 7. – Species-area regression analyses: mean species diversity versus log(woodland area) for (A) total carabid species per sampling series, (B) number of species per 100 ind. (rarefaction), mean number of (C) open landscape species and (D) stenotopic woodland species (arrows indicate distinct outliers, further explained in the text).

The general conclusion from these results is that most woodlands in Flanders have been impoverished to a high degree in terms of their stenotopic woodland beetle fauna.

Ancient forest carabids: distribution and dispersal power

Stenotopic carabids from ancient forests apparently have become increasingly rare and now show a highly discontinuous distribution in Flanders. Nowadays, some of these species have probably disappeared entirely from this region (*e.g. Carabus intricatus*), or are only known from one (*Leistus piceus*) or very few relatively large forests (*Abax ovalis, Carabus auronitens, Cychrus attenuatus, Molops piceus*) (DESENDER *et al.*, 1995). All these species indicate(d) a habitat type which can no longer be found in our region: large, dark, cool forest without human interference, with an undisturbed soil and natural decay processes related

to abundant dead wood. In Flanders, there is not a single forest left where all of the aforementioned ground beetle species still occur together, not even the large «Zoniënwoud», and yet all of these species co-occurred in the archaeological assemblages, many in vast numbers. These unique archaeological samples show that it is highly improbable to invoke purely ecological or biogeographic reasons for the recent absence of these stenotopic woodland carabids in Flanders, at least in a region with similar edaphic conditions. Some other stenotopic woodland carabid beetles still appear to survive in a higher proportion of forests in Flanders, all of which are classified as «ancient». In several sites, such species are known only from relatively small populations (possibly as a result of decreased habitat quality and/or increased edge effects due to forest fragmentation) and they are therefore probably close to extinction (*e.g. Abax parallelus, Carabus problematicus*).

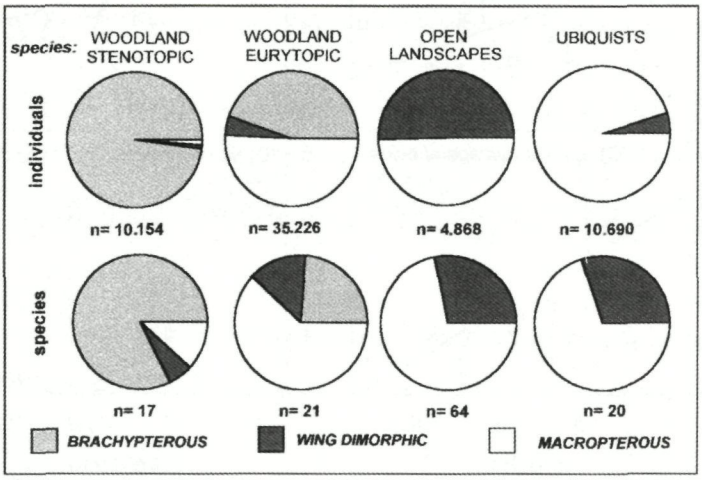


Fig. 8. – Dispersal power and habitat preference in woodland inhabiting ground beetles, based on the total data-set for the studied 13 woods (more than 60.000 carabids, belonging to 122 species). Species are classified into 4 habitat preference categories; dispersal power clearly increases from left to right.

Several of the stenotopic woodland ground beetles have recently been categorised as indicators of ancient woods in other regions also, *e.g.* in many parts of Germany (ASSMANN, 1994, in press; VOSSEL & ASSMANN, 1995), France (BUREL, 1989; TIBERGHIEN, 1981) and the UK (LUFF, 1998). The degree of forest fragmentation, however, is much higher in Flanders, and, as a result, eventual future recolonisation of rehabilitated forests will not easily occur in the region. Most of these stenotopic woodland carabids indeed are constantly brachypterous (cf. Fig. 8) and avoid living near woodland edges, thereby further reducing chances for natural colonisation (ASSMANN, in press). In north-west Germany, for example, woodland cover has increased considerably during the last 200 years (ASSMANN, in press). In the same study, at least some of these typical woodland species appeared to have been able to recolonise recent forest, contiguous to ancient woods. Detailed studies on *Carabus*

auronitens (NIEHEUS *et al.*, 1996; SCHWÖPPE *et al.*, 1998) in the same region have provided population genetic as well as experimental evidence (by translocation experiments) for the historical ecological influence on the actual distribution of this species.

Genetic diversity and differentiation in the woodland carabid *Abax ater*: preliminary results

A simple measure of genetic diversity is compared for the 12 investigated populations of *Abax ater* in Fig. 9. Genetic erosion in fragmented forests cannot (yet?) be concluded from this data, although there are somewhat higher mean values for the ancient woods as compared to the others. The absence of clear evidence for genetic erosion could be due to the eurytopy of this woodland carabid species. Indeed, *Abax ater* has been observed in high population densities in all kinds of forest, also in small fragments. This means that effective population size for such a species will not easily fall below threshold values enhancing the chances for genetic drift (and resulting genetic erosion). It is therefore necessary to enlarge the data-set, if possible with data from even smaller populations. Actual isolation of forests (instead of size *per se*) might also be a more relevant influencing factor for the comparison with genetic diversity.

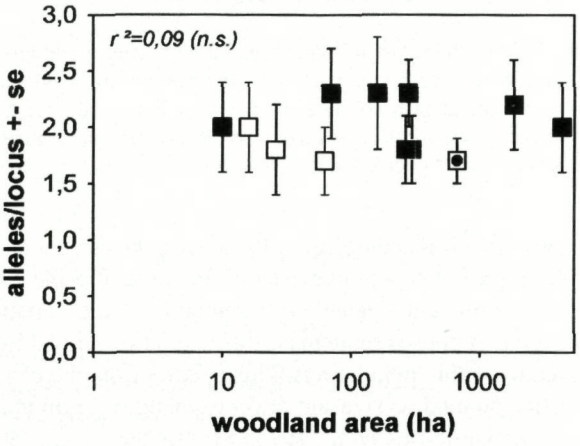


Fig. 9. – Estimates of genetic diversity in *Abax ater* (12 populations; mean number of alleles per locus; 19 alleles for 5 allozymes), plotted against log(woodland area); exploitation woods (open symbols), recent wood (open dotted symbol), ancient forests (black symbols).

The population genetic structure of *Abax ater*, compared between 12 of the woods in this study, shows an overall significant genetic differentiation (for 4 of the 5 allozymes: X^2(MPI)=50.3, p=0.0005; X^2(PEP)=25.6, p=0.0075; X^2(G6PDH)=102.0, p=0.0000; X^2(PGI)= 21.4, p=0.93; X^2(PGM)=87.47, p=0.0000), although the associated Fst-value is low, amounting only to 0.03. Fig. 10 shows a dendrogram based on Rogers' similarity between allele frequencies for the 12 populations studied. Significant differentiation between pairs of populations (test-results added on Fig. 10) was consistently found

between each of the three forests near Brussels (Zoniënwoud, Meerdaalwoud and Walenbos) and each of 6 other woods investigated in this study. This suggests isolation by distance (reduced gene flow), but only on a relatively large geographic scale (exceeding the size of single forests). Whether this result is linked to the more eurytopic habitat preference of *Abax ater* (which still occurs in many, sometimes very small, forests) or to the influence of a small data-set, remains to be answered.

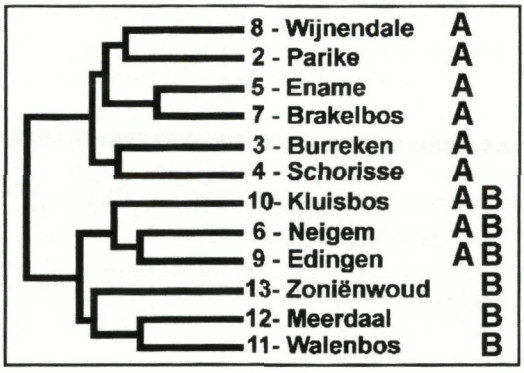


Fig. 10. – UPGMA-dendrogram based on genetic similarity (Rogers' similarity, 5 allozymes) for *Abax ater* from 12 populations; woodland sites, followed by the same letter code, are not significantly different (Bonferroni-corrected pairwise comparisons between all populations).

Because of obvious historical ecological influences on the current distribution of many woodland species (see above), one would expect to find at least some effects of woodland history and fragmentation on actual genetic differentiation and diversity in these beetles. In theory, fragmentation is supposed to increase differentiation among isolated sites and to decrease genetic diversity within populations. This follows from the combined or separate effects of lower effective population size and fewer exchange of individuals (reduced gene flow) (for Flanders, *e.g.*: DESENDER *et al.*, 1998; MATTHYSEN *et al.*, 1995; VAN DONGEN *et al.*, 1994, VAN DONGEN, 1997). Our preliminary results for the eurytopic woodland ground beetle *Abax ater* are not yet conclusive in this respect. Another recent population genetic study of the same species, on a small geographic scale in a region of Germany, yielded a comparable degree of genetic differentiation between populations (BUTTERWECK, 1998). *Abax ater*, although being constantly wingless, has been observed to move from forests into hedgerow networks (CHARRIER *et al.*, 1997). This is an additional explanation as to why population genetics could be less influenced by fragmentation than would be expected for more stenotopic woodland beetles. Where possible, the population genetics of some of these more stenotopic species should now also be studied. Only then will it be possible to evaluate more generally whether historical ecology has influenced the currently observed population genetics of woodland beetles in Flanders.

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A SINGLE TOOTH REPLACEMENT PATTERN GENERATES DIVERSITY IN THE DENTITION IN CICHLIDS OF THE TRIBE ERETMODINI, ENDEMIC TO LAKE TANGANYIKA (TELEOSTEI: CICHLIDAE)

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Abstract. Cichlids from the tribe Eretmodini, endemic to Lake Tanganyika, provide a unique example of diet-associated differences in dentition (especially tooth shape) within a group of closely related species. Here, we examine the tooth pattern and sequence of tooth replacement in four representative eretmodine taxa, as a starting point of a new study that will focus on the mechanisms responsible for ontogenetic and phylogenetic divergence of tooth shape. New teeth are formed in adjacent positions labial to but alternating with older ones in waves that sweep from mesial to distal. Only a minor shift (different spacing of newly developing germs) is necessary to produce the different dental arcades observed in eretmodine cichlids. The position and state of development of the replacement teeth, as well as localized growth and resorption of the jaw bone, add histological evidence in support of the replacement pattern described for the four taxa. The tooth replacement pattern proposed here is uncommon among teleosts.

Key words: teeth, dentition, tooth replacement, cichlids, Lake Tanganyika.

INTRODUCTION

Vertebrate teeth provide exciting material to biologists of disciplines as diverse as paleontology, taxonomy, functional morphology and developmental biology. Morphological characteristics, and in particular shape, of vertebrate teeth have been considered to have a high heritability and have therefore been widely used as taxonomic characters defining species in many taxa of toothed vertebrates (*e.g.*, GREENWOOD, 1981; THENIUS, 1989). Yet, recent studies have shown that dental characters of vertebrates that replace their teeth throughout life, may be susceptible to environmentally-induced variation (*e.g.*, HUYSSEUNE, 1995). Moreover, phylogenies based on taxonomic characters such as tooth shape have turned out to be in conflict with molecular-based phylogenies as is nicely illustrated by recent studies of cichlids of the tribe Eretmodini, endemic to Lake Tanganyika (East-Africa) (VERHEYEN *et al.*, 1996; RÜBER, 1998).

The tribe Eretmodini, as defined by POLL (1986), comprises four nominal species currently assigned to three genera: *Eretmodus cyanostictus* Boulenger, 1898, *Spathodus erythrodon* Boulenger, 1900, *S. marlieri* Poll, 1950, and *Tanganicodus irsacae* Poll, 1950. The shape of the oral teeth is the main defining character to delineate taxa within this tribe (POLL, 1986). The teeth of *Eretmodus* are spatula-shaped with a slender neck region, those of *Spathodus* are cylindrical-shaped with flattened and truncated crown, and those of *Tanganicodus* are slender and pointed. In a recent phylogenetic study of the tribe Eretmodini using mitochondrial DNA (mtDNA) sequences, six genetically distinct lineages were observed (lineages A-F) (RÜBER, 1998). Genera and species are polyphyletic, suggesting the occurrence of cryptic species in this tribe, and the need for a reconsideration of the generic classification based mainly on tooth shape. The results have further indicated that the resemblance in tooth shape between some lineages might be the result of parallel evolution rather than common ancestry. This claim is further substantiated by genetic and morphological differences between *E. cyanostictus* and *E.* cf. *cyanostictus*. The eretmodine cichlids thus provide an excellent model to study what causes the apparent conflict between high heritability and strong adaptive potential of teeth. Before such a study can be undertaken, however, and before any experiment can be conceived, it is necessary to understand how, and in what order, new teeth arise in their dentitions, i.e., to know their replacement pattern (cichlids, like most teleosts, replace their teeth throughout life).

The anatomy of the feeding apparatus, the feeding behaviour and the evolution of the Eretmodini has been studied before by LIEM (1979). YAMAOKA *et al.* (1986) have examined the dentition and ecomorphology of three of the four nominal eretmodine species and have come to the conclusion that differences in dental morphology (as well as the position of the mouth and the morphology of the dental arcade) are related to trophic differences. Dietary differences range from algae scraping in *E.* cf. *cyanostictus* and *S. marlieri* to invertebrate picking in *T. irsacae*, whereas *S. erythrodon* is thought to have a more intermediate feeding behaviour (POLL, 1956; YAMAOKA *et al.*, 1986). In the same paper, YAMAOKA *et al.* (1986) present, in a schematic way, the manner in which teeth are replaced in *E.* cf. *cyanostictus* (the taxon that was investigated in their study) and *S. marlieri*, and state that *T. irsacae* replaces its teeth in a different, yet unexplained, way. A critical examination of the schemes proposed by YAMAOKA *et al.* (1986) reveals, however, that they contain features unlikely to occur during tooth replacement in any cichlid.

We therefore reexamined the tooth replacement pattern and collected additional histological data of the replacement teeth in four eretmodine taxa: *E. cyanostictus*, *E.* cf. *cyanostictus*, *S. erythrodon*, and *T.* cf. *irsacae*. The results of this study will form the starting point for future investigations that will focus on the mechanisms responsible for ontogenetic and phylogenetic divergence of tooth shape within the Eretmodini.

MATERIAL AND METHODS

Natural populations were sampled in 1996 during an expedition along the western Lake Tanganyika shorelines. The specimens were fixed and stored in 80% ethanol. The following, adult, specimens were used:

– *Eretmodus* cf. *cyanostictus* (lineage A): 3 specimens (46.5, 51.5 and 52.5 mm standard length, SL);
– *E. cyanostictus* (lineage C): 3 specimens (51.5, 53.0 and 56.5 mm SL);
– *Spathodus erythrodon* (lineage F): 3 specimens: 50.0, 53.0 and 53.5 mm SL);
– *Tanganicodus* cf. *irsacae* (lineage E): 3 specimens: 41.5, 44.0 and 44.5 mm SL).

The four lineages studied here are derived from a mtDNA-based phylogeny that defined six genetically distinct lineages within eretmodine cichlids (RÜBER, 1998; RÜBER *et al.*, unpublished data).

The oral jaws were examined both before and after dissection. Some jaws were rinsed to eliminate the ethanol and were fixed and decalcified in 1.5 % paraformaldehyde-glutaraldehyde in 0.1M cacodylate buffer, containing 0.1M EDTA. Subsequently, they were rinsed in the same buffer, dehydrated in a graded series of ethanol, cleared in propylene oxide and embedded in epon. Sections of 2 µm thickness were cut with a glass knife and stained with toluidine blue. The terminology used here is represented schematically in Fig.1.

RESULTS

On the oral jaws, teeth are present on the premaxillary and dentary bones. Since the characteristics of the oral jaw dentition have been described by YAMAOKA *et al.* (1986), only the data relevant for the interpretation of the tooth replacement pattern are presented here.

In all four taxa studied, both the premaxillary and the dentary teeth are implanted in what we have called tooth groups, arranged in adjacent oblique tiers (Figs 1, 2a-f, 3a,b, 4a,b, 5a). The number of tooth groups differs between the taxa, between specimens of a taxon, and even between left and right side of an individual. It ranges from 3 to 5 in *E.* cf. *cyanostictus*, from 5 to 7 in *E. cyanostictus*, and from 3 to 5 in *S. erythrodon*. In *T.* cf. *irsacae*, there are usually 2 or 3 well identifiable groups. YAMAOKA *et al.* (1986) reported the presence of only 2 to 4 groups in *E.* cf. *cyanostictus*.

The angle between the aligned teeth within each tooth group and the occlusal surface of the bone differs among the taxa (Figs 1, 2a-f, 3a,b, 4a,b, 5a). It is steep in *E. cyanostictus* and *E.* cf. *cyanostictus* (Figs 2a,b, 3a,b) and shallow in *S. erythrodon* (Figs 2c,d, 4a,b) and *T.* cf. *irsacae* (Figs 2e,f, 5a). Because of this difference in angle, the most lingual position within a tooth group in *S. erythrodon* and *T.* cf. *irsacae* is more mesial than in *Eretmodus*. The groups consist of maximally three erupted teeth in *Eretmodus* (Fig. 3b) and maximally four erupted teeth in *S. erythrodon* (Fig. 4b) and *T.* cf. *irsacae* (Fig. 5a). These data fit with those of YAMAOKA *et al.* (1986). In addition, tooth germs are visible by transparancy within the medullary cavity of the bone in all three species.

Within each tooth group, the tooth located at the highest level of the bone (the occlusal surface) is found in a lingual position. The teeth in lingual positions show various degrees of wear (as shown by the reduced amount of orange-coloured enameloid) and can be considered to be the functional teeth. The other teeth within a group are of the same size as the functional teeth, except in *T.* cf. *irsacae*, where tooth size within a group diminishes labially. Unlike the functional teeth, they appear to be unworn, and the degree to which they protrude from the bone diminishes in a labial direction (rather distal in *S. erythrodon* and *T.* cf. *irsacae*). These

teeth can be considered to represent erupted replacement teeth. Teeth of more posterior groups are smaller than those of anterior groups; this is more pronounced in *T.* cf. *irsacae*, where tooth size distinctly decreases from the second group onwards. Functional teeth and erupted replacement teeth all show separate perforations of the oral mucosa.

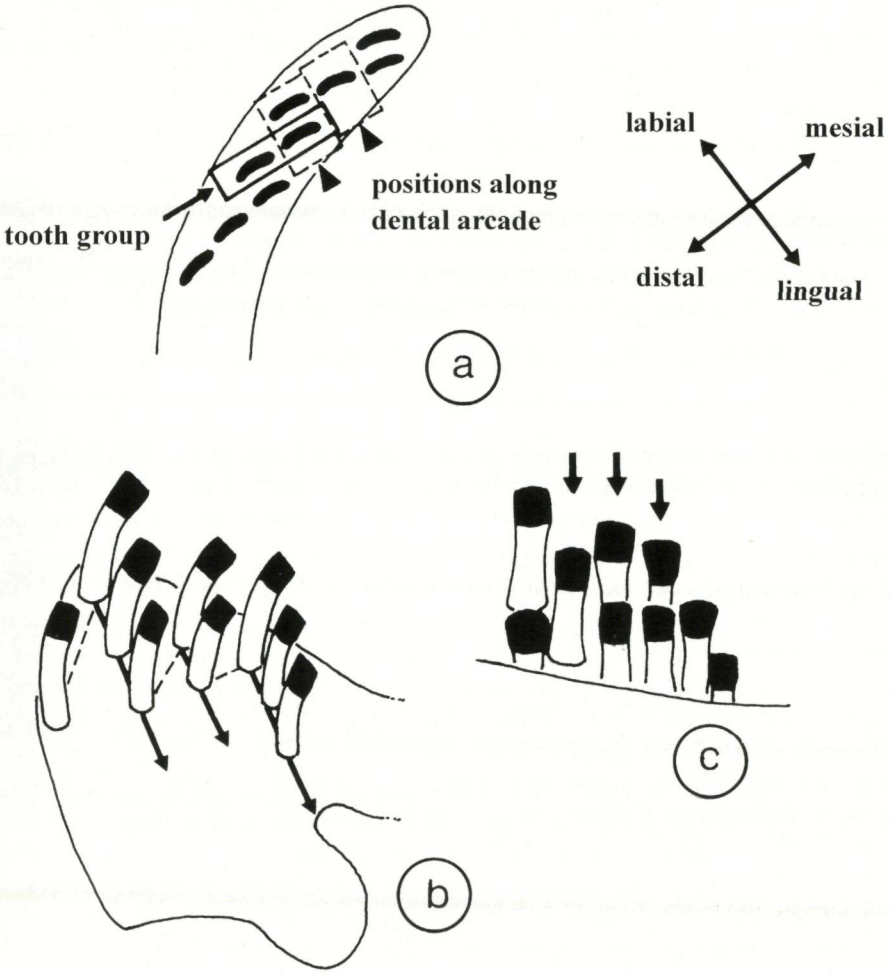


Fig. 1. – Orientation terminology used for the oral dentition of the eretmodines, shown on a schematic representation of an *E.* cf. *cyanostictus* left dentary dentition. (a) occlusal view; (b) labial view; (c) frontal view (*in situ*). In (b) broken lines connect teeth located on the same position along the dental arcade; arrowed lines interconnect teeth of a single tooth group. Arrows in (c) indicate adjacent tooth positions along the dental arcade.

Tooth replacement

For every single specimen of each of the four taxa, and for all four bones in each specimen (two premaxillaries, and two dentaries), the tooth positions, along with the state of

development at a particular position (non-erupted germ visible in transparancy, or erupted tooth) were recorded on a two-dimensional chart. On these charts, tooth groups were set out on lines, and tooth positions were evenly spaced within a group. From these individual charts, we have deduced a general chart for each of the four taxa, which is discussed below (Fig. 6a,b). Ascending numbers on the general charts reflect the mere order of development of the teeth, and are significant only as a ranking device to compare individual teeth; they do not reflect a true number in the dentition (tooth n° 1 is not the first tooth ever-formed in the animal's life, but the oldest possible tooth of a dentition at a given life-stage of an individual). Moreover, individual dentitions never express the full range of this general chart (meaning that not all the tooth numbers ever develop in a dentition). In all cases examined, however, numbers that are lacking occur only at the margins of the dentition, i.e., in mesial, distal and labial positions, in accordance with the extent of the dentition (i.e., the number of tooth groups). When the general chart is used as a template, individual charts are found to match the general chart in different areas. We fitted the individual chart as much as possible into the area with lowest numbers, to make comparisons easier (see Figs 3c, 4c, 5b).

E. cyanostictus and *E.* cf. *cyanostictus*

Fig. 6a shows the general chart as deduced from the observation of 24 bones of the two taxa with *Eretmodus*-like spatula-shaped teeth. According to this chart, starting from tooth n° 1, the next teeth to form lie labial to this tooth in an anterior (mesial) and posterior (distal) direction (teeth n° 2 and 3, respectively). New teeth will develop in the same relative positions with respect to teeth n° 2 and n° 3, yielding teeth n°s 4, 5 and 6. This process is repeated and produces the pattern depicted in Fig. 6a. If this chart is used as a template onto which the chart of a particular bone is grafted, tooth numbers on the template can be copied on the individual chart. Such a superimposition of individual and general (template) chart is shown for a left dentary bone of an *E.* cf. *cyanostictus* specimen in Fig. 3c. In nearly all the jaw bones of the specimens examined, the tooth that is, by ranking, the oldest tooth according to the template chart, also shows the heaviest degree of wear. Such teeth are always lingually situated in a tooth group, but do not necessarily belong to the most anterior tooth group. They most likely represent the first teeth to be shed. *E.g.*, in Fig. 3c, the oldest tooth (tooth n° 3) is placed lingually in the third tooth row; upon inspection of the dentition, it appears that this tooth is also the most worn tooth as indicated by the severely reduced amount of orange-coloured enameloid (Fig. 3a).

From the general chart, it appears that new teeth develop in adjacent positions along oblique lines, with teeth on one oblique line alternating in position with those of a more lingual line. If the oblique lines are considered waves of tooth development, labial waves are initiated after more lingual ones. The oldest teeth are found lingually, the youngest teeth along the labial side of the dentition.

The individual charts of both *E. cyanostictus* and *E.* cf. *cyanostictus* can be grafted on the same template chart. A wider area of the template is, however, expressed in *E. cyanostictus*, because these specimens contain more tooth groups. In the same way, although left or right bone, or premaxillary or dentary bone of one side, do not necessarily show the

same pattern, their individual charts can still be grafted on the same general chart. The observed differences relate to losses of lingually placed teeth and/or a delay in appearance of germs on the labial side.

S. erythrodon

The chart drawn after the observation of 12 bones of *S. erythrodon* (*e.g.*, Fig. 4a,b) differs slightly from the chart for *Eretmodus* (compare Fig. 6b with 6a). The major difference resides in a larger (mesio-distal) distance between newly developing germs. Therefore, the oblique lines that connect adjacent teeth are less steep than in *Eretmodus*. For this taxon too, tooth positions on every single bone can be grafted onto the general chart, and the oldest positions in the template correspond to the most heavily worn teeth in the dentition (*e.g.*, Fig. 4c).

T. cf. *irsacae*

The observations on 12 bones of *T.* cf. *irsacae* (*e.g.*, Fig. 5a) fit with a chart that is identical to the one for *S. erythrodon*. In each of the 12 bones examined, tooth size and degree of eruption diminish in a labial (distal) direction within each tooth group. This matches the order of appearance as suggested on the general chart (Fig. 5b, 6b). As for the other three taxa, the teeth on the « oldest » positions on the general chart are the most heavily worn teeth.

Histological observations of the jaws

Light microscopical observations on serial sections through the premaxillary and dentary bones of the four taxa studied revealed the presence of replacement teeth in various phases of development within the medullary cavity of the bone. In the two taxa with the *Eretmodus*-like tooth shape, numerous germs are present (Fig. 7a). In *Tanganicodus*, only a few replacement teeth are observed in the medullary cavity. The position of the germs, and their state of development, fits with what can be expected from the general chart (compare Figs 6a and 7a). The tips of the germs are located labially in the medullary cavity; however, their proximal ends lie more lingually.

Collars of attachment bone (ovals in the section shown) are found at different levels in the medullary cavity (Fig. 7a). They consist of a compact bone ring firmly anchored to a mass of cancellous bone that fills this part of the medullary cavity (Fig. 7a). Collars still in the process of deposition lie deepest with respect to the oral epithelium. They support fully-grown teeth, the tips of which erupt more labially (and less apically) along the jaw bone than do those supported by attachment bone that lies nearer to the surface. So far, we have not been able to trace the origin of the replacing teeth, whether it is from a dental lamina associated with the predecessor, or whether it is totally separate from the oral epithelium.

Depending on the place along the jaw bone, specialized cell types line the bone tissue. Numerous osteoclasts engulf the free bone margins below the oral mucosa (Fig. 7b). At the opposite side of the jaw bone, numerous intensively basophilic osteoblasts surround the end of the free bone spicules or are arranged in a pseudo-epithelial manner along the bone surface (Fig. 7c).

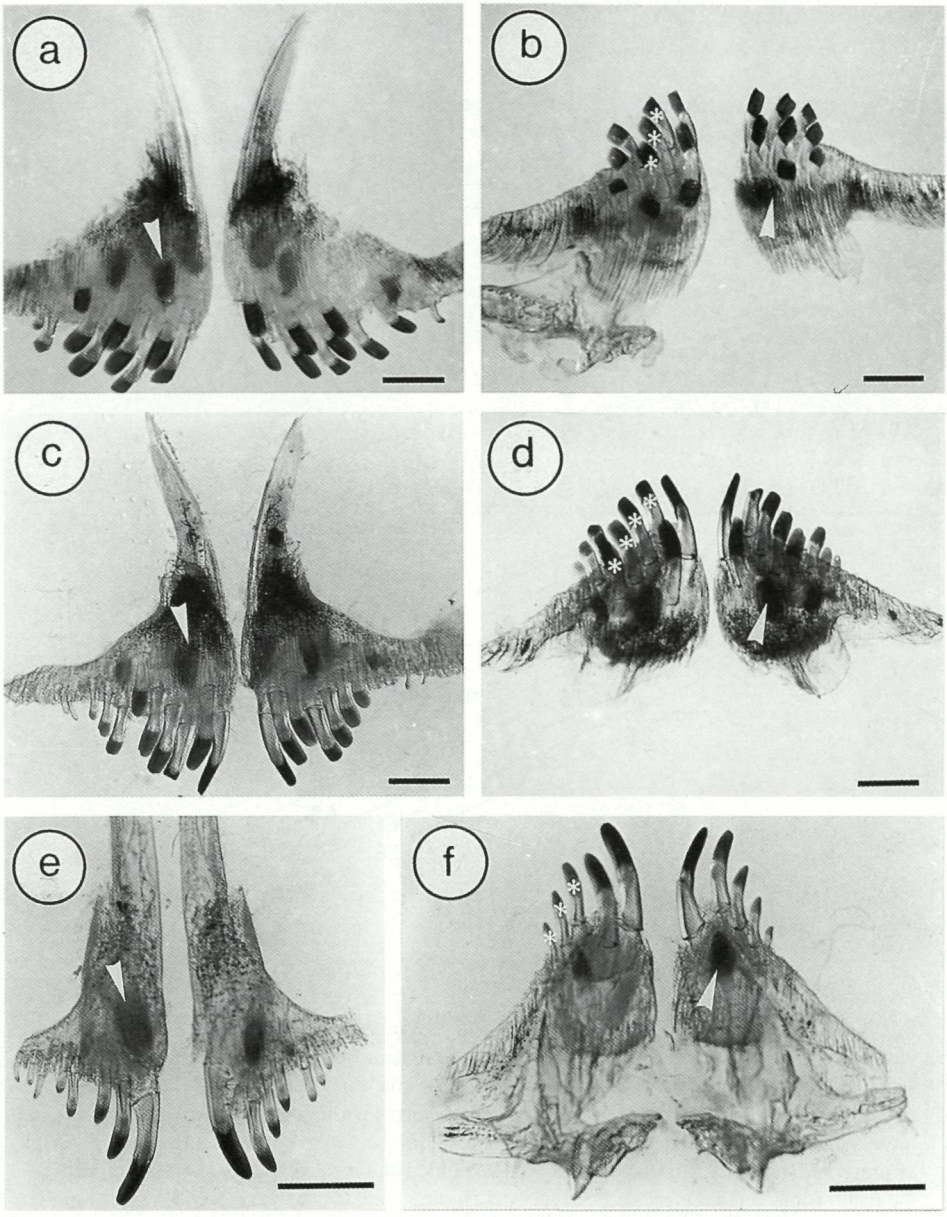


Fig. 2. – Labial view of dissected premaxillaries (a,c,e) and dentaries (b,d,f) of *E.* cf. *cyanostictus* (a,b), *S. erythrodon* (c,d), and *T.* cf. *irsacae* (e,f). The dark tips of the teeth are the (orange-coloured) enameloid caps. Note the presence of tooth germs in the medullary cavity, visible in transparancy (arrowheads). In (b), (d) and (f), asterisks indicate teeth belonging to a single tooth group. Scale bars in (a) to (f) = 1mm.

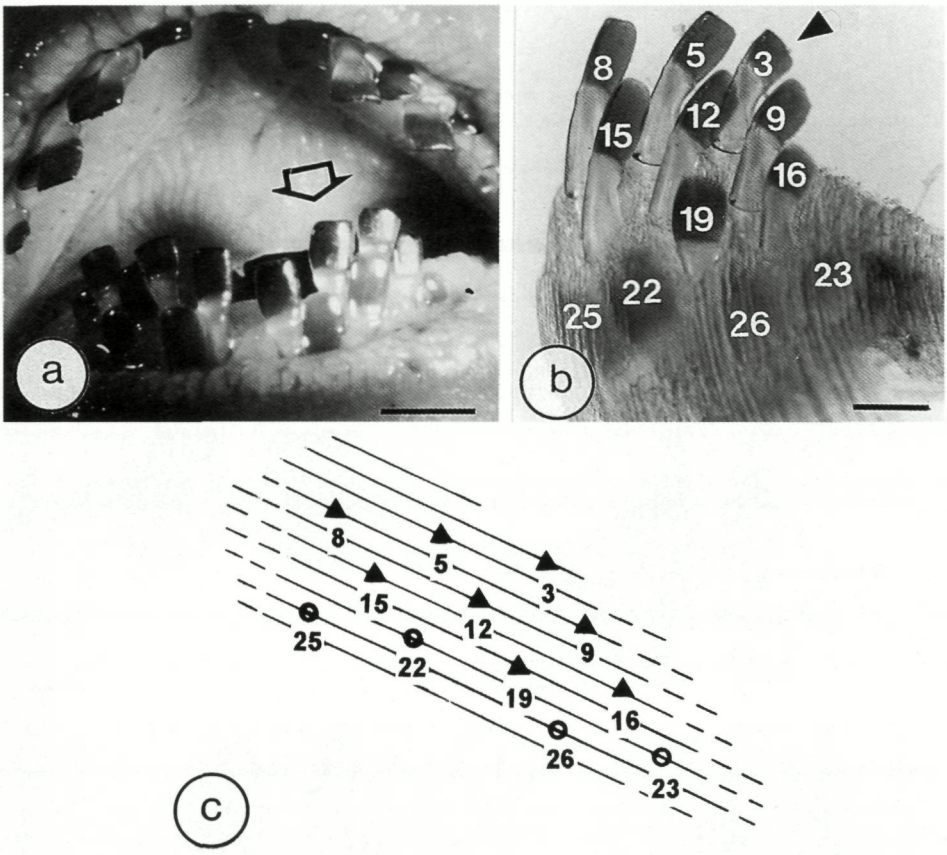


Fig. 3. – Left dentary of an *E.* cf. *cyanostictus* (51.5 mm SL), shown *in situ* (a, arrow; frontal view) and after dissection (b, labial view). In (c) the dentition of this left dentary is depicted schematically to show how the scheme of the replacement pattern relates to actual morphology. Erupted teeth are indicated by triangles, tooth germs within the medullary cavity (at least those visible in transparancy) by circles. The teeth are set out according to the general chart as presented in Fig. 6a. Tooth numbers are then added and reflect the order of development of the teeth. Lines interconnect successively developing teeth. In (b) an arrowhead points to the most heavily worn tooth on the lingual side of the dentition. Scale bar in (a) = 1 mm, in (b) = 0.5 mm.

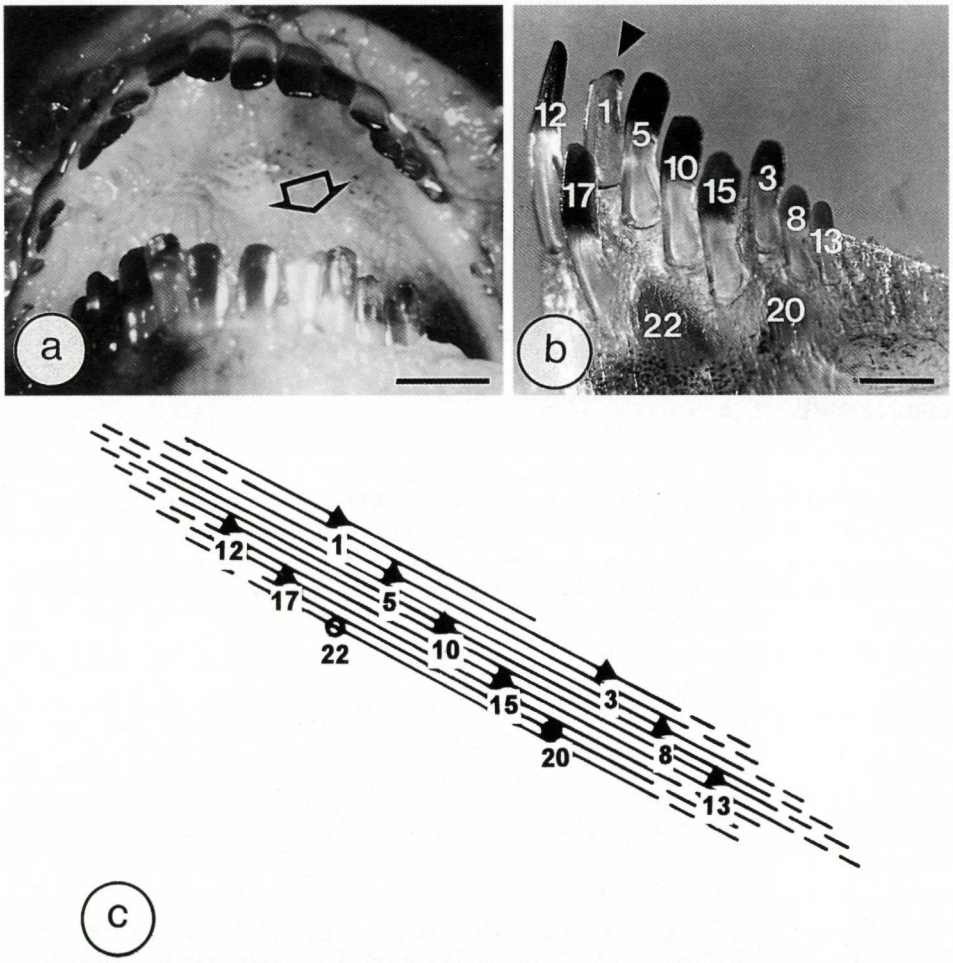


Fig. 4. – Left dentary of a *S. erythrodon* (53 mm SL), shown *in situ* (a, arrow) and after dissection (b). In (c) the dentition is shown schematically and grafted onto the general chart shown in Fig. 6b in the same way as was done for Fig. 4c. In (b) an arrowhead points to the most heavily worn tooth (note the reduced amount of enameloid). Scale bar in (a) = 1 mm, in (b) = 0.5 mm.

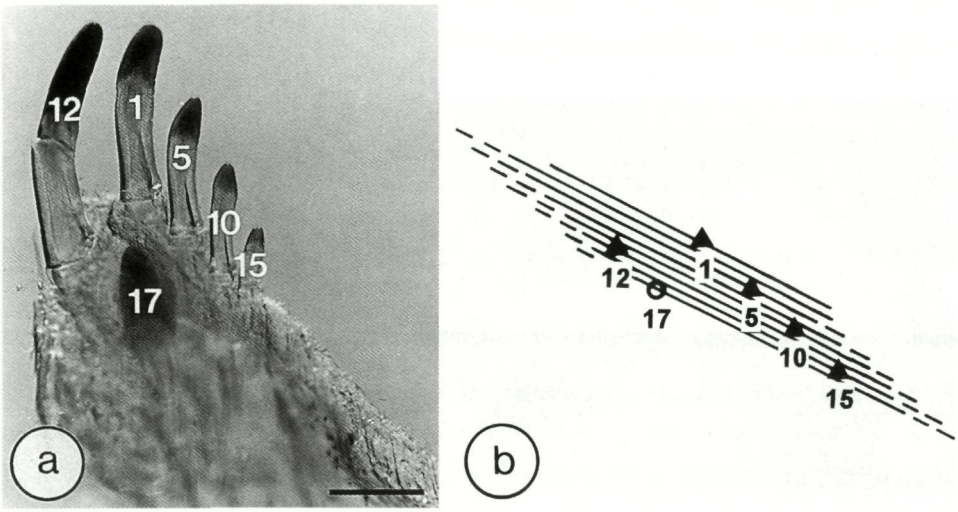
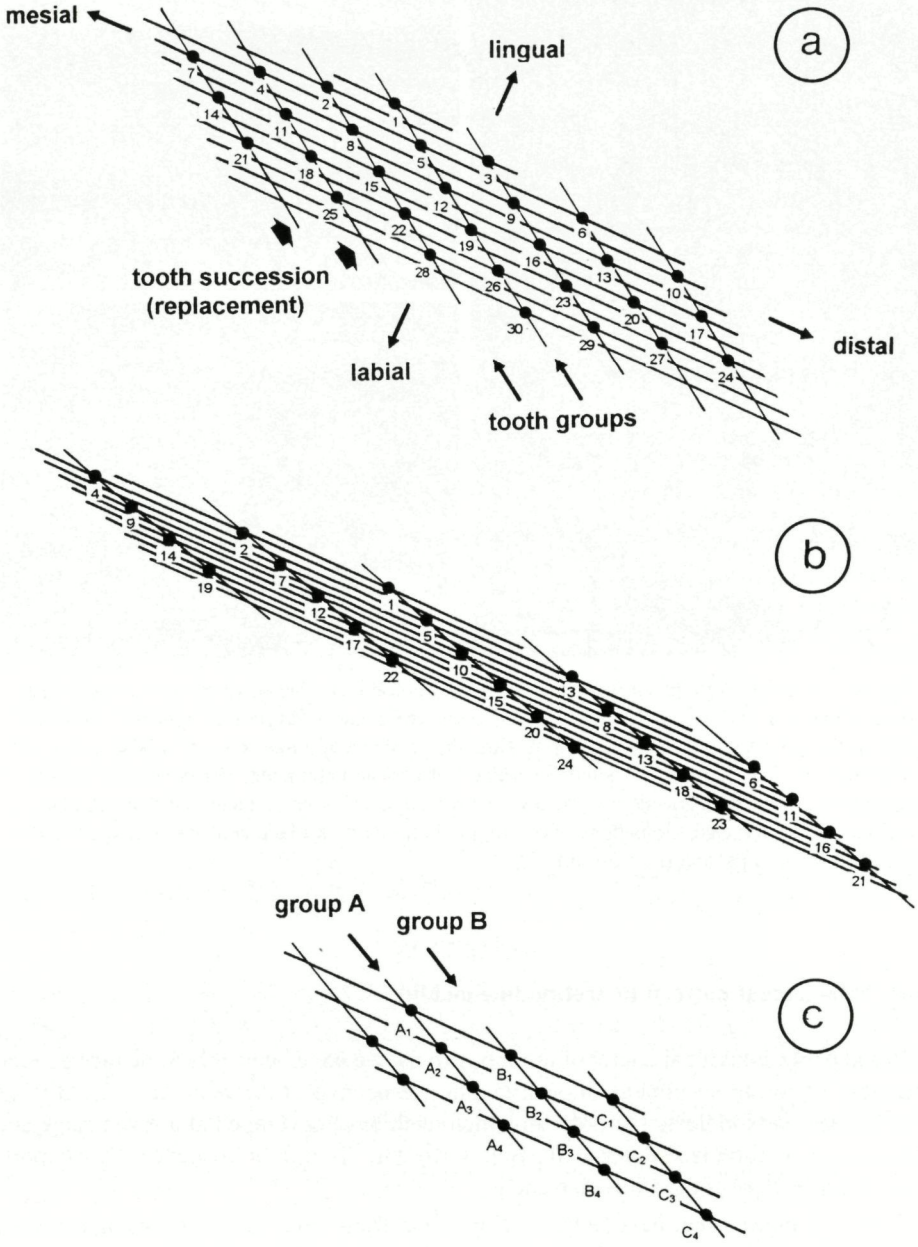


Fig. 5. – (a) Left, dissected, dentary of a *T.* cf. *irsacae* (44 mm SL). The most anterior tooth was broken during preparation. The same dentary, and its contralateral counterpart, are also shown on Fig. 2f. In (b) the dentition is shown schematically and grafted onto the general chart shown in Fig. 6b in the same way as was done for Fig. 4c. Scale bar in (a) = 0.5 mm.

Legend to the figure (see opposite page)

Fig. 6. – General charts of the adult dentition in *Eretmodus* (a), and in *S. erythrodon* and *T.* cf. *irsacae* (b). The figures are applicable to left premaxillary and dentary, and the mirror images to right premaxillary and dentary. Teeth (unerupted and erupted) are indicated by circles. Oblique lines connect successively developing teeth. The number of tooth groups on charts (a) and (b) corresponds to the maximal number of tooth groups observed in the respective taxa (7 in *E. cyanostictus*, 5 in *S. erythrodon*). In (c), the replacement pattern in *E.* cf. *cyanostictus*, as erected by YAMAOKA *et al.* (1986), is redrawn to match the graphical representation used here. According to this scheme, A_3 succeeds to A_2 which itself succeeds to A_1.


mesial
lingual
a
tooth succession
(replacement)
labial
distal
tooth groups
b
group A
group B
c

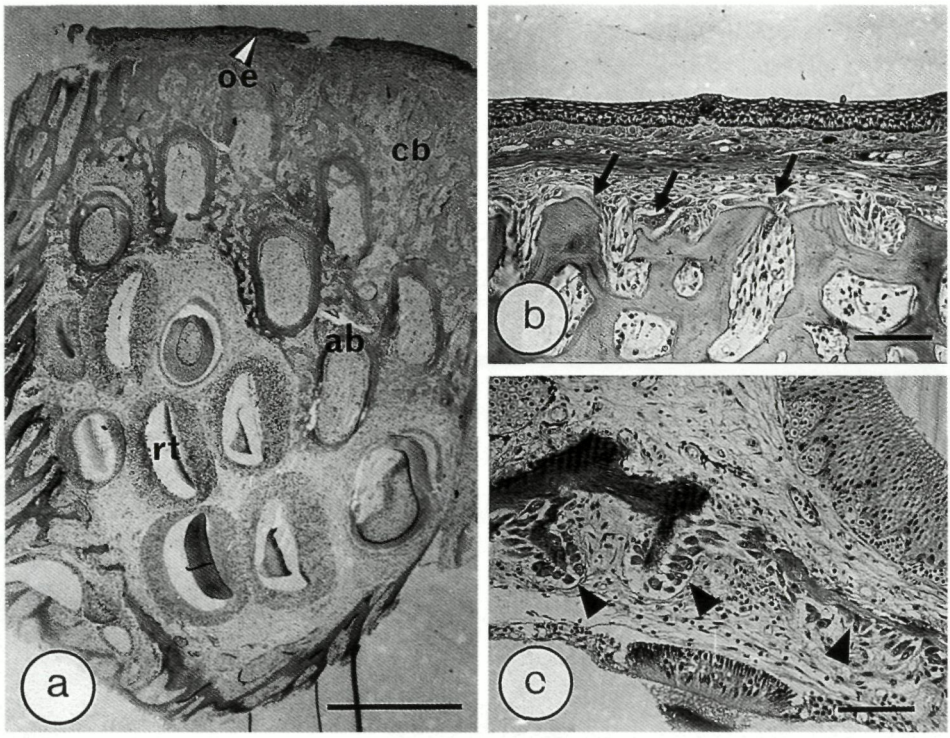


Fig. 7. – Microphotograph of a vertical section parallel to the mesio-distal axis through the right dentary of an *E.* cf. *cyanostictus* (a) and details of the occlusal (b) and opposite, basal (c) side of the jaw bone. Mesial is to the left. Note the presence of numerous osteoclasts (arrows) resorbing the free margins of the bone surface (arrows) and abundant, intensively basophilic osteoblasts on the opposite side of the jaw (arrowheads). ab : young attachment bone of labial, erupted tooth ; cb : cancellous bone ; oe : oral epithelium ; rt : replacement tooth. Scale bar in (a) = 0.5 mm ; in (b) and (c) = 50 µm.

DISCUSSION

Tooth replacement pattern in eretmodine cichlids

Based on 48 individual charts of tooth positions, we have been able to deduce general charts that fit for all specimens of each taxon, irrespective of the bone considered (Figs 6a,b). These charts indicate the order in which teeth have developed at a given stage and in which they will replace older teeth. Below we will discuss the evidence that supports the proposed patterns of tooth replacement.

Firstly, it appears that new teeth develop labial from older ones. The youngest tooth germs are found along the labial side of the dentition, the oldest, functional, teeth along the lingual side of the dentition. These observations agree with our histological findings, indicating that tooth germs develop and erupt labial to older teeth. The labial formation of new germs is uncommon among teleosts ; usually teeth develop lingually from functional

teeth (*e.g.* in *Amia*: MILLER & RADNOR, 1973; in *Salmo gairdneri*: BERKOVITZ, 1977; in *Serrasalmus*: BERKOVITZ & SHELLIS, 1978; see also FINK, 1981). In cichlids the most common situation is that teeth, at least those of the outer row, are replaced within the same row and that replacement teeth develop from below (SNOEKS, personal communication). In rare cases, such as in *Prionurus,* replacement teeth develop both lingual and labial to the functional teeth (WAKITA *et al.*, 1977).

Secondly, if one assumes that the speed of development of germs is constant along the mesio-distal axis of the dental arcade, the order in which teeth become functional should be a reflection of the order of tooth development. Since it is likely that functional teeth are more or less equally submitted to wear, teeth showing the heaviest wear should therefore represent the oldest teeth. Our observations show that this is the case: the teeth showing the heaviest wear always coincide with the oldest teeth. These may well be in the posterior region of the dentition, *e.g.* in a situation when teeth n°s 1 to 5 are shed, and tooth n° 6 is the oldest persisting tooth in the dentition, as observed on several occasions.

The oblique lines that connect the positions of successively forming teeth represent successive waves of tooth development. New germs alternate along these waves with respect to teeth along a previous (older) wave. If the space between teeth of a single tooth group is considered to be constant, as in Fig. 6a,b, the place along the dental arcade where new germs will form along the oblique lines depends on the slope of these lines. In the *Eretmodus*-like dentitions, germs form successively at every third position along the dental arcade. In *S. erythrodon* and *T.* cf. *irsacae*, where the slope is very shallow, new germs arise at only every seventh position along the dental arcade. Inevitably, this raises the question of whether and how teeth in a particular position are replaced. Interestingly, the sections reveal that erupted teeth, even those that emerge low along the labial side of the jaw bone (*e.g.* the third or fourth tooth of a tooth group), are firmly anchored to attachment bone deep within cancellous bone of the medullary cavity, and that they have separate perforations through the oral mucosa. It is highly unlikely that teeth will become detached from this attachment bone and move upward to take a more apical position on the bone, *i.e.*, more towards the occlusal surface. In such a process, the tooth would repeatedly become loose and again attached. It is unlikely that tooth replacement would proceed in such an inefficient way. We suggest that the most likely explanation for our observations is that the tooth does not move once its attachment bone has been deposited. Rather, the jaw bone is remodeled to expose the most apical tooth in a tooth group once an even more lingually placed tooth of that tooth group has been shed. The presence of numerous osteoclasts indicating severe resorption along the apical (occlusal) side of the bone, and intense osteoblastic activity indicative of new bone formation along the opposite side, support this hypothesis. We therefore propose that, as waves are lost lingually through shedding of the teeth, the next wave will deliver the next functional teeth, without these having to move but with bone remodeling instead to expose them. That teeth become functional in such a passive way by exposure resulting from bone resorption progressing apically and lingually, and deposition basally and labially, appears to be a novel mechanism, to our knowledge not previously reported in any bony fish. It requires that the rate of bone remodeling be tuned to the rate of tooth formation. This can be tested by injecting bone markers in living fish.

After three waves have been shed in the *Eretmodus*-like dentitions (seven in *Spathodus* and *Tanganicodus*), a tooth eventually ends up in the position along the dental arcade previously taken by a tooth of a more posterior group; *e.g.* in Fig. 6a, tooth n° 15 will eventually succeed n° 5 on that position along the dental arcade. A tooth in a certain position is thus « replaced » by a tooth of an anterior tooth group (*i.e.*, between-group replacement). Within-group replacement (*e.g.*, in the same example, tooth n° 15 succeeding n° 8) is unlikely because teeth would have to move continuously into more mesial positions. Should this occur, replacement teeth should often be found in intermediate positions along the dental arcade; this is not the case (*e.g.* on Fig. 6a, teeth n°s 5 and 15, or 3 and 12, are aligned in one position along the dental arcade, compare with the *in situ* view on Fig. 3a). In addition, within-group replacement cannot explain other features such as the presence of incomplete tooth groups (not possessing the full number of erupted teeth). Evidence for between-group replacement can also be drawn from left-right comparisons. If between-group replacement is operating, the large tooth (tooth n° 1) on the dentition shown in Fig. 5a, will be replaced by tooth n° 17. This is exactly what is found on the contralateral side (cf. Fig. 2e). This observation also suggests that a phase difference may exist between both sides. The tooth germs which succeed each other at a given position along the dental arcade constitute tooth families *sensu* REIF (1982, 1984) (*i.e.*, a functional tooth and its successors). So far, we have not been able to trace any epithelial links between these tooth germs, unlike what could be expected from teeth of a single tooth family (the germs are separated by three, or even seven, waves).

In most bony fish, the successor expands beneath or around the base of the tooth in function, resulting in its resorption. The mechanism of shedding in the eretmodine oral jaws is clearly different. Although we have observed wearing of functional teeth down to below the level of the enameloid cap, it is unlikely that this is the only way for the tooth to be shed. Probably resorption along the lingual side of the premaxillary or dentary bone affects the attachment bone as well. Further histological studies are needed to clarify this point.

While successive waves are lost, tooth groups migrate in a posterior direction. Tooth groups are temporary assemblages, the composition of which changes as successive waves deliver the functional teeth. Depending on the number of waves that have been shed, tooth groups that lie anteriorly along the dental arcade, will eventually end up in the middle of, or even beyond the middle of the dental arcade. The mechanism of between-group replacement explains (i) why left and right halves of the dentition often contain different numbers of tooth groups: this simply depends on which wave has been lost, and this does not need to be synchronous left and right; (ii) why the dental arcade may show only half a group at its anterior or posterior end, and (iii) why, upon first inspection of the dentition, some tooth groups overlap each other with one, others with two teeth.

The general charts represent the state of the dentition in adults. At present, it is unknown to what extent the pattern of replacement relates to the order of tooth appearance in the larva, in other words whether the pattern of succession is established upon the first appearance of the teeth in the larva. Such a correspondance was found earlier in piranhas by BERKOVITZ & SHELLIS (1978), but is not always the case. In cyprinids, for example, distinct differences between larval and adult dentitions reflect differences in tooth replacement patterns (NAKAJIMA, 1984).

Different models have been proposed to explain tooth replacement patterns in lower vertebrates (OSBORN, 1984). The most important models are the field model of Zahnreihen (EDMUND, 1960, 1969), and the clade model of local inhibition (first defined by OSBORN, 1971, 1978, but later refined by WEISHAMPEL, 1991). Our observations seem to be at variance with both models. At first sight, the tooth groups as defined in this study could correspond to Zahnreihen. However, the Zahnreihe model postulates the emission of signals at the rostral tip of the jaw, eliciting tooth formation at regular intervals from the front to the back of the jaw. The observation that teeth are formed anterior to existing teeth is incompatible with Zahnreihen, an argument already raised by WEISHAMPEL (1991). On the other hand, although local inhibition may well account for the pattern seen here, it does not explain the gradual size decrease of the teeth towards the distal side of the dentition. A decrease in the number of cells with odontogenic potential from mesial to distal along the dental arcade is compatible with Zahnreihen. Clearly, the pattern described for the eretmodines must be seen as the empirical outcome of a tooth generating mechanism which is still not fully understood.

As described above, the proposed tooth replacement pattern allows the frequently observed differences between left or right bone, or between premaxillary or dentary bone, to be understood in terms of a phase difference in tooth development between the jaw quadrants. It is interesting to note that LIEM (1979) found that the morphologically symmetrical muscular apparatus can act with pronounced asymmetrical firings of multiple muscles. This asymmetric firing could provoke differences in wear between left and right jaw bones and result in a phase difference in the replacement of the dentition. Nevertheless, tooth replacement has repeatedly been shown to be independent, to a marked extent, in the different jaw quadrants (BERKOVITZ & MOORE, 1974, 1975; REIF, 1976; BERKOVITZ & SHELLIS, 1978).

To compare the tooth replacement patterns proposed here with those from YAMAOKA *et al.* (1986), we have redrawn their scheme for *Eretmodus* in a way that matches our type of presentation (Fig. 6c). First, they suggested that the most lingually placed tooth of each tooth group has the same age. Our observations on the overall organisation of the dentition, and the state of wear of these particular teeth, suggest that this is not the case. Secondly, YAMAOKA *et al.* (1986) suggested that a tooth group consists of a functional tooth and its successors. Inevitably, they failed to explain how a tooth would « move up » (their expression) along the bone as is required according to their tooth replacement scheme. According to the tooth replacement pattern described here, teeth do not « move up » since the replacement teeth are already positioned where they will replace the current functional tooth.

Inter(generic) taxon comparisons

The chart onto which the dentitions of *S. erythrodon* and *T.* cf. *irsacae* are easily grafted, is essentially identical to the chart for *E. cyanostictus* and *E.* cf. *cyanostictus*. The only difference is the larger distance between successively initiated germs in *S. erythrodon* and *T.* cf. *irsacae* compared to the *Eretmodus*-like dentitions. This results in a pattern where tooth groups are shifted more with respect to each other, because of the smaller

angle of tooth groups with respect to the occlusal surface. As a result, it seems that, although there is superficial resemblance between the *Spathodus* dentition and that of *Eretmodus* (at least with respect to tooth size and shape), the *Spathodus* dentition is actually more similar to the *Tanganicodus* dentition.

It appears that a single pattern explains the different dentitions observed among the eretmodines. Differences between taxa can be related to differences in extent of the theoretically possible dentition being expressed: *e.g.* when the two *Eretmodus* type dentitions are superimposed on the general chart, *E. cyanostictus* appears to express a far more extensive part of the scheme compared to *E.* cf. *cyanostictus*. The same reasoning can be applied to the intraspecific differences that are sometimes found: these always concern positions at the mesial or distal margin of the dentition, and can be explained in terms of smaller parts of the theoretical pattern being expressed.

In conclusion, the different dental arcades in these closely related species, which allow them to utilize different food resources, are the outcome of a single, regular pattern of tooth formation and replacement. The difference in angle of the tooth groups with respect to the occlusal surface, the different number of tooth groups, and number of teeth within a tooth group, can all be related to a small shift in spacing of the newly developing germs. The challenge for future studies will be to understand how different tooth shapes are generated and genetically controlled within this group of closely related species.

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THE CERVICAL MUSCULATURE IN HELODERMATID LIZARDS

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Abstract. The evolution of an independently moveable craniocervical system is a key feature in the evolution of amniote organisms. The cervical system not only plays a crucial role during the orientation of the head towards external stimuli (*e.g.* visual, auditive stimuli), but is also of major importance during the inertial components of feeding in amniotes. Although the cervical system and its musculature are generally well studied in mammals, archosaurs (birds and crocodiles) and turtles, very little is known about the cervical system in some of the most primitive amniotes (lepidosaurians). In a first step towards elucidating the evolution of the cervical system, we examined the neck musculature in lizards of the family Helodermatidae. In general, the bauplan of the cervical muscular system appears stable within closely related phylogenetic groups. Nevertheless, preliminary data suggest that the evolutionary shift from a lingual-based to a predominantly inertial feeding system within lizards coincides with an increase in the complexity of mainly the epaxial components of the cervical musculature. A "new" comprehensive nomenclature of lizard neck muscles (based on the work of NISHI, 1916) is proposed, and should enable future workers to interprete the neck musculature in an evolutionary context.

Key words : cervical musculature, lizard, functional morphology, Helodermatidae, inertial feeding.

INTRODUCTION

The evolution of an independently moveable cranial and cervical system is a key novelty in the evolution of amniote organisms (GANS, 1992). About 300 million years ago (JOUFFROY, 1992), the first two vertebrae (atlas and axis) became modified, which permitted bending and rotation between the cranial and cervical system, and thus movements of the head relative to the rest of the body. This independent head movement was crucial for the first land vertebrates as it increased their sensory abilities, and opened new avenues to orient towards, or away from, stimuli (visual, auditive, olfactory), discover food sources, catch prey, etc. The cervical system not only plays a crucial role during orientation of the head, but it is also of major importance during the inertial components of feeding (see GANS, 1969) in terrestrial amniotes such as lizards (SMITH, 1982), crocodiles (CLEUREN & DE VREE, 1992 ; CLEUREN, 1996), birds (ZWEERS, 1982), and mammals (GORNIAK & GANS, 1980). Although the cervical system and its musculature are generally well studied in mammals (*e.g.* RINKER, 1954 ; RICHMOND & ARMSTRONG, 1988 ; JOUFFROY, 1992), archosaurs [birds (*e.g.* BOAS, 1929 ; VANDEN BERGHE & ZWEERS, 1993 ; ZWEERS *et al.*, 1994) and

crocodiles (*e.g.* SEIDEL, 1978; FREY, 1988; CLEUREN, 1996)] and turtles (*e.g.* GEORGE & SHAH, 1954, 1955; SHAH, 1963; SCANLON, 1982), little is known about the cervical system in some of the most primitive amniotes such as lizards (NISHI, 1916; VALLOIS, 1922; OELRICH, 1956). The presence, position and orientation of neck muscles are, however, used as systematic characters in the classification of lizard groups (RIEPPEL, 1980).

The aim of the present study is to compare the neck musculature of a derived group of lizards (helodermatids) with previously published data on other lizards, and to re-evaluate the present nomenclature of the cervical musculature. With increased knowledge about the complexity of the lizard cervical system, an attempt can be made to characterise evolutionary patterns of the cervical musculature within lizards, and to elucidate functional correlates of these patterns during feeding.

MATERIAL AND METHODS

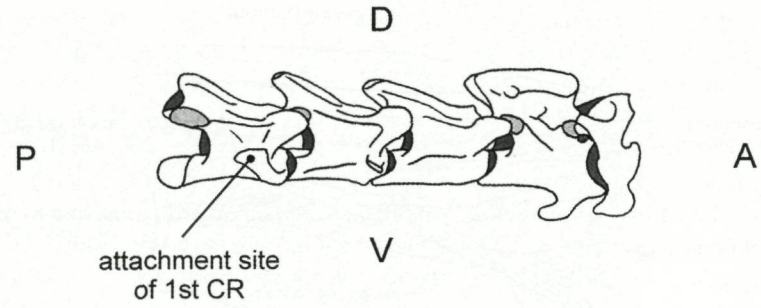
One specimen of *Heloderma horridum*; Wiegmann, 1829 (Smithsonian Institution) and one *Heloderma suspectum* Cope, 1869 (Carnegie Museum of Natural History) were used for the detailed morphological analyis.

All specimens examined were dissected and stained (BOCK & SHEAR, 1972) to characterise the cervical musculature. Drawings were made of all stages of the dissection using a Wild M5 dissecting microscope, equipped with a camera lucida.

RESULTS

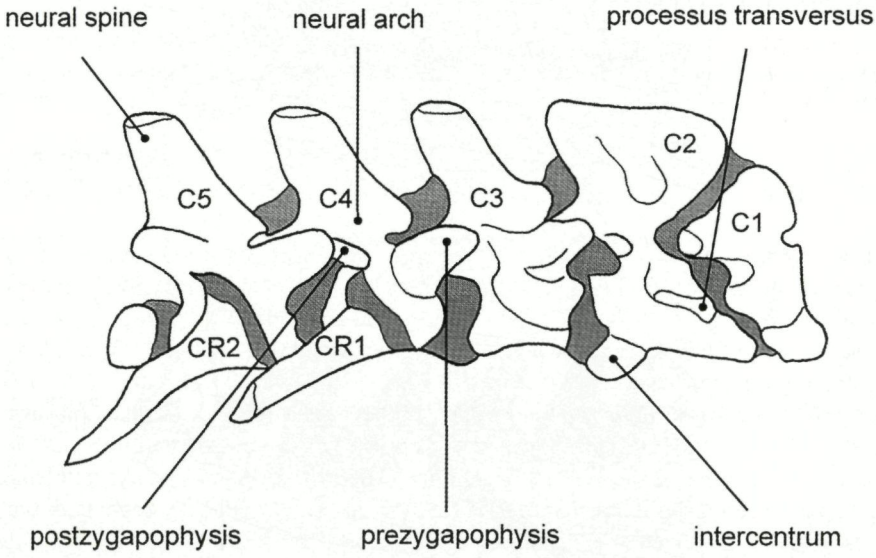
The structure of the cervical vertebrae in reptiles has been discussed previously, so it is not discussed in detail here. For an excellent overview of the complexity and diversity of the cervical vertebrae in lizards we refer to HOFSTETTER & GASC (1969). Only some striking differences between the representatives of the groups examined here are mentioned. Whereas most lizard groups possess eight cervical vertebrae, varanids have one extra. Notable is the remarkable reduction of the hypapophyses in Helodermatidae (Fig. 1). Cervical ribs occur in all specimens studied. Whereas the first cervical ribs attach to the fourth cervical in the Iguanidae, Scincidae, and Helodermatidae examined here, in the Agaminae and Leiolepidinae the first pair of cervical ribs attaches to the fifth cervical vertebra (Fig. 2). In varanids the first cervical ribs usually attach to the sixth cervical vertebrae (see HOFFSTETTER & GASC, 1969). The nomenclature of the various parts of the cervical vertebra is indicated on Fig. 1.

The cervical musculature in *Heloderma* is described briefly, and compared with that in representatives of other lizard families. The cervical system of *Heloderma* was chosen as it shows the full complexity observed within lizards. In general, the muscle nomenclature of NISHI (1916) and OELRICH (1956) is used, unless mentioned otherwise. Some muscles associated with the pectoral girdle and situated in the cervical region are also described for clarity. For these muscles the terminology of SANDERS (1870, 1872, 1874) is used.



A: *Uromastix acanthinurus*

1 cm



B: *Heloderma horridum*

1 cm

Fig. 1. – *A*: first five cervical vertebrae of *Uromastix acanthinurus*. Note that the first cervical rib has been removed. – *B*: ligamentous preparation of the first five cervical vertebrae in *Heloderma horridum*.

The shaded area represents connective tissue. Both drawings are at the same scale (6.5 x magn.) and oriented with the cranial side to the right. A, anterior; C, cervical vertebra; CR, cervical rib; D, dorsal; P, posterior; V, ventral.

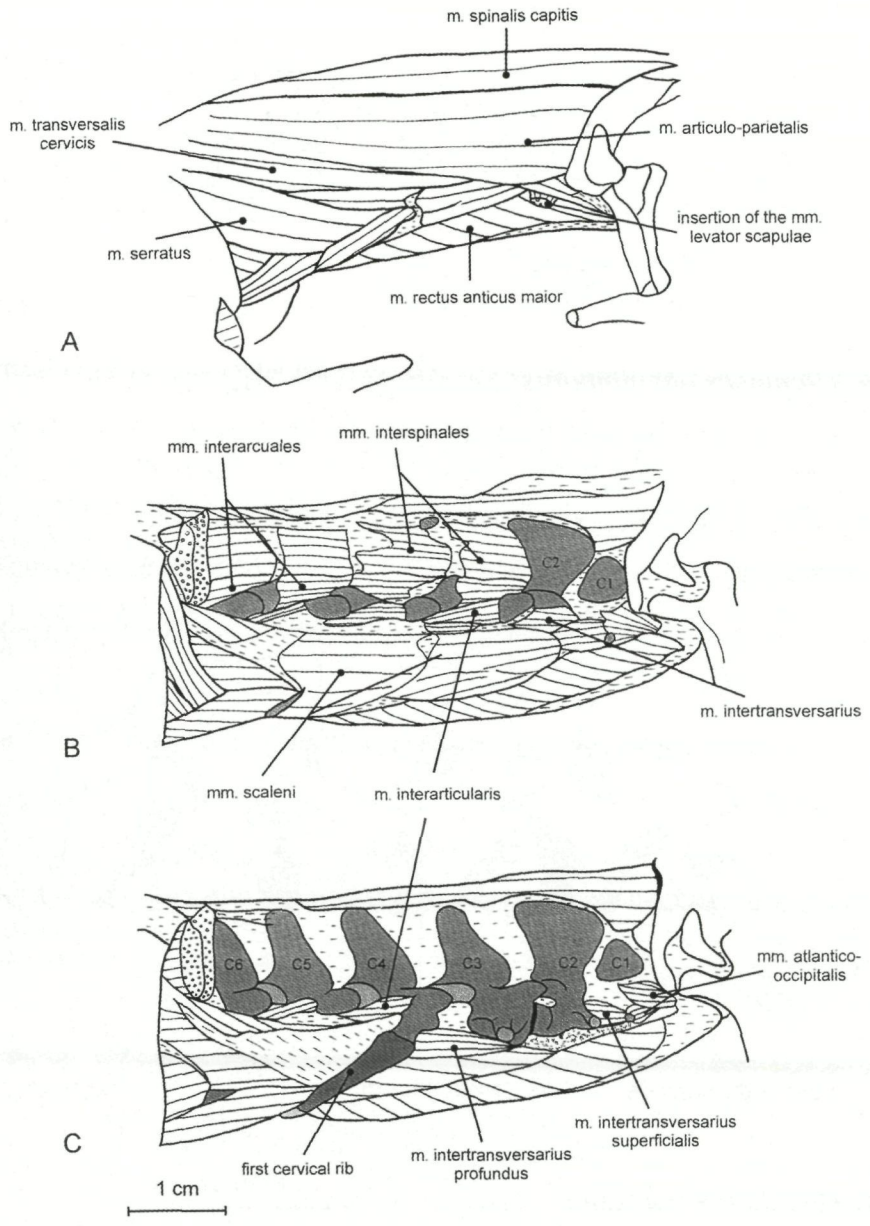


Fig. 2. – Neck musculature. – *A*: superficial lateral view on the neck musculature in *Heloderma suspectum*. The MLSS, MLSP, MEM, MT, the m. depressor mandibulae, the m. cervicomndibularis and the m. constrictor colli have been removed. – *B*: *Heloderma horridum*, deeper level of dissection after removal of the MSCe, the MSSCe, the MOCa, the MICoCa, the MICoCe, the right MSCa and the MLCeCa complex. – *C*: as in B, but after removal of the MIS, the MIARC, the MS and some MIART and MIT (for an explanation of abbreviations, see results).

The transverso-spinalis complex

* The *m. spinalis capitis* (MSCa), the dorsal-most neck muscle, lies adjacent to the ligamentum nuchae, and mesially with respect to all other neck muscles. The muscle originates at the dorsolateral side of the neural spines of cervical vertebrae 2 to 5 (C2-C5). The fibres run anteriad and insert at the mediocaudal aspect of the parietal bone. A small slip of this muscle, arising at the C1-C2 junction, runs a little ventrad and inserts at the occipital crest of the supraoccipital bone (see OELRICH, 1956).

* The *mm. spinalis* and *semispinalis cervicis* (MSCe; MSSCe) are the craniocervical parts of the mm. spinalis and semispinalis dorsi. Both muscles are inseparable and will be discussed as one complex. The complex arises on the lateral side of the prezygapophyses of the first three thoracal (T1-T3) and last five cervical (C3-C8) vertebrae. The muscles run anterodorsad and insert at the dorsocaudal aspect of C6-C2.

* The *mm. interarticulares* (MIART) are short intervertebral muscles occuring from C2 downwards. These muscle slips run between the ventral side of the zygapophysis near the articulation, to the slightly more dorsal aspect of the previous vertebrae.

* The *mm. interarcuales* (MIARC) are also very short intervertebral muscles running from the neural arch of the cervical vertebrae (from C2) to the lateral side of the neural arch of the previous vertebra.

* The *mm. interspinales* (MIS) are similar to the MIARC but run from the neural spine to the lateral aspect of the neural spine of the previous vertebrae.

The occipito-vertebral group

* The *m. obliquus capitis* (MOCa) is situated in the anterior neck region, laterally with respect to the MSCa and dorsally to the MLCe. Usually two disctinct parts can be discerned: the MOCa magnus and the underlying MOCa inferior. The magnus part originates at the laterodorsal side of the second (partly tendinously) and the third (fleshy) cervical vertebrae. Whereas the smallest and dorsal-most part of the MOCa magnus inserts at the lateral-most aspect of the quadrate process of the parietal, the bulk of the fibres run outwards and insert at the dorsocaudal edge of the paraoccipital process. The MOCa inferior arises at the dorsal side of C2, and its fibres run anteriad to insert at the dorsocaudal side of the postzygapophysis of the atlas (C1).

* The *m. rectus capitis* (MRCaP) is one of the deep neck extensors and lies ventral to the MSCa, and mesial to the MOCa. The MRCaP can also be subdivided into two parts: a lateral part (pars maior; NISHI, 1916), originating at the dorsal side of C2 and inserting at the dorsal aspect of the supra- and exoccipital bones; and a medial part (pars minor; NISHI, 1916) originating at the C1-C2 junction and inserting just laterally to the ventral slip of the MSCa at the dorsal aspect of the supraoccipital bone.

The longissimus cervico-capitis complex

The *longissimus cervico-capitis* complex is the cervical extension of the m. longissimus dorsi. The complex originates at the level of the last cervical and first four thoracic

vertebrae at the lateral edge of the prezygapophyses. The fibres run anteriad and converge at the level of C5-C4 to form a strong "zwischensehne" (NISHI, 1916) that inserts at the postzygapophysis of the atlas. From the C5-C4 level upwards, several clearly individualised bundles can be identified:

* The *m. transversalis cervicis* (MTCe) consists of two parts: a first one originating at the lateral side of the prezygapophyses of C3 and C4, and a second part originating on the ventrolateral side of C1-C3. The dorsal part inserts together with the first muscle belly of the m. illiocostalis cervicis at the lateral side of the postzygapophysis of the atlas. The more ventrally situated part inserts with the m. rectus anticus, and the m. illiocostalis capitis at the tuberculum spheno-occipitale at the ventral side of the basi-occipital.

* The *m. articulo-parietalis* (MAP) is situated dorally in the neck and arises at the level of C4 on the "zwischensehne". From their origin, the fibres radiate anterolaterally and insert at the posterodorsal aspect of the quadrate process of the parietal, ajacent to the insertion of the MSCa, and dorsal to that of the MOCa.

* The *m. transversalis capitis* (MTCa) is located dorsolaterally in the neck and originates at the "zwischensehne" at the level of C2-C6. The fibres turn outwards, converge, and insert ventral to the insertion of the MOCa at the laterocaudal edge of the paraoccipital process, and lateral of the MAP at the lateralmost edge of the posterior side of the quadrate process of the parietal.

The rectus anticus

* The *m. rectus anticus* (MRA) is the ventralmost neck muscle and best-developed neck flexor. It arises at the ventral sides of the processus transversi of C2-C6, and from the medial, proximal side of the third and fourth cervical rib. The fibres run anteriad and outwards at an angle of about 45°. Laterally the fibres converge to form a strongly developed tendon that inserts at the tuberculum spheno-occipitale.

The ventral and lateral monoarticular muscles

* The *mm intertransversarii* (MIT): these small intervertebral muscles are situated at the deepest level of the ventrolateral side of the neck. The first MIT is the one running inbetween C1 and C2. The m. intertransversarii can be subdivided into two separate parts. A more superficial part runs from the caudal side of the processus transversus to the anterior side of the processus of the next vertebra, while the deeper part originates at the ventral side of the processus transversus and runs to the anteroventral side of the processus transversus of the next vertebra.

* The *mm atlantico-occipitales* (MAO) are two short muscles similar to the mm. intertransversarii, but attaching to the skull instead. The dorsal-most of the two originates at the ventral side of the atlas and runs to the tuberculum spheno-occipitale; the somewhat more ventrally originating muscle inserts medial to the other MAO at the basioccipital.

The illicostalis complex

* The *m. illiocostalis cervicis* (MICoCe) forms, together with the m. illiocostalis capitis, the cervical extension of the m. illiocostalis dorsi (NISHI, 1916). Both muscles of the complex are situated at the lateral side of the neck, lateroventral with respect to the m. longissimus cervicis. The MICoCe consists of four distinct bellies. The posterior two are the smallest and originate at the fascia dorsi at the level of C7. Both muscles insert tendinously at the diapophyses of C5 and C4. The next belly (more cranially) is somewhat bigger and originates at the dorsolateral edge of the m. longissimis cervicis at the level of C6. The fibres run anteroventrad and insert by means of an aponeurosis at the diapophysis of C3. The cranial-most belly is the largest, and originates in a bipartite manner at the dorsolateral edge of the m. longissimus cervicis at the level of C5. Both parts unite and insert tendinously at the postzygapophysis of the atlas.

* The *m. illiocostalis capitis* (MICoCa) originates at the level of C4-C3 at the dorsolateral edge of the *m. longissimus cervicis* and inserts with the *m. longissimus capitis* at the ventral side of the basioccipital.

The scalenus anticus

* The *m. scalenus anticus* (MSA) originates at the hypapophyses, and the ventral aspect of the processus transversi of C1-C3. The fibres run posteriad and insert at the first cervical rib.

The musculature associated with the pectoral girdle

* The *m. episternocleidomastoideus* (MEM) originates at the connective tissue associated with the clavicle. The fibres curve anterodorsad around the neck and insert at the connective tissue at the posterolateral side of the parietal, just ventral to the origin of the m. depressor mandibulae.

* The *m. serratus* (MS) consists of three bellies originating at the posteromedial side of the suprascapula. The fibres run anteroventrad and insert at the cervical ribs of C3 and C4.

* The *m. levator scapulae superficialis* (MLSS) is, with the exception of the MEM, the lateral-most muscle in the cervical region. The muscle originates tendinously at the ventrolateral side of the diapophysis of the atlas. Its fibres run posterodorsad and insert at the anterolateral side of the suprascapular bone.

* The *m. levator scapulae profundus* (MLSP) lies just ventral, and adjacent to the superficial part. Its origin is similar to that of the superficial part. The fibres run posterodorsad and insert at the ventral anterolateral side of the suprascapula, and at the dorsal side of the clavicle.

* The *m. trapezius* (MT) originates at the lateral side of the clavicle and inserts, just dorsal to the suprascapula at the fascia dorsalis.

* The *m. trapezius anterior* (MTA) also originates at the dorsal aspect of the clavicula. Two distinct parts can be discerned, based on differences in the insertion : the anteriormost part inserts at the lateral edge of the fascia dorsalis, just posterior to the MEM ; the fibres of the more posterior part on the other hand run directly dorsad, cover the first part of the MLS, and insert at the fascia dorsalis.

DISCUSSION

The only other study dealing with the cervical musculature in helodermatids is that of RIEPPEL (1980). A comparison of these data with ours is, however, difficult as RIEPPEL (1980) discussed only parts of the cervical system, and used a different nomenclature. For example, the longissimo cervico-capitis complex as described here is not recognised as such by RIEPPEL (1980). The most important differences are situated in the MSCa and the MRA. In the animals examined here, no slip of the MSCa running outward to the paraoccipital process was present. This character was, however, used to distinguish helodermatids from other platynotan lizards (RIEPPEL, 1980). Presumably this slip as described by RIEPPEL (1980) corresponds to a part of the MAP as described here. The other important difference lies in the MRA. Whereas RIEPPEL (1980 ; following the terminology of OELRICH, 1956) discerned two separate muscles (the mesial m. rectus capitis anterior and the lateral m. longissimus capitis), in the animals examined in the present study these two clearly form a single muscle : the fibres coming from the mesial part (= m. rectus capitis anterior of RIEPPEL, 1980) converge at the ventrolateral side of the neck to form one muscle (= m. longissimus capitis of RIEPPEL, 1980).

Despite the importance of the cervical system, the available information on neck musculature in lizards in general, is scarce, incomplete, and sometimes even erroneous. For example, in most accounts (DE VIS, 1884 ; JENKINS & TANNER, 1968 ; AVERY & TANNER, 1971) fewer muscles are described than are actually observed for closely related representatives (pers.obs.). Nevertheless, the data gathered in this study indicate that, in general, the bauplan of the cervical musculous system appears stable within closely related phylogenetic groups. For both the *Heloderma* specimens examined here, few or no interspecific differences in neck musculature were observed. Similarly, preliminary data on agamid (*Plocederma stellio* (Blyth,1854) and *Hydrosaurus amboinensis* (Kaup,1828)) and closely related uromastycine lizards indicate a general within-group conservatism.

In lizards, the feeding process can be categorised as lingual or inertial (BRAMBLE & WAKE, 1985). In inertial feeding, prey transport is accomplished without the intervention of the tongue ; instead, a backward acceleration is imparted upon the prey when it is released from the teeth. This acceleration can only be achieved by moving the head relative to the body of the animal. Clearly the neck plays a most crucial role in this respect. However, within lizards the purely kinetic inertial feeding mode has only been described for varanid and helodermatid lizards (SMITH, 1982 ; HERREL *et al.*, 1997a). A comparison of the musculature in *Heloderma* with that of varanids (NISHI, 1916) indicated few differences. This observation can be related to the fact that both groups of lizards include more (*Varanus* ; SMITH, 1982) or less (*Heloderma* ; HERREL *et al.*, 1997a) inertial components in their feeding mechanism. The fact that, in general, few differences in neck musculature

were noted in the whole group of anguinomorph lizards (RIEPPEL, 1980) may indicate that: (1) all anguinomorphs use (at least partially) some inertial components in their feeding system or (2) the complexity of the cervical musculature is a primitive trait for the whole group and has been exapted in relation to inertial feeding in *Heloderma* and *Varanus*.

The neck muscles also play an important role during prey processing for non-inertial feeders. In all lizards examined so far, jaw opening is achieved partially by an elevation of the neurocranium, which is mediated by activity of the dorsal cervical musculature (HERREL *et al.*, 1995, 1997b; CLEUREN, pers. comm.). Similarly, during jaw closure the ventral cervical musculature can aid in biting by actively depressing the neurocranium (GANS *et al.*, 1985). Consequently, the MSca, MAP on the dorsal side, and the MRA on the ventral side of the neck are strongly developed, at least in agamid and scincid lizards (pers. obs.).

Preliminary data on other lizards (geckoes and lacertids) indicate that the evolutionary shift from a lingual-based to a predominantly inertial feeding system within lizards apparently coincides with an increase in the complexity of mainly the epaxial components of the cervical musculature. This should be tested, however, by a thorough examination of the cervical musculature in representatives of these and other lizard groups within a strict phylogenetic framework. The nomenclature of lizard neck muscles as proposed here (based on the work of NISHI, 1916), which encompasses the full compexity of the system, should enable future workers to interprete the neck musculature in an evolutionary context.

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THE USE OF INTEGUMENTAL PORE SIGNATURE IN THE CHARACTERISATION OF SPECIES OF THE GENUS *THERMOCYCLOPS* KIEFER, 1927 : THE CASE OF *THERMOCYCLOPS EMINI* (MRÁZEK, 1895) (CRUSTACEA : COPEPODA : CYCLOPOIDA)

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Abstract. The possibility to use the integumental pore signature as a tool in the identification of cyclopoid copepods is explored. A full classical description of female specimens of *Thermocyclops emini* (Mrázek, 1895) is supplemented by mapping of the integumental perforations. These are bilaterally symmetrical, and most occupy constant geometrical positions. On the metasome and urosome the pattern changes from one segment to another, and differs in ventral and dorsal position (on the urosome). The total number of perforations varies between 193 and 202.

Key words : Copepods, *Thermocyclops emini* (Mrázek, 1895), integumental pore pattern, characterisation.

INTRODUCTION

Following the investigations of FLEMINGER (1973), the number and position of integumental organs became used as taxonomic tools to classify calanoid copepods. Many species of copepods are now known to possess integumental perforations ; these pores, with their underlying soft tissue, form integumental organs that are usually arranged in a discrete pattern (KOOMEN, 1992). The perforations can be made visible after a preparation of the cuticle that includes digestion of all tissues and staining. Investigating the calanoid *Paramisophria platysoma* Susumu & Mitsuzumi, 1990 by Scanning Electron Microscopy (S.E.M), SUSUMU & MITSUZUMI (1990) found that the integumental organs of the female cephalothorax were nearly symmetrical in distribution.

In their habitats, copepods migrate, feed, breed and perform different socio-behavioural activities (MAUCHLINE, 1977) for which these eye-less animals must rely on chemical and vibrational communication. Integumental organs are probably essential to locate and correctly identify a potential mate or predator without visual aids (FLEMINGER, 1973).

Such monitoring and interpretation suggests a variety of organ types, such as sensory receptors and glands (MAUCHLINE, 1977), with a possibly species-specific distribution across the body surface. Indeed, it is now known that each species of the genus Eucalanus Dana, 1853 has a distinct pore signature (FLEMINGER, 1973; MAUCHLINE, 1977). MALT (1983) successfully used integumental pore patterns to separate two Poecilostomatoida of the genus *Oncaea* Philippi, 1843: *O. ornata* Giesbrecht, 1902 and *O. englishi* Heron, 1977, which otherwise have a similar morphology.

Unfortunately information on the taxonomic value of the pore signature is still limited, and little is known of cyclopoids. Therefore, we aimed in the present study to provide a basis for investigations on the taxonomic value of the pore pattern of a variety of cyclopoid species and genera.

We studied the pore signature of the genus *Thermocyclops* Kiefer, 1927. As a test case, *Thermocyclops emini* (Mrázek, 1895) is here redescribed using classical morphological characters, including mouth parts and thoracopods, but its pore signature is also mapped. Mapping was carried out on the dorsum and sides of the rostrum and cephalosome; the dorsum of the metasome; the dorsum, ventrum and sides of the urosome. Perforations occur across the whole length of an animal and occupy bilaterally symmetrical positions. They are either simple, double or in groups of three. The preparation for routine work made it impossible to differentiate the integumental organs between sensilla (hair, cone, peg, pit) and gland openings. Thus, this work is limited to mapping the number and position of these organs.

MATERIAL AND METHODS

Dr. L. Mwebaza Ndawula collected material in 1990 from Lake Victoria. Samples were preserved in 4% formalin. Only adult females were used as no male was present in the studied material. Specimens were identified on the evidence of their elongated shape, antennules, long furcal rami, and dorsal setae of furcal rami, and sorted from the sample. For routine morphometrical analysis, unstained specimens were used. After dissection under a dissecting microscope, parts were mounted in glycerine on a covered slide. For the digestion of tissues, staining and washing procedure, the method of FLEMINGER (1973) was used: specimens were first heated at 80-100°C for four hours in KOH 10%. The remaining exoskeletons were first kept for some minutes in distilled water and in 70% alcohol for washing. Clean animals were then stained in Chlorazol black in lactophenol. Parts were studied separately except for the urosome that was studied intact. Dissections were done using tungsten needles electrolytically sharpened in KOH solution. Perforations were analysed and drawn under immersion oil using a camera lucida on a Medilux-12 microscope. For each part investigated (rostrum, cephalosome, each metasomal somite and urosome), a minimum of five specimens was used. Scanning Electron Microscopy (S.E.M) micrographs of critical point dried, gold-coated specimens were taken to complete optical images. Total body length excluded the furcal setae and was measured from the anterior basis of the rostrum to the posterior edge of the caudal rami. The width of the cephalosome was measured at its widest part.

The terminology used in VAN DE VELDE (1984), HUYS & BOXSHALL (1991) was adopted. All measurements are in µm and abbreviations are as follows: T. L: Total Length, L: Length, W: Width, Spi: Internal spine, Spe: External spine, Si: Internal seta, Smi: Median internal seta, Sme: Median external seta, Se: External seta, Sd: Dorsal seta, Sl: Lateral seta, SD: Standard Deviation.

RESULTS

Redescription of females

Measurements are given in Table 1. The animals are elongate with a length range of 828-928 µm, mean 878.8 µm (n=10). DUSSART'S (1982) and EINSLE'S (1970), material from the same Lake measured 1.000-1.100 µm and 900-946 µm, respectively. In his original material, Mrázek (1895) cited 990 µm. Cephalosome and genital somite longer than broad; ratios 1.28 and 1.43, respectively. On Enp_3P_4, Spi 2.43 times as long as Spe, and segment 1.15 times as long as Spi, Furca 3.52 times as long as broad, Smi 1.47 times as long as Sme, Sd 1.14 as long as Si, Sd 4.80 as long as Sl, Sd 3.03 as long as Se, Si 2.65 as long as Se and Si 1.9 as long as Furca.

Antennule (Fig. 2: N): Long and reaching the 4th metasomal somite, 17 segmented, with a row of minute spinules on first segment, 10th and 13th segments devoid of setae; segments 16th and 17th with a relatively well developed hyaline lamella (Fig. 6: B, C, D, E).

Antenna: Spine pattern on frontal and caudal sides of basipodite as in Fig. 5: H, I. Second endopodite with four setae (Fig. 5: I).

Labrum: Ventrally with eleven sharp teeth, long hair dorsally surrounding two strong median teeth; lateral edges rounded (Fig. 5: C, D).

Mandible: Pars molaris with series of sharp teeth, mandibular palp provided with three unequal setae: a small and naked seta plus two long and feathered setae (Fig. 5: A).

Maxillule (Fig. 5: G).

Maxilla (Fig. 5: B): Inner side precoxa with two strong and feathered setae. Coxa with a median seta, provided with strong spines. Outgrowth of coxa with two unequal setae, the strongest feathered, with spinules at the end. A stout claw-like seta bearing a strong seta prolongates the maxilla. Stout seta provided with variable strong spines. Endopodite one-segmented with two spinous setae and three strong feathered setae with spinules at the end.

Maxilliped: Basal segment (fusion of precoxa and coxa) provided with three feathered setae, frontal side glabrous (Fig. 5: E), caudal side with spinules (Fig. 5: F). Basis of maxilliped with two feathered setae, frontal side with two groups of minute spinules (Fig. 5: E); caudal side with long, strong spines (Fig. 5: F). Endopodite two-segmented. Caudal side of proximal segment with a group of spines, segment bearing a long feathered seta. Distal segment with three unequal setae, the longest two feathered.

Thoracopods P_1-P_4: Spine formula: 2-3-3-3, armature of the segments (Figs 3: B; 4: B) outgrowths of the connecting lamellas naked.

P_1: Inner distal margin of basipodite with a spine, inner part of basipodite with setules (Fig. 3: A, D).

TABLE 1

Measurements of the females of Thermocyclops emini *(Mrázek, 1895) from Lake Victoria*

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Mean*	*SD*
Total length	T. l	915.00	928.00	853.00	881.00	859.00	896.00	878.00	900.00	850.00	828.00	878.80	29.92
Cephalosome	L	321.00	337.00	325.00	325.00	328.00	343.00	343.00	328.00	318.00	315.00	328.30	9.31
	W	259.00	243.00	243.00	262.00	256.00	275.00	275.00	262.00	253.00	243.00	257.10	11.43
	L/W	1.24	1.39	1.34	1.24	1.28	1.25	1.25	1.25	1.26	1.30	1.28	0.05
Thorax	L	243.00	237.00	243.00	240.00	231.00	240.00	234.00	237.00	228.00	209.00	234.20	9.60
Genital Somite	L	128.00	128.00	109.00	131.00	115.00	115.00	128.00	140.00	115.00	112.00	122.10	9.64
	W	84.00	87.00	84.00	84.00	84.00	87.00	81.00	87.00	87.00	87.00	85.20	1.99
	L/W	1.52	1.47	1.30	1.56	1.37	1.32	1.58	1.61	1.32	1.29	1.43	0.12
Enp3P4	L	60.00	62.00	59.00	59.00	58.00	64.00	54.00	64.00	63.00	58.00	60.10	3.01
	W	17.00	17	17	18	15	18	18	17	17	18	17.20	0.87
	L/W	3.53	3.65	3.47	3.28	3.87	3.56	3.00	3.76	3.71	3.22	3.49	0.25
	Spi	53.00	52.00	53.00	50.00	53.00	51.00	48.00	54.00	53.00	52.00	51.90	1.70
	Spe	25.00	20.00	21.00	20.00	21.00	23.00	20.00	21.00	20.00	24.00	21.50	1.75
	Spi/Spe	2.12	2.60	2.52	2.50	2.52	2.22	2.40	2.57	2.65	2.17	2.43	0.18
Abdomen	L	275.00	275.00	281.00	256.00	228.00	250.00	259.00	265.00	231.00	228.00	254.80	19.11
Furca	L	75.00	75.00	71.00	68.00	78.00	78.00	71.00	71.00	75.00	78.00	74.00	3.38
	W	21.00	21.00	21.00	21.00	21.00	21.00	21.00	21.00	21.00	21.00	21.00	0.00
	L/W	3.57	3.57	3.38	3.24	3.71	3.71	3.38	3.38	3.57	3.71	3.52	0.16
Furcal setae	Si	137.00	146.00	134.00	153.00	146.00	140.00	140.00	156.00	150.00	150.00	145.20	6.87
	Smi	281.00	300.00	293.00	290.00	291.00	287.00	290.00	287.00	271.00	296.00	288.60	7.66
	Sme	181.00	190.00	203.00	203.00	203.00	193.00	203.00	203.00	196.00	187.00	196.20	7.72
	Se	53.00	56.00	59.00	53.00	56.00	59.00	50.00	56.00	50.00	56.00	54.80	3.06
	Sd	162.00	156.00	171.00	171.00	156.00	171.00	165.00	171.00	159.00	178.00	166.00	7.14
	Sl	31.00	37.00	34.00	37.00	37.00	37.00	31.00	34.00	31.00	37.00	34.60	2.62
	Smi/Sme	1.55	1.58	1.44	1.43	1.43	1.49	1.43	1.41	1.38	1.58	1.47	0.07
	Sd/Si	1.18	1.07	1.28	1.12	1.07	1.22	1.18	1.10	1.06	1.19	1.14	0.07
	Sd/Sl	5.23	4.22	5.03	4.62	4.22	4.62	5.32	5.03	5.13	4.81	4.80	0.38
	Sd/Se	3.06	2.79	2.90	3.23	2.79	2.90	3.30	3.05	3.18	3.18	3.03	0.18
	Si/Se	2.58	2.61	2.27	2.89	2.61	2.37	2.80	2.79	3.00	2.68	2.65	0.21

P_4: Inner margin of the basipodite and both sides of the outgrowths of connecting lamella glabrous; distribution of setules on sides of coxopodite as in Fig. 4: C, G. Enp_3, 3.49 times as long as broad, Spi 2.43 times as long as Spe and 1.15 times shorter than the segment.

P_5: Setae on terminal segment almost equal (Fig. 4: E).

P_6: Three setae on thoracopod: two strong, dwarfed, naked setae next to a relatively long, feathered seta (Fig. 6: G). A variable number of openings on distal area of the implantation of P6, as described in *Mesocyclops leukarti* (Claus, 1857) by Van de Velde (1984).

Lateral margins of last metasomal somite glabrous (Figs1: I, J, K, L; 5: H).

Genital somite and receptaculum seminis: Somite 1.43 times as long as broad.

Lateral arms of the receptaculum almost straight, as described in DUMONT *et al.* (1981) and Einsle (1970). Copulatory pore and pore canal in the middle of receptaculum; a large «gland opening» (Figs 1: J, K; 6: A, G, H) at proximal edge of integument, near distal edge of last metasomal somite. P6 implanted laterally on genital somite (Fig. 6: G).

Last (anal) somite: Ventrally, distal edge with a row composed of a variable number of spines (Fig. 1: K; 6: A), naked dorsally (Fig. 1: I, 6: F).

Integumental pore pattern

Rostrum and cephalosome

Rostrum (Fig. 1: A, C, D.): Eight perforations present. One in the middle, another at the limit of the rostrum and cephalosome. The other six surround two sieve plates. In five investigated specimens, there was no variation in number or position of perforations.

Cephalosome: Maximum 91 pores were counted. Seventy-seven are in dorsal position (Figs 1: B, Ca, E; 2: A, B), 14 in lateral position (Fig. 1: C; a). Their distribution delimits three zones (Fig. 1: B, C, E; I, II, III). The first includes a maximum of 37 perforations, arranged symmetrically. Thirty-six of them are geometrically disposed around a single mediocentral one. Half of fifteen specimens analysed had double perforations at some positions as highlighted in Fig. 1: B & E (arrow in Fig. 2: Photograph A), they were simple in the other half (Fig. 1: B, E).

The second area has 26 perforations. Twenty two, symmetrically disposed, surround four mediocentral perforations.

The third zone is composed of 14 perforations and lacks median pores.

Metasome

First somite: Integument with nine pores, geometrically disposed (Fig. 1: F).

Second somite: The integument has between 24 and 26 perforations. Two of them are exactly on the longitudinal axis. A position in front of the posterior perforation can hold either a symmetrical perforation or not, as in Figs 1: G; 2: C. On the same somite median pores occur, either single or double (highlight in Fig. 1: G & M, arrow in Fig. 2: C). Seven out of ten checked animals presented double perforations at this position.

Third somite: Integument with 21 pores (Fig. 1 : H ; 2 : D). The number and positions were constant in five investigated specimens.

Fourth somite: Figs 1 : I, L ; 2 : E. The perforation number varied from four to eight.

Urosome

Genital somite (*First somite*) (Figs 1 : I, J, K ; 2 : F) : Dorsally with 10 perforations, three on the anterior middle of the somite, six in a transverse row near the posterior perforation and one on the longitudinal axis, close to the distal edge. Ventral side with four perforations.

Second somite (Figs I, J, K ; 2 : G) : Seven perforations are present dorsally ; six on the sides and one on the longitudinal axis. In ventral position, only two perforations are seen.

Third somite (Figs 1 : I, J ; 2 : G) : One perforation only on dorsum, ventral side without any pores.

Fourth (anal) somite (Figs 1 : 1 : I, J, K ; 2 : G) : six pores present dorsally, bilaterally symmetrical around the longitudinal axis. In ventral position, two pores near the distal edge of the somite.

Furcal ramus

With six perforations : two on the dorsum and four on the ventrum (Fig. 1 : I, J, K).

CONCLUSION

To date, only morphology and morphometrical analysis have been used to identify species within *Thermocyclops* Kiefer, 1927. The distribution of the integumental perforations, sites of the integumental organs, herein used to redescribe *Thermocyclops emini* (Mrázek, 1895), revealed limited variation in total pore number (from 193 to 202). Most positions were fixed. Further work will seek to clarify whether this conclusion can be generalised to other populations, and whether the pore signature of *Thermocyclops emini* (Mrázek, 1895) is unequivocally distinct from that of its congeners.

ACKNOWLEDGEMENT

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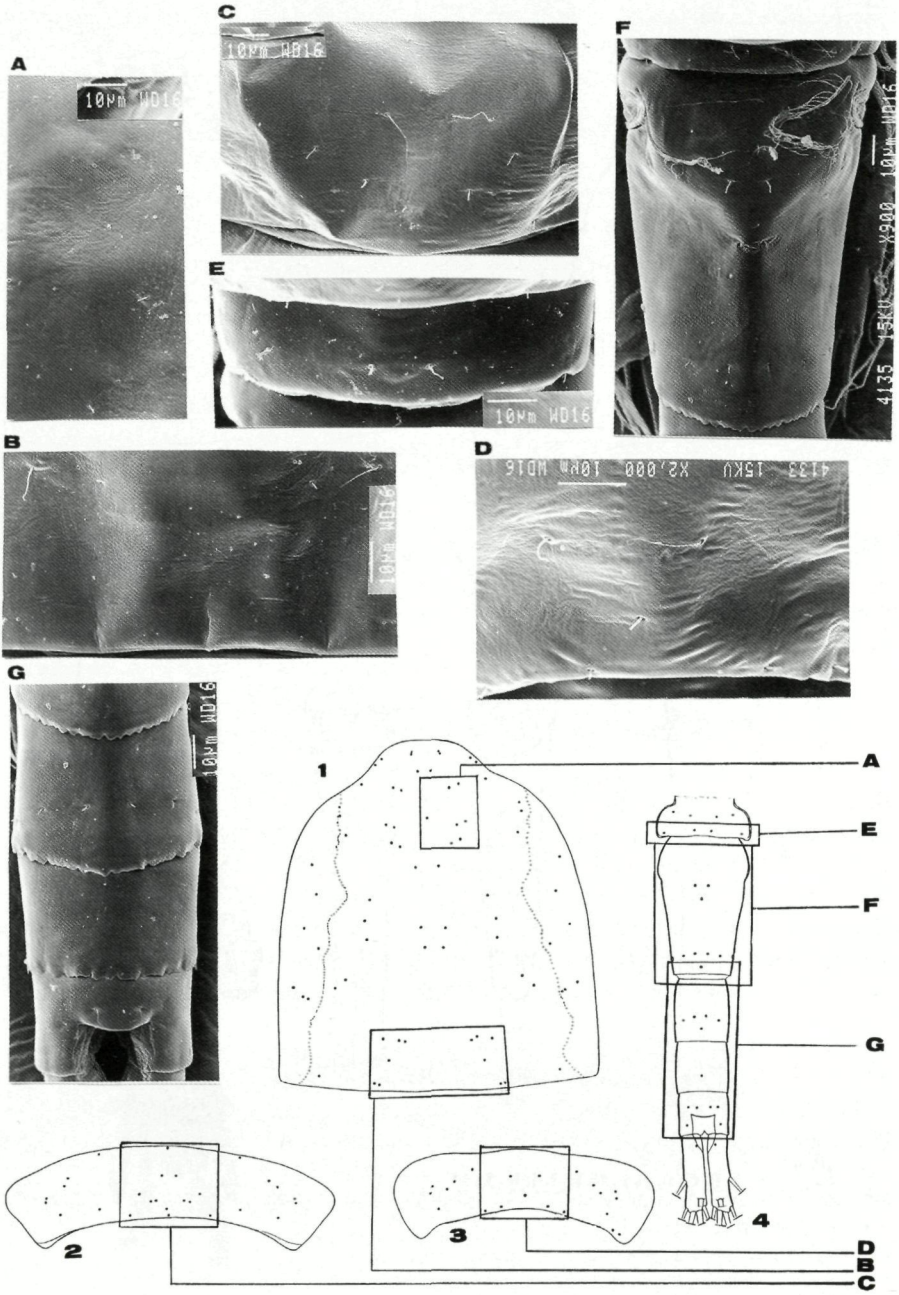

Fig. 1. – *Computer-edited S.E.M micrographs.* – 1. : Cephalosome : Photos A and B ; 2. : Second metasomal somite : Photo C ; 3. : Third metasomal somite : Photo D ; 4. : Urosome and last metasomal somite : Photos E, F, G.

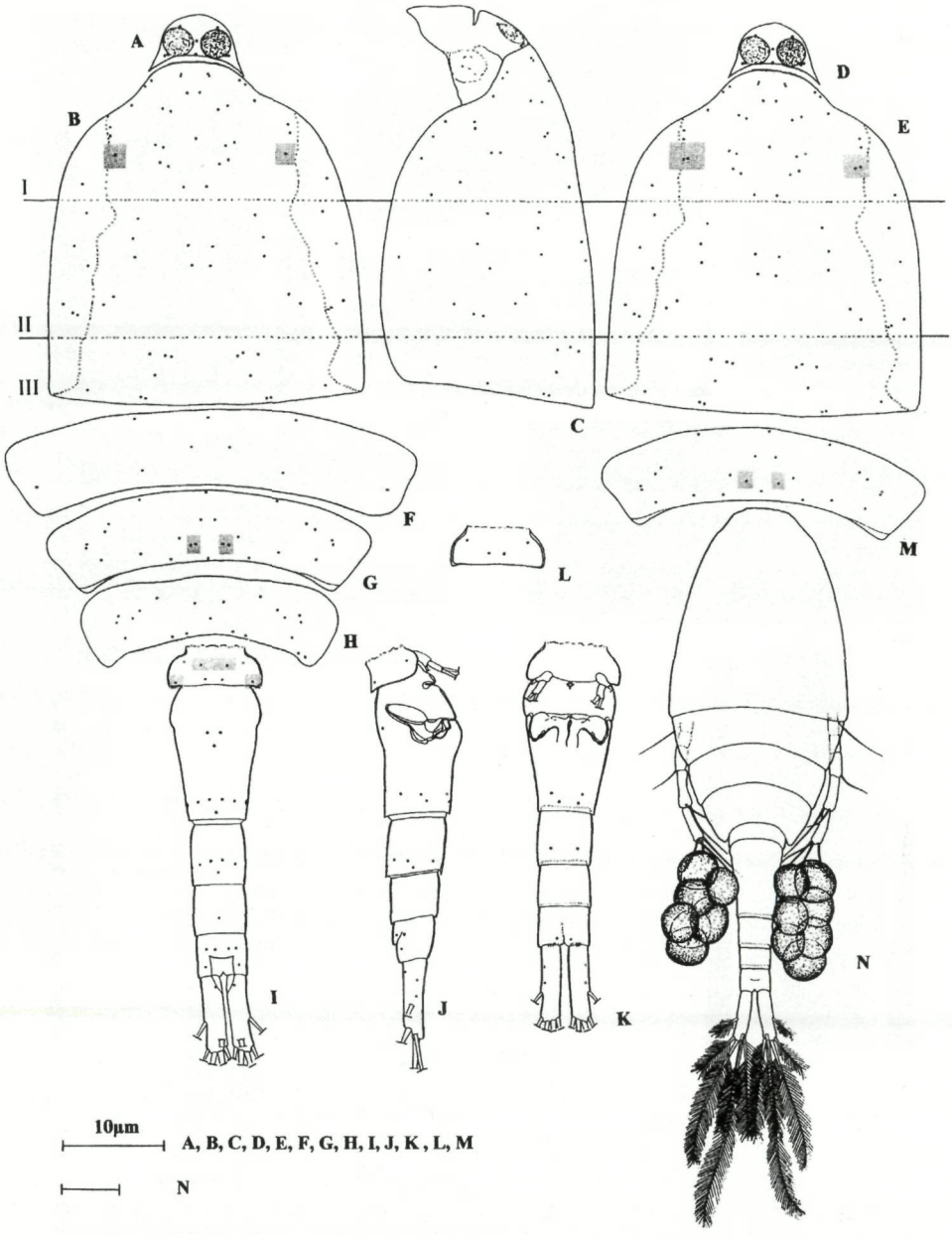

Fig. 2. – *Integumental perforations and habitus.* – A, D : Rostrum ; B, E : Cephalosome (dorsal) ; C : Cephalosome (lateral) F : First thoracic segment ; G, M : Second thoracic segment ; H : Third thoracic segment ; I : Urosome (dorsal) with last thoracic segment ; J : Urosome (lateral) ; K : Urosome (ventral) ; L : Last thoracic segment ; N : Habitus. Scale bars = 10 µm.

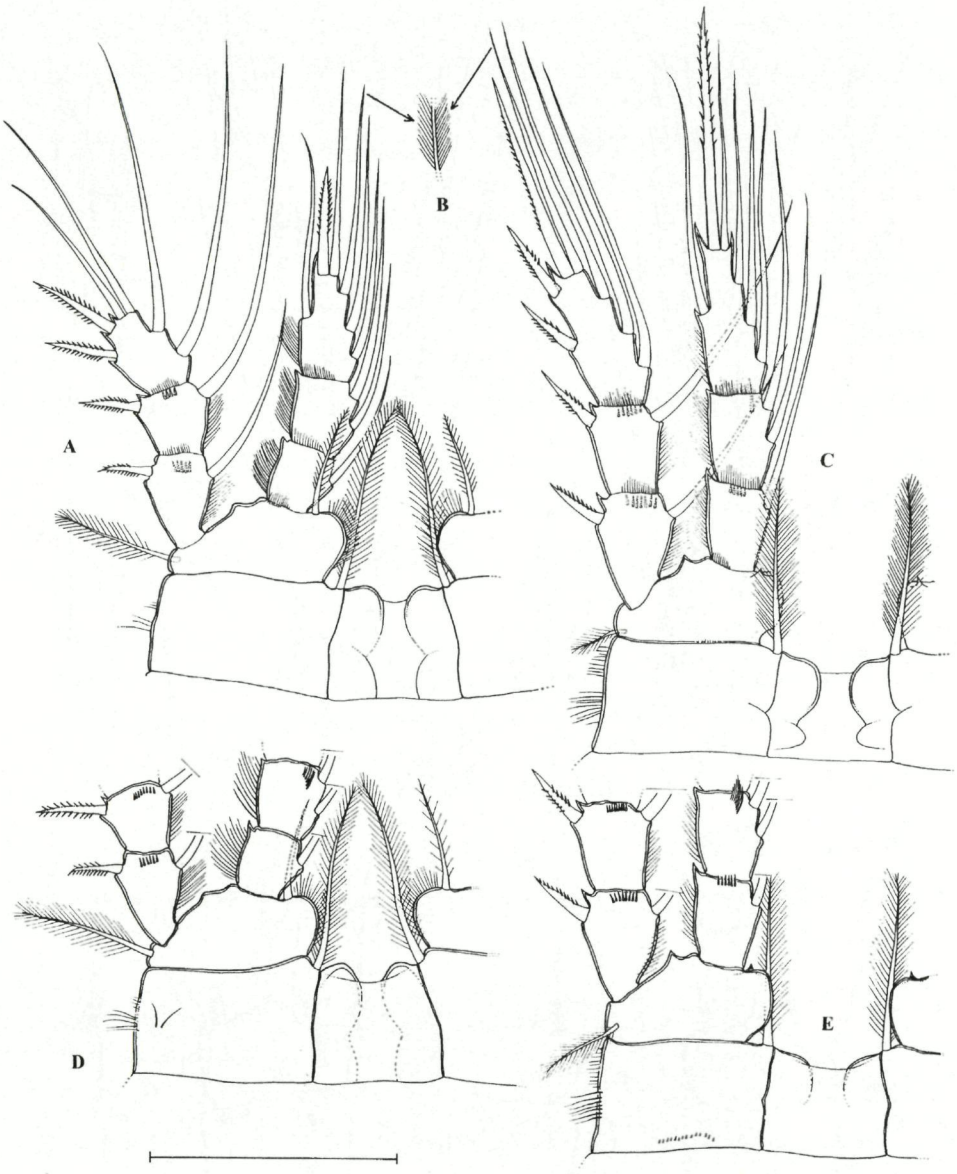

Fig. 3. – *Thoracopods.* – A, C : P_1-P_2 frontal side ; D, E : P_1-P_2 caudal side ; B : Ornamentation of setae of endo. and exopodites. Scale bars = 10 µm.

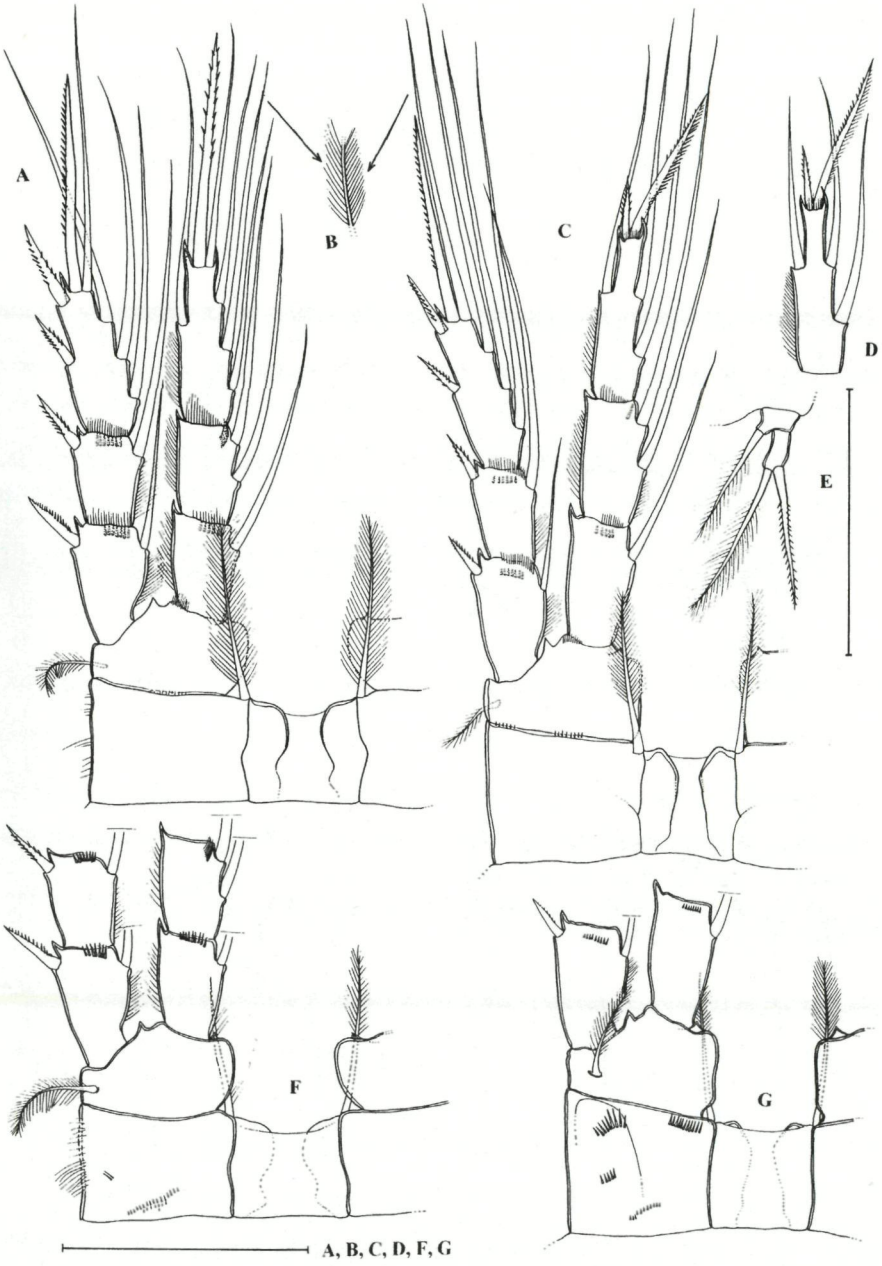

Fig. 4. – *Thoracopods.* – A, C : P_3-P_4 frontal side ; F, G : P_3-P_4 caudal side ; D : Enp_3P_4 ; E : P5 ; B. – Ornamentation of setae of endo. and exopodites. Scale bars = 10 µm.

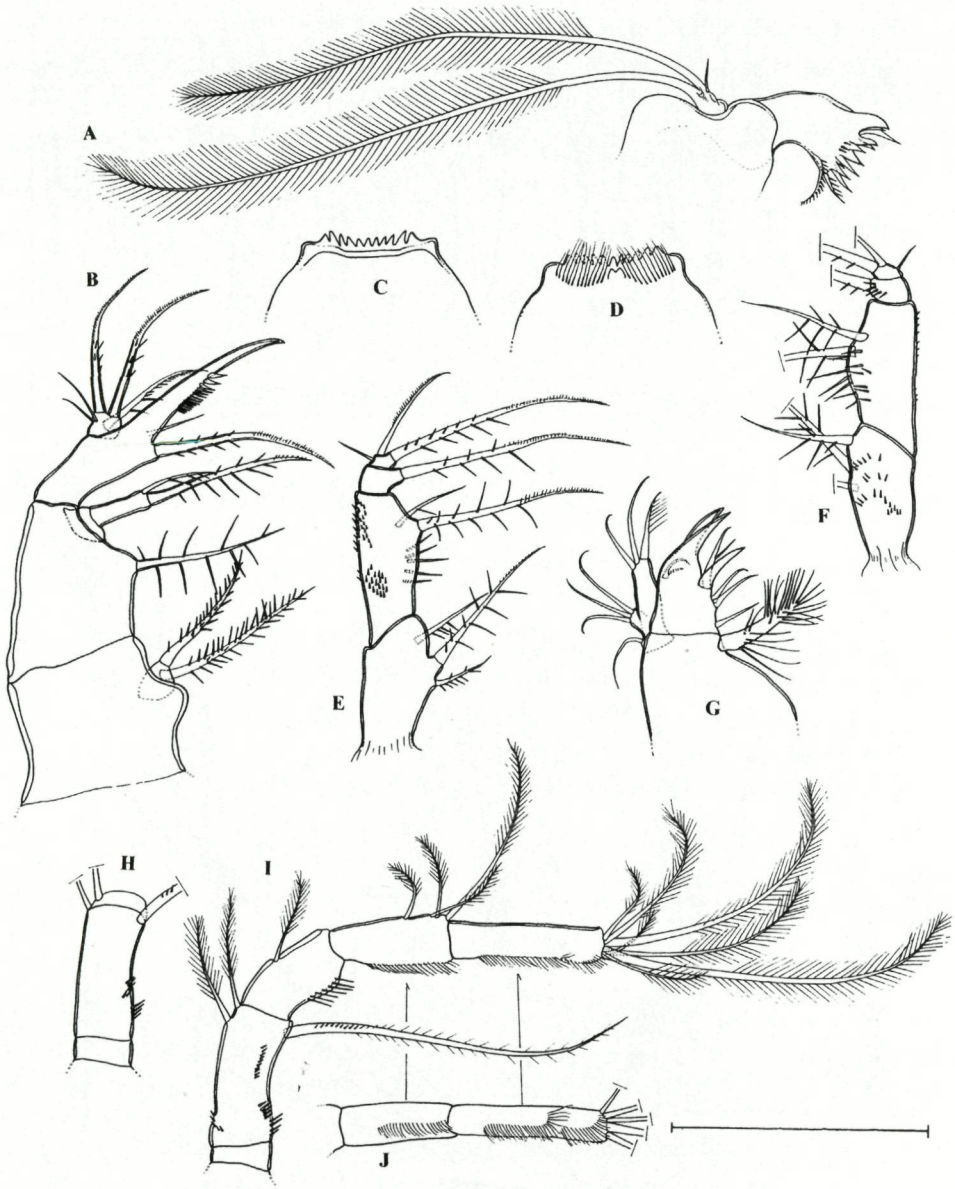

Fig. 5. – *Mouth parts and antenna.* – A : Mandible ; B : Maxilla ; C, D : Labrum (C : ventral, D : dorsal) ; E, F : Maxilliped ; G : Maxillule ; H : Basipodite A_2 (frontal side) ; I : Antenna (caudal side) ; J : $Enp_{2,3}$ Antenna (postero-ventral). Scale bars = 10 µm.

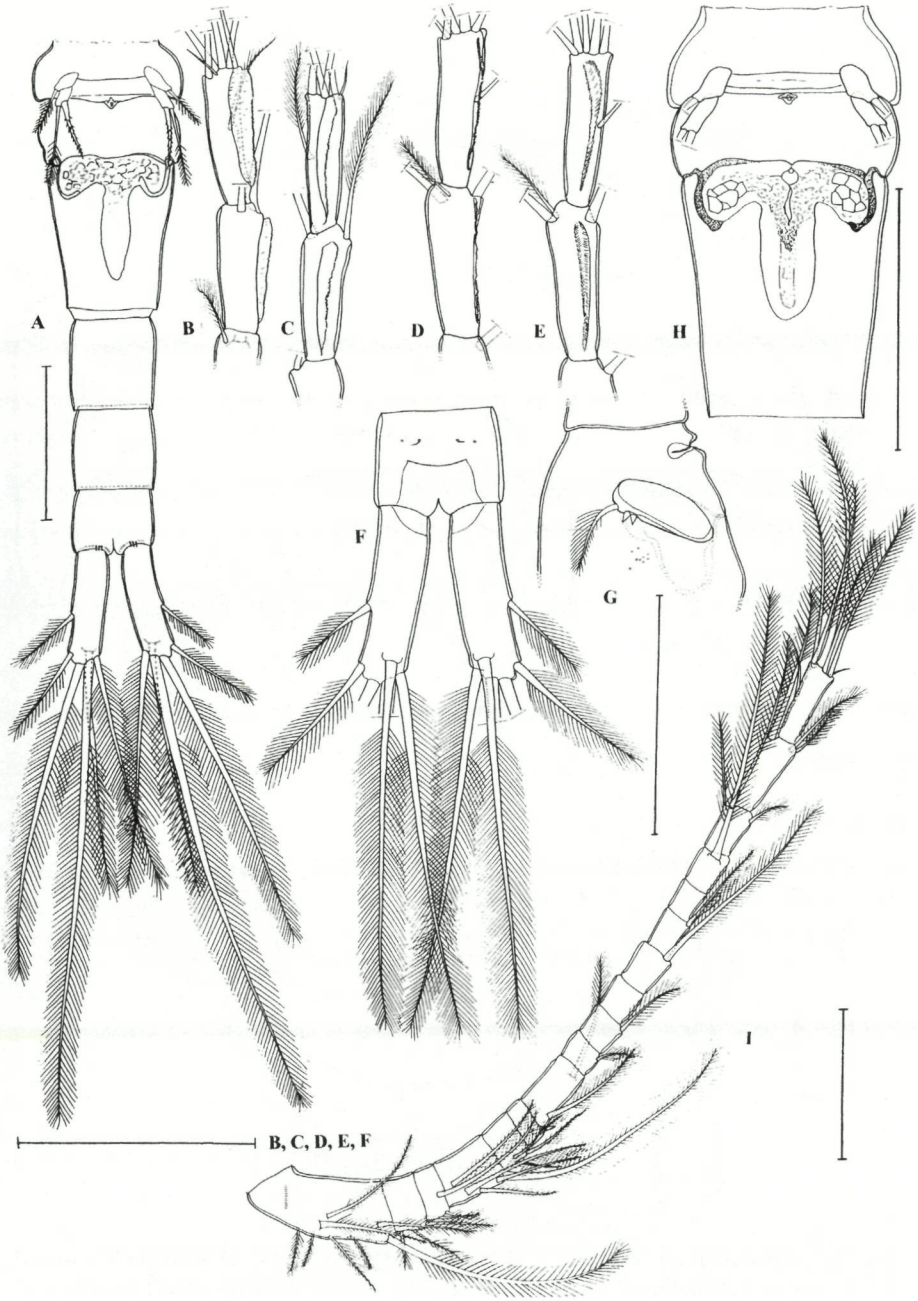

Fig. 6. – *Urosome, furca and antennule.* – A : Urosome and furca (ventral), B, C, D, E : Hyline lamella ; F : Anal segment and furca (dorsal side), G : Antennule. Scale bars = 10 µm.

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FUNCTIONAL MORPHOLOGY AND EVOLUTION OF A CARPENTER'S PLANE-LIKE TOOL IN THE MANDIBLES OF TERMITE WORKERS (INSECTA ISOPTERA)

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Abstract. The left mandible of termite workers possesses just in front of the molar plate a characteristic « premolar tooth » that, in most species, is partly or wholly hidden under the mandible. The position, structure and size of this tooth were observed and compared from a functional point of view in 46 wood-feeder species belonging to all termite families and in 33 soil-feeder species belonging to 4 different clades of Termitidae.

In all wood- and other plant matter-feeder species observed the premolar tooth resembles the chisel of a carpenter's plane-like device. It is suited to cut superficial fragments out of the wood before ingestion.

In all soil-feeder species observed the premolar tooth has lost one or several functional features that characterise wood feeders. It assists the other teeth in the gathering of soil particles towards the mouth before ingestion.

In the left mandible of termite workers, the premolar tooth thus shows clear morphological adaptations to the species' diet.

Key words : functional morphology, adaptation, mandibles, termites, diet, food processing.

INTRODUCTION

This work comes into the general scope of functional and comparative morphology of insects.

In chewing insects, mandibles are the most voluminous mouthparts, the hardest ones and those that are moved by the most powerful muscles. They play a key role in the taking and in the first mechanical processing of the food. From one group of insects to another, the morphology of mandibles may vary, particularly as regards their inner margins, which come into direct contact with food. Besides the marked differences between the mandibles of carnivorous and plant-feeder insects, very precise morphological adaptations to different plant matter-diets have been shown in some groups, notably grasshoppers (CHAPMAN, 1964). Such observations inspired the present work.

In termites, the soldier and worker mandibles play crucial roles in defence and feeding. Their morphology may vary from one genus or even from one species to another and is therefore commonly used for the systematic description of termite species. Furthermore, since the beginning of the century, they have proved to be of great interest in the study of phylogenetic relations between termite taxa, as illustrated by the works of HOLMGREN (1911 ; 1912), HARE (1937), AHMAD (1950) and KRISHNA (1970).

Several functional morphological adaptations to diet have been observed in the mandibles of termite workers, notably in the molar plate (or *mola*), i.e. the proximal masticatory part of their inner margin. This *mola* has flattened ridges for grinding in the workers of xylophagous species but becomes smooth and hollow like a spoon in soil-feeder species, as shown by SANDS (1965) for Nasutitermitinae and by DELIGNE (1966) for other families and subfamilies.

Despite the interest in mandibles for systematic and phylogenetic purposes, and their functional importance, only the external outlines of their upper sides have generally been described and represented in taxonomic papers, with a few notable exceptions (*e.g.* SANDS 1972 ; 1992 ; 1998).

The external outline of the mesal margin always shows an « apical tooth », a few « marginal teeth » and the « molar plate » (Fig. 2). During the course of an earlier work (DELIGNE 1970) my attention was drawn to a tooth situated in front of the molar plate and partly or wholly hidden under the left mandible. Due to its position we called it a « premolar tooth » (DELIGNE & PASTEELS 1969) while KRISHNA (1968), SANDS (1972 ; 1992) and MATHEWS (1977) gave it other names as discussed below.

As the premolar tooth of termite workers has not been studied so far from a functional point of view, I compared the position, structure and size of this tooth in wood-feeder and soil-feeder species, in order to detect possible adaptations to diet.

MATERIAL AND METHODS

The study is based on alcohol preserved termites belonging to 79 species and 62 genera, representing all 6 families and most of the subfamilies of termites. The list of examined species is given below.

For each species, from 2 to 5 workers were observed. The mandibles were first handled *in situ* under a stereomicroscope to enable analysis of their relative movements. The adductor apodemes were first cut with micro-scissors. The mandibles were then dissected and observed along different orientations with a stereomicroscope ; to facilitate this multidirectional observation, they were secured on a bed of thin sand covered with alcohol. Some were mounted whole on slides in Canada balsam *with the ventral side upwards* for observation with a light microscope. To observe the mesal cutting edge at right angels to the optic axis, the slide was slightly tilted, as much as necessary, under a stereomicroscope. For most species, mandibles were also prepared for the scanning electron microscope *with their denticulate inner margin upwards.* The mandibles were not cleaned before observation because the distribution of food parcels in different parts might be indicative of the role of these parts.

As a complement to morphological descriptions, the maximal length of the premolar tooth was measured in surface view using an ocular micrometer, and expressed as percentage of the total mandible length (TML, measured from the point of the apical tooth to the most proximal point of the molar plate). Although the proportions of mandible parts generally show very little variability among the workers of a given species, I consider that too few specimens have been measured to calculate a mean value. The values given are therefore rough estimations (*e.g.* >20%, >30% etc). More complete data will be published in a later paper.

The diet is established by examining the workers' intestinal contents with the help of a polarizing microscope, which improves the observation of mineral elements and plant fibres (DELIGNE, 1966). These data were checked and supplemented with other published data (notably NOIROT & NOIROT-THIMOTHÉE, 1969, GRASSÉ, 1986 and SANDS, 1998). The main diet of the major taxa is summarised in the list of examined species. As generally recognised, a wood- and other plant tissue-diet characterises all termite families, except a part of Termitidae among which a soil-diet appeared at least 4 times in the course of evolution.

List of examined species

– [W] means « mainly wood and other plant tissue feeders »
– [S] means « mainly soil feeders »
– The reference of the sample is put in quotation marks
– All cited collecting sites in Gabon are located within a radius of 80 km around Makokou

Mastotermitidae [W]

– *Mastotermes darwiniensis* Frogatt 1896, « TD 31 », North Queensland, Australia

Kalotermitidae [W]

– *Kalotermes flavicollis* (Fabricius 1793), « TD 32 », Banyuls, France
– *Neotermes desneuxi* (Sjöstedt 1904), « Mad 4 », Mandraka forest, Madagascar
– *Cryptotermes havilandi* (Sjöstedt 1900), « TN 541 », Ivory Coast
– *Glyptotermes parvulus* (Sjöstedt 1907), « TD 33 », Banco forest, Ivory Coast
– *Postelectrotermes amplus* (Sjöstedt 1925), « TD 34 », Mandraka forest, Madagascar

Termopsidae [W]

Termopsinae

– *Zootermopsis angusticollis* (Hagen 1858), « TD 35 », Philipsville, California, USA

Stolotermitinae

– *Stolotermes africanus* Emerson 1942, « N 23 », South Africa

Porotermitinae

– *Porotermes planiceps* (Sjöstedt 1904), « N 14 », South Africa

Hodotermitidae [W] ([1])

– *Microhodotermes viator* (Latreille 1804), « SAf 5 », South Africa
– *Anacanthotermes ochraceus* (Burmeister 1839), « T 500A », Beni Abbès (Algeria)

([1]) Termopsidae and Hodotermitidae are considered as separate families according to GRASSÉ (1986).

Rhinotermitidae [W]

Psammotermitinae

– *Psammotermes allocerus* Silvestri 1908, « SAf 50 », South Africa
– *P. hybostoma* Desneux 1902, « T 205 », Tamanrasset, Algeria

Heterotermitinae

– *Heterotermes sp.* Frogatt 1896, « TC 49 », Santa Cruz Island, Galapagos
– *Reticulitermes lucifugus* (Rossi 1792), « TD 38 », Banyuls, France

Coptotermitinae

– *Coptotermes silvaticus* Harris 1968, « 1145 », Belinga, Gabon

Rhinotermitinae

– *Schedorhinotermes putorius,* (Sjöstedt 1896) « TD 17 », Mayela, Gabon
– *S. lamanianus* (Sjöstedt 1926), « T 617 », Bossembele, Centr. Afr. Rep.

Termitidae [W] or [S]

Macrotermitinae [W]

– *Pseudacanthotermes militaris* (Hagen 1858), « 1056 », Makokou, Gabon
– *P. spiniger* Sjöstedt 1900, « TC 7 », Kisangani, D. R. Congo
– *Acanthotermes acanthothorax* (Sjöstedt 1898), « 1929 », Ekowong, Gabon
– *Protermes prorepens* (Sjöstedt 1907), « 1099 », Madjime, Gabon
– *Sphaerotermes sphaerothorax* (Sjöstedt 1911), 1134-3, Madjime, Gabon
– *Macrotermes nobilis* (Sjöstedt 1911), « 1160 B », Belinga, Gabon
– *Macrotermes natalensis* (Haviland 1898), « TC 55 », Kinshasa, D. R. Congo
– *Odontotermes simplicidens* (Sjöstedt 1899), « 1108 », Madjime, Gabon
– *O. terricola* (Sjöstedt 1902), « 1183 », Makokou, Gabon
– *Microtermes sp.* Wasmann 1902, « 1027 », Mayela, Gabon

Apicotermitinae [S]

– *Eburnitermes grassei* Noirot 1966, « TN 261bis », Anguédedou forest
– *Labidotermes celisi* Deligne &Pasteels 1969, « TC 89 », Lubero Territory, Kivu, D. R. Congo
– *Ateuchotermes ctenopher* Sands 1972, « 1003/2 », Edzamangen, Gabon
– *Speculitermes cyclops* Wasmann 1902, « TD 39 », Ohaver, Mysore, India
– *Allognathotermes hypogeus* Silvestri 1914, « TD 25 », Dabou, Ivory Coast
– *Apicotermes gurgulifex* Emerson 1956, « TC 58 », Kinshasa, D. R. Congo
– *Jugositermes tuberculatus* Emerson 1928, « 1107 », Madjime, Gabon
– *Rostrotermes cornutus* Grassé 1943, « TN 1017 », Dakpadou, Ivory Coast

Termitinae A (genera with biting soldiers) [W] or [S]

1° mainly wood and other plant matter feeders [W]

– *Amitermes evuncifer* Silvestri 1914, Ndili River, D. R. Congo
– *Cephalotermes rectangularis* (Sjöstedt 1899), « 1089 », Belinga, Gabon
– *Microcerotermes fuscotibialis* (Sjöstedt 1896), « 1006 », Madjime, Gabon
– *Microcerotermes progrediens* Silvestri 1914, « 1002 », Endoumavion, Gabon

2° soil feeders [S]

– *Foraminitermes tubifrons* Holmgren 1912, « TN1018 », Dakpadou, Ivory Coast
– *Thoracotermes macrothorax* (Sjöstedt 1899), « 1162 B », Belinga, Gabon
– *Crenetermes albotarsalis* (Sjöstedt 1897), « 1003 », Edzamangen, Gabon
– *Ophiotermes grandilabius* (Emerson 1928), « 1070 », Mekob, Gabon

– *Ophiotermes sp.* Sjöstedt, « 1102 », Madjime Gabon
– *Furculitermes winifredi* Emerson 1960, « 1166 », , Amyéré, Gabon
– *Cubitermes gaigei* (Emerson 1928), « 1132 », Madjime, Gabon
– *Cubitermes heghi* Sjöstedt 1924, « 1017 », Madjime, Gabon
– *Noditermes indoensis* Sjöstedt 1926, « 1120bis » , M'Vadhi, Gabon
– *Proboscitermes tubuliferus* (Sjöstedt1907), « TD 15bis », Mayale, Gabon
– *Basidentitermes malelaensis* (Emerson 1928), « 1153 B 2 », Belinga, Gabon
– *Orthotermes mansuetus* (Sjöstedt 1911), « 1134/2 », Madjime, Gabon
– *O. depressifrons* Silvestri 1914, « 1003/3 », Edzamangen, Gabon

Termitinae B (« *Termes* » group : genera with snapping soldiers) [W] or [S]

1° mainly wood and other plant matter feeders [W]

– *Neocapritermes sp.* Holmgren 1912, « TC5/2 », Rio de Janeiro, Brazil
– *Termes langi* (Emerson 1928), « 1020 », Ngote, Gabon

2° soil feeders [S]

– *Tuberculitermes bycanistes* (Sjöstedt 1926), « 1077 », Mekob, Gabon ([2])
– *Cavitermes sp.* Emerson 1925, « 41C », Guajara Mirim, Brazil
– *Pericapritermes magnificus* Silvestri 1912, « 1103 », Madjime, Gabon
– *Discupiditermes incola* (Wasmann 1893), « TD 40 », Dhorwar, Mysore, India

Nasutitermitinae [W] or [S]

1° mainly wood and other plant matter feeders [W]

– *Syntermes dirus* (Burmeister 1839), « TC4, TC10 & TC16 », Rio de Janeiro, Brazil
– *Procornitermes triacifer* (Silvestri 1901), « TC 39 », Alto Araguaia, Mato Grosso, Brazil
– *P. araujoi* Emerson 1952, « TC 62 », Ribeirão Prêto, Sao Paulo, Brazil
– *Cornitermes cumulans* (Kollar 1832), « TC 22 », Panloc Dumont, Mato Grosso, Brazil
– *Rhynchotermes nasutissimus* (Silvestri 1901), « TC 24 », Felixlandia, M. Grosso, Brazil
– *Nasutitermes diabolus* (Sjöstedt 1907), « 1066 », Badi Gabon
– *N. elegantulus* (Sjöstedt 1911), « Be 1149 », Belinga, Gabon
– *N. fulleri* Emerson 1928, « 1048 », Abor, Gabon
– *N. latifrons* (Sjöstedt 1896), « 1093 », Belinga, Gabon
– *Nasutitermes schoutedeni*, (Sjöstedt 1924), « 1021 », Ngota, Gabon
– *Constrictotermes cyphergaster* (Silvestri 1901), « TC 33 », Tres Marias, Mato Grosso, Brazil
– *Leptomyxotermes doriae* (Silvestri 1912), « Be 1129 », Belinga, Gabon

2° soil feeders[W]

– *Labiotermes labralis* (Holmgren 1906), « TC 41 A », Guajara Mirim, Brazil
– *L. pelliceus* Emerson & Banks 1965, « TC 47 », Guajara Mirim, Brazil
– *Armitermes* sp. Wasmann, « TC 34bis », Belo Horizonte, Mato Grosso, Brazil
– *Eutermellus convergens* Silvestri 1912, « 1158 B », Belinga, Gabon
– *Subulitermes sp.* Holmgren 1910, « TC 47bis », Guajara Mirim, Brazil
– *Postsubulitermes parviconstrictus* Emerson 1960, « 1098 », Belinga, Gabon
– *Verrucositermes tuberosus* Emerson 1960, « 1165 », Belinga, Gabon
– *Verrucositermes hirtus* Deligne 1983, « TC 242 », Lome forest, near Kribi, Cameroon ([3])

([2]) *Tuberculitermes* has been placed in the *Termes* group according to DELIGNE (1971).

([3]) *Verrucositermes* is considered here not as a monotypic genus (SANDS, 1998) but as including at least 2 species (DELIGNE, 1983).

RESULTS

The results are presented in the following way. (1) The position and structure of the premolar tooth as well as observations related to its actions are presented for a species chosen as an example of wood feeder and (2) the other wood feeders are then compared to this species. (3) The same data are given for a species chosen as an example of soil feeder and (4) the other soil feeders are similarly presented in a comparative way.

An example of a wood feeder : *Zootermopsis angusticollis*

The premolar tooth is situated at the ventral side of the left mandible.

In a ventral surface view (Fig. 2), it appears as a narrow blade that overlaps the posterior half of the 3d marginal as well as the front part of the molar plate. Its length amounts to more than 20% of the TML (total mandible length). It is completely situated behind an imaginary line (A-M3) passing through the point of the apical tooth and that of the 3d marginal. With very slight differences in the lateral tilt of the specimen, it is entirely seen on the ventral surface of the mandible or as slightly intersecting the indentation separating the 3d marginal from the molar plate. Its mesal edge is long and straight. Its apical edge is rounded and fused with the ventral surface of the mandible, while its ventral proximal edge forms a more angular outline with the long mesal one.

In tangential view (Fig. 21), it appears as a straight and sharp blade which is clearly out of alignment with the marginal teeth and the dorsal edge of the molar plate. Along the dorsal side of the premolar tooth there is thus an elongated « premolar hollow » partially edged frontwards by the 3d marginal tooth and rearward by the edge of the molar plate.

When the mandibles are manipulated under the stereomicroscope and flexed along their natural articulation axis, one can observe how they work during the chewing movements. The two mandibles cross each other, with the left sliding above the right one. Any bit of wood brought into contact with them is thus necessarily held tightly between the ventral surface of the left mandible and the dorsal surface of the right one.

Due to its position and structure the premolar tooth then slightly juts out between the two surfaces (Fig. 1). While moving, it must thus exert shearing forces on the wood bits that are in its way and is perfectly suited for cutting superficial slices or fragments out of them.

This working is analogous with that of a carpenter's plane as discussed below.

Other wood and plant matter feeders

In the termite families that are entirely wood-feeder or phytophagous all the observed species show a premolar tooth with the same general features as those described above for *Zootermopsis angusticollis*. This point is illustrated in the plates for Mastotermitidae (Fig. 20), Kalotermitidae (Figs 5, 22), Termopsidae (Figs 2, 3, 4, 21), Hodotermitidae (Fig. 6), and Rhinotermitidae (Figs 7, 23).

The premolar tooth is always either straight or slightly convex and always long, its length amounting to more than 20% of the TML. It even exceeds 30% of the TML in most Kalotermitidae and Rhinotermitidae observed.

It the case of Termitidae, the 28 wood-feeder or other plant matter-feeder species observed (belonging to 19 genera), all present a similar « plane device », with some differences from one group to the other.

1° In the 10 observed species of Macrotermitinae (belonging to 7 genera), the following features were noted. In surface view, the cutting edge of the premolar tooth shows a nearly straight or slightly convex edge, as illustrated for *Macrotermes nobilis* (Fig. 8). When a concavity is also present in this edge, it is very faint and restricted to the most proximal part of its length (Fig. 9). The tooth always lies behind the line A-M3 as defined above. It generally intersects the indentation separating the 3d marginal tooth from the molar plate. In some species it is at a distance from the inner margin of the mandible and is therefore totally situated under the ventral surface of the mandible. This back position is particularly pronounced in Macrotermes (Fig. 9) and Odontotermes species.

In tangential view the premolar tooth appears as a straight sharp blade (Fig. 24). It is always clearly out of alignment with the edge of the marginal and molar teeth.

The length of the premolar tooth amounts to about 20-25% of the TML, except in Odontotermes (~16%) and *Protermes prorepens* (only 13%).

2° In the group of Termitinae with biting soldiers, the 4 wood-feeder species observed belong to the genera *Amitermes*, *Cephalotermes* and *Microcerotermes*. In these 4 species the premolar tooth presents, either in surface view or in tangential view, the same general features described above for the other wood-feeder species. These features are illustrated for *Microcerotermes* (Figs 12, 25). The premolar tooth amounts to about 20-25% of the TML, except in *Amitermes evuncifer* (~17%).

3° In the group of Termitinae with snapping soldiers, the 2 wood-feeder species observed, i.e. *Termes langi* and *Neocapritermes sp.* (Figs 18-19) also exhibit the same features. In both species, the premolar tooth intersects the indentation between the 3d marginal and the molar plate and its length amounts to about 20% of the TML.

4° The observed wood-feeder Nasutitermitinae belong to 12 species and 7 genera. These species are either Neotropical or African but they all show a premolar tooth with the general morphology described in the previous wood-feeder groups. This point is illustrated for *Procornitermes* (Figs 15, 26) and for *Nasutitermes* (Fig. 27). The premolar tooth however has a more variable position, since it either crosses the A-M3 line (in Neotropical genera *Syntermes*, *Procornitermes*, *Cornitermes* and *Rhynchotermes*), or stays behind this line (in *Constrictotermes*, *Nasutitermes* and *Leptomyxotermes*) and intersects to a variable extent the marginal-molar indentation. Its length amounts to 20-30% of the TML.

An example of a soil feeder : *Thoracotermes macrothorax*

In surface view (Fig. 11), the premolar tooth is no longer situated at the ventral side of the mandible : it reaches the same mesal level as the marginal teeth and its mesal edge crosses the A-M3 line. It is quite short, its length amounting to only about 15% of the TML,

and its apical part hardly overlaps the 3rd marginal tooth. Its mesal edge shows a distinct concave outline, its point is rounded and its proximal edge is a continuation of the apical slope of the molar depression. In surface view this proximal edge overlaps the large dorsal molar prominence.

In tangential view (Fig. 28), the premolar tooth appears with a curved and blunt edge, in the same general alignment as the marginal teeth. Its dorsal side is still adjacent to a « premolar hollow » and its proximal end is enlarged, thus flanking the apical part of the molar depression.

When the mandibles are manipulated under the stereomicroscope and flexed along their natural axis, the left mandible slides over the right one and the premolar tooth matches the opposite dorsal part of the right mandible. Due to its blunt edge it does not appear to be suited for cutting hard plant tissue but rather for pushing soft material with its rounded dorsal side.

Other soil-feeder species

The 33 soil-feeder species observed belong to 4 groups. They include 8 species (from 8 different genera) of Apicotermitinae, 13 species (10 genera) of Termitinae with biting soldiers, 4 species (4 genera) of Termitinae with snapping soldiers and 8 species (6 genera) of Nasutitermitinae. With the exception of some Nasutitermitinae they all present most of the features described above for *Thoracotermes macrothorax*.

The case of Nasutitermitinae is presented separately.

In the three first groups, the observed species all share the following characteristics. In ventral surface view (Figs 10, 11, 13, 14), the premolar tooth reaches the same mesal level as the marginal teeth, or nearly so, and crosses the A-M3 line. Its length amounts to less than 20% of the TML. In tangential view (Figs 28-30), its edge is blunt and lies in continuity with that of the 3d marginal tooth.

Besides these common features the following particularities can be noted.

1° In the observed species of soil-feeder Apicotermitinae, the premolar tooth is very short, amounting to 5 to 15 % of the TML, except in *Eburnitermes* (15-19%). The mesal edge of the premolar tooth is straight in the cases of *Eburnitermes* (Fig. 10), *Allognathotermes*, *Apicotermes* (Fig. 30) and *Jugositermes*. It shows a distinct concave outline in *Labidotermes* and *Ateuchotermes*.

2° Among the observed species of soil-feeder Termitinae with biting soldiers, the premolar tooth has a concave outline in most of the cases. It is straight in *Ophiotermes* and *Furculitermes*.

3° Among the observed soil-feeder species of Termitinae with snapping soldiers, the premolar tooth is straight in *Tuberculitermes bycanistes* and concave in *Cavitermes*, *Pericapritermes* and *Discupiditermes*.

As for soil-feeder Nasutitermitinae, in the observed Neotropical species of *Labiotermes* (Figs 16, 17, 31) and *Armitermes* the premolar tooth does show the same general features as in *Thoracotermes*. In the small African species belonging to 4 close genera (*Verrucositermes*, *Postsubulitermes*, *Subulitermes* and *Eutermellus*), the premolar

tooth is short (~ 15% of the TML) and blunt but it differs from that of *Thoracotermes* in being straight and out of alignment with the 3d marginal tooth.

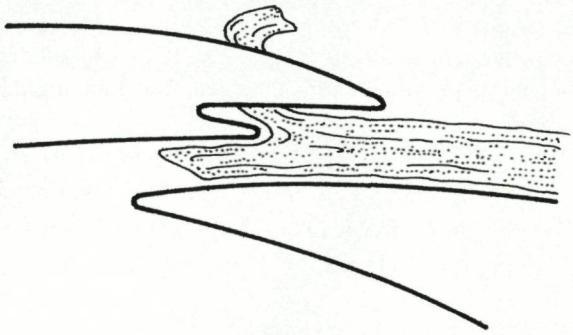
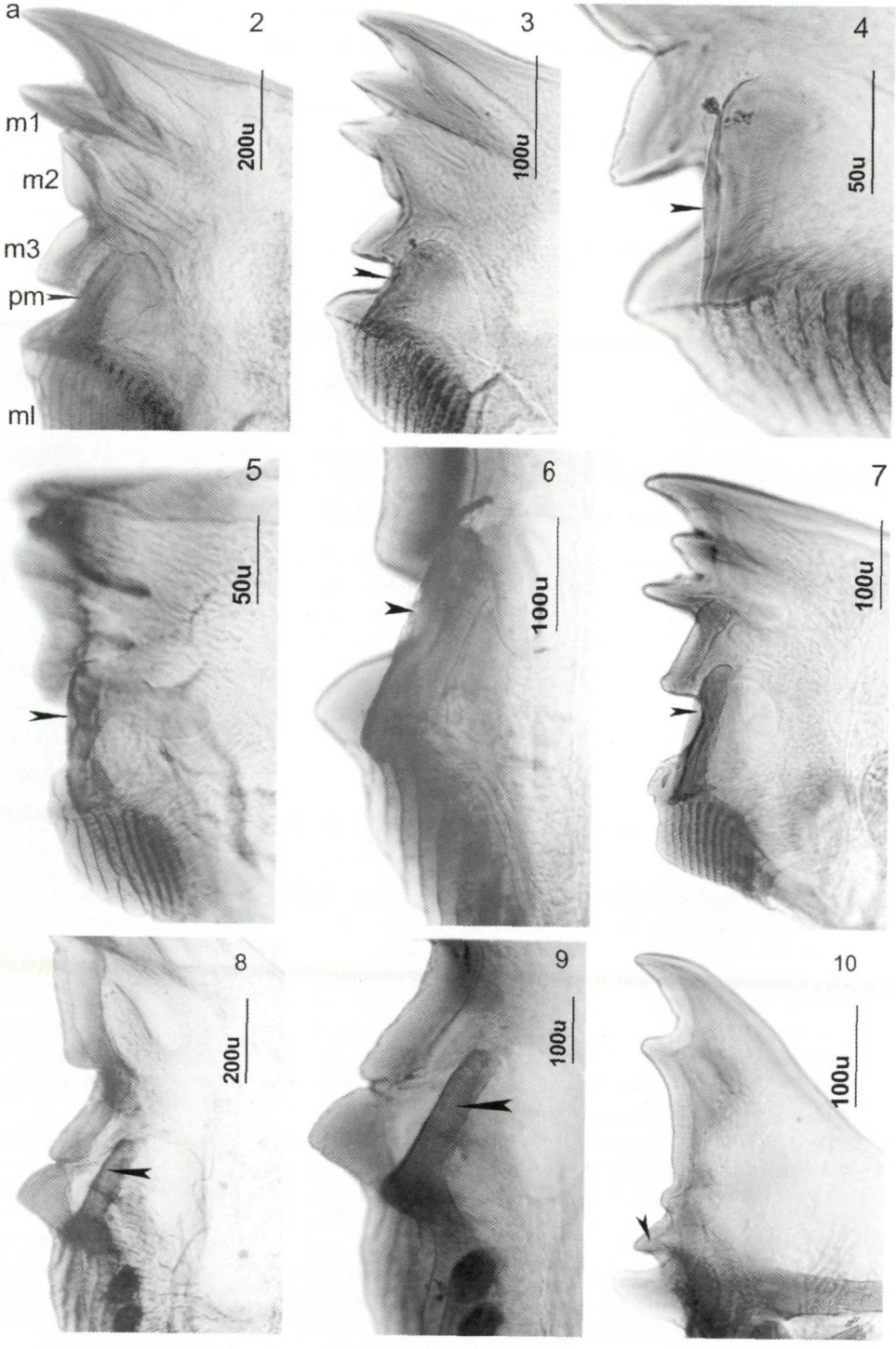


Fig. 1. – Schematic functioning of the premolar tooth of the left mandible of a wood-feeder worker of termite. Left and right mandibles are seen in cross section from their proximal part. The left mandible slides above the right one and ingested bits of wood are held tight between the two mandibles. The premolar tooth slightly juts out between the two mandibles; while moving it exerts shearing forces on the wood bits which are in its way and can cut superficial fragments out of them.

Legend to the figures (see pages 210-214)

Figs 2-17. – Microphotographs of the left mandible of termite worker seen in ventral surface view to show the position and form of the premolar tooth (arrow). – Fig. 2. *Zootermopsis angusticollis.* a : apical tooth ; m1, m2, m3 : first, 2d, 3d marginal tooth ; ml : molar plate ; pm : premolar tooth.- Fig. 3. *Stolotermes africanus*. Fig. 4. *Stolotermes africanus* (detail). Fig. 5. *Kalotermes flavicollis*. Fig. 6. *Microhodotermes viator*. Fig. 7. *Heterotermes sp*. Fig. 8. *Macrotermes nobilis*. Fig. 9. *Macrotermes nobilis* (detail). Fig. 10. *Eburnitermes grassei*. Fig. 11. *Thoracotermes macrothorax*. Fig. 12. *Microcerotermes fuscotibialis*. Fig. 13. *Pericapritermes magnificus*. Fig. 14. *Pericapritermes magnificus* (detail). Fig. 15. *Procornitermes striatus*. Fig. 16. *Labiotermes labralis*. Fig. 17. *Labiotermes labralis* (detail).

Figs 18-31. – SEM micrographs of the left mandibles of worker termites seen in tangential view from the inner side to show the position and form of the premolar tooth (arrow). – Fig. 18. *Neocapritermes sp.* Fig. 19. *Neocapritermes sp.*(detail). Fig. 20. *Mastotermes darwiniensis.* Fig. 21. *Zootermopsis angusticollis*. Fig. 22. *Kalotermes flavicollis.* Fig. 23. *Reticulitermes lucifugus*. Fig. 24. *Acanthotermes acanthothorax*. Fig. 25. *Microcerotermes fuscotibialis* (detail). Fig. 26. *Procornitermes araujoi*. Fig. 27. *Nasutitermes lujae.* Fig. 28. *Thoracotermes macrothorax.* Fig. 29. *Pericapritermes magnificus*. Fig. 30. *Apicotermes gurgulifex*. Fig. 31. *Labiotermes labralis* (detail).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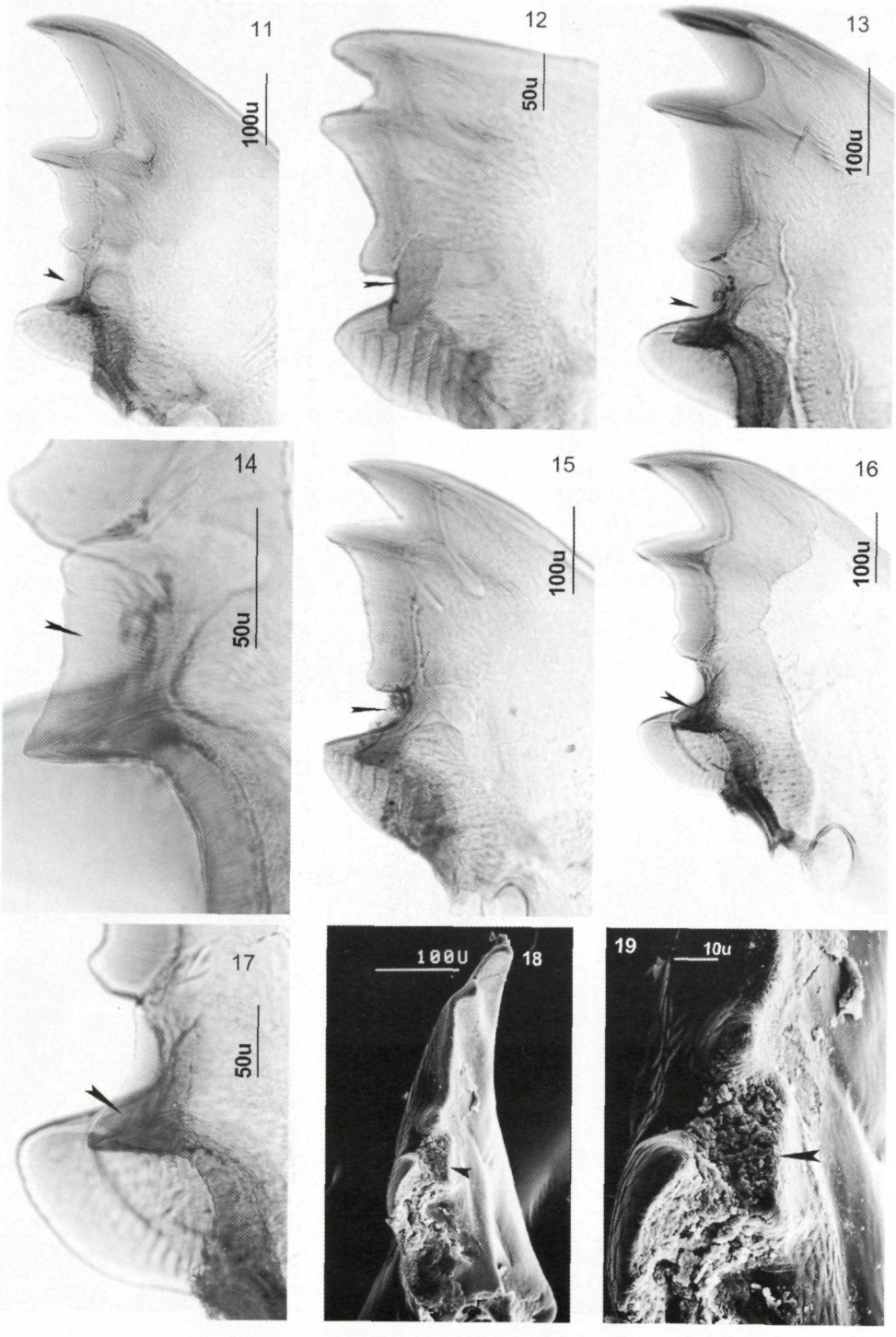
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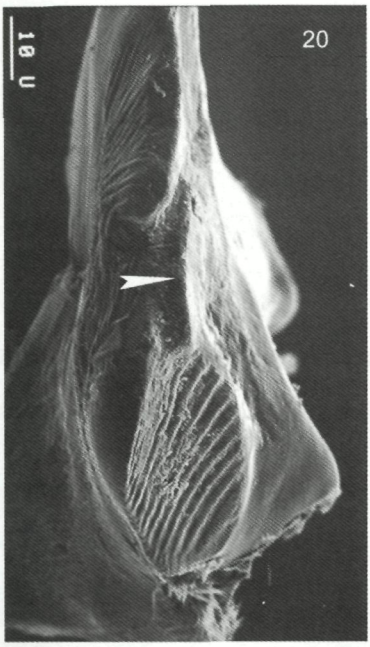
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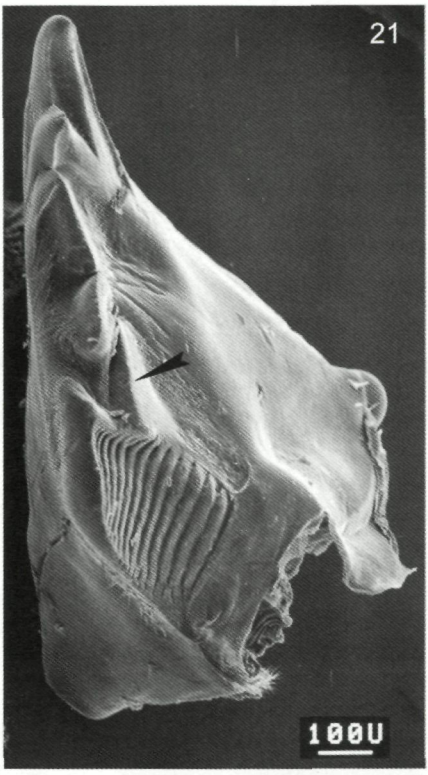
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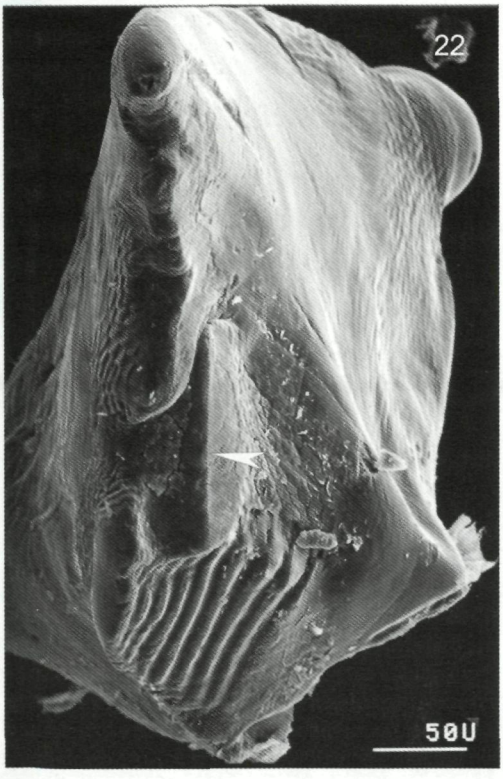
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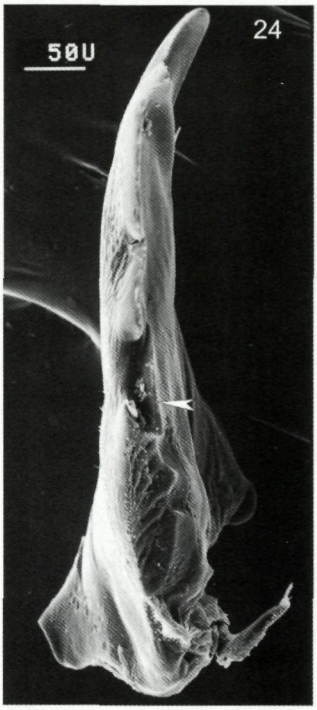
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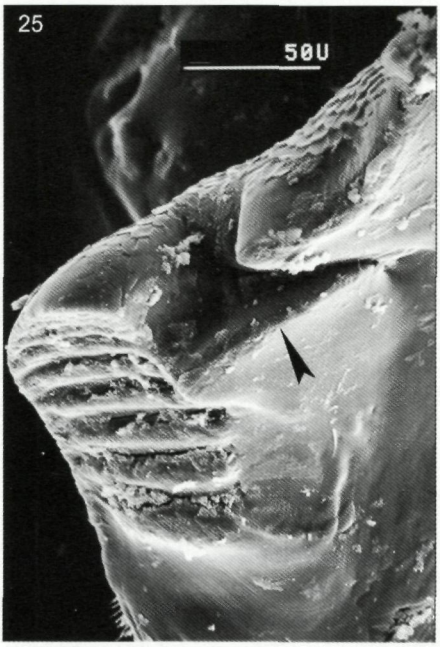
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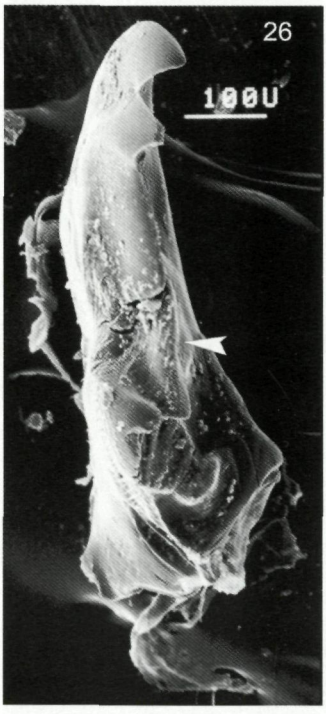
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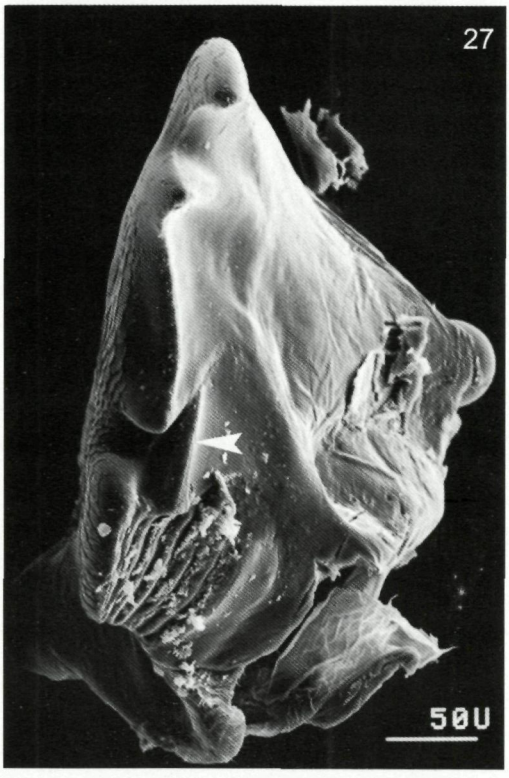
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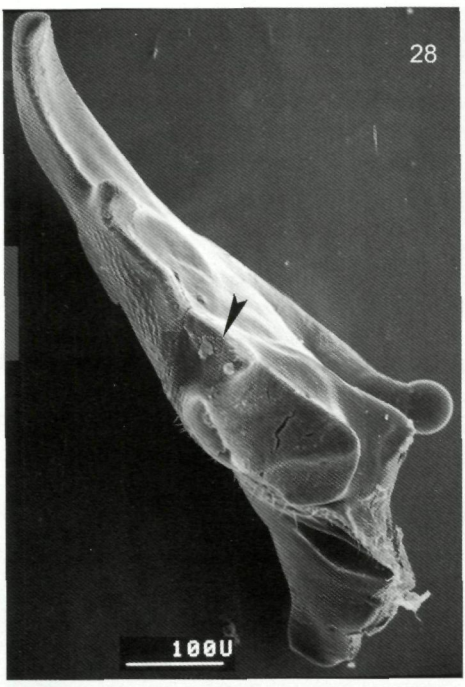
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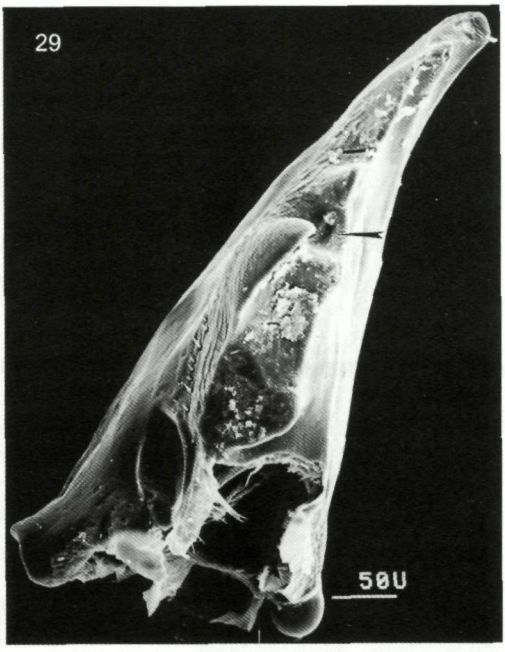
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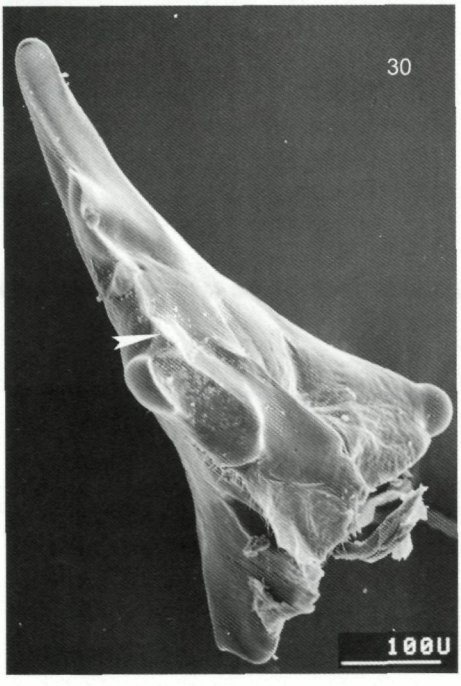
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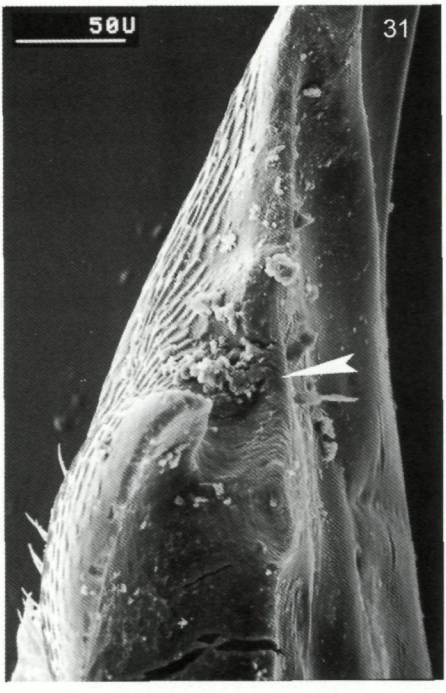
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DISCUSSION

The premolar tooth is an asymmetrical structure only present in the *left* mandible of termite workers. It has escaped the notice of earlier morphologists, probably because it is generally more or less hidden *under* the mandible. Since the late '60s, it has been described under the names of « molar tooth » (KRISHNA, 1968), « premolar tooth » (DELIGNE & PASTEELS, 1969; DELIGNE, 1970), « subsidiary marginal tooth » (SANDS, 1972), « submolar tooth » (MATHEWS, 1977) and « 4th marginal tooth » (SANDS, 1992). Among these names, I consider both adjectives « molar » and « subsidiary marginal » to be ambiguous, the former because it may induce a confusion with the molar plate and the latter because the same terms designate a different tooth in the right mandible. The adjective « submolar » may also be ambiguous or inexact. The tooth under discussion is actually often situated under the dorsal molar prominence but never under the molar plate itself. Furthermore in many Termitidae genera the tooth is wholly situated in front of the molar region and not under any part of this region. Similarly the name « 4th marginal » does not seem appropriate because, in most families and subfamilies, the tooth is *not* situated at the inner margin of the mandible but lies instead more or less far from that margin. The name « premolar tooth » seems better because the tooth is always in front of the molar plate and in close connection with it. Furthermore its sensillae are innervated by the same nerve branch as the molar plate and not by a marginal branch (DELIGNE & PASTEELS, 1969). Finally as it differs in structure and position both from molar plate and from marginal teeth in most families and subfamilies, it seems preferable to also give it a specific name. For all these reasons I retain the name of « premolar tooth ».

Until recently the morphology of the premolar tooth had only been described in a few groups of termites. SANDS (1998) published an outstanding systematic synthesis on the termite genera from soils of Africa and the Middle East, in which the « 4th marginal » (i.e. the premolar tooth) was described for many African species. Even though the information given in the present work is partly based on the same material as Sands', it is not redundant because of the use of different observation techniques. Indeed the observation in ventral view of the mandible, as proposed here in Figs 2-17, allows a more accurate description of the premolar tooth than the dorsal observation used for systematic purposes. The observation in tangential view, as depicted here in SEM Figs 18-31, is necessary in order to observe the precise form and connections of the premolar tooth. I have also observed termites belonging to families (Mastotermitidae, Kalotermitidae), to subfamilies (Termopsinae, Stolotermitinae, Porotermitinae) and to non-African genera or species, which are not included in Sands' material. Furthermore the scope of this work is rather functional morphology than systematics.

Concerning functional morphology, the premolar tooth of wood-feeder species always shows a similar position and structure, as described for *Zootermopsis*. It is suited for exerting shearing forces on bits of wood held tightly between the two mandibles and for cutting superficial slices or fragments out of them (Fig. 1). As briefly mentioned above this function is similar to that of a carpenter's plane. The analogy is as follows. With the plane (*or* with the premolar region of the mandible), wood is held tight between the sole of the plane (*or* the ventral surface of the left mandible) and the workbench (*or* the dorsal sur-

face of the right mandible), while the slightly jutting chisel of the plane (*or* the premolar tooth) cuts superficial shavings out of the wood.

In a very schematic way, Fig. 32 suggests how the resulting fragments may be moved. Under the pressure of newly cut fragments, the first fragments are pushed into the premolar hollow and, from there, towards the molar region (dotted arrow), where they are further rasped. The connection between the premolar hollow and the molar rasp can be clearly observed on Figs 20-27 and the movement of fragments from one part to the other is suggested by Figs 18-19. In the species where the premolar tooth is near the inner margin of the mandible or even intersects the indentation between the 3d marginal tooth and the mola, the cut fragments of wood can also be pushed at the level of this indentation towards the dorsal surface of the left mandible (continuous arrow).

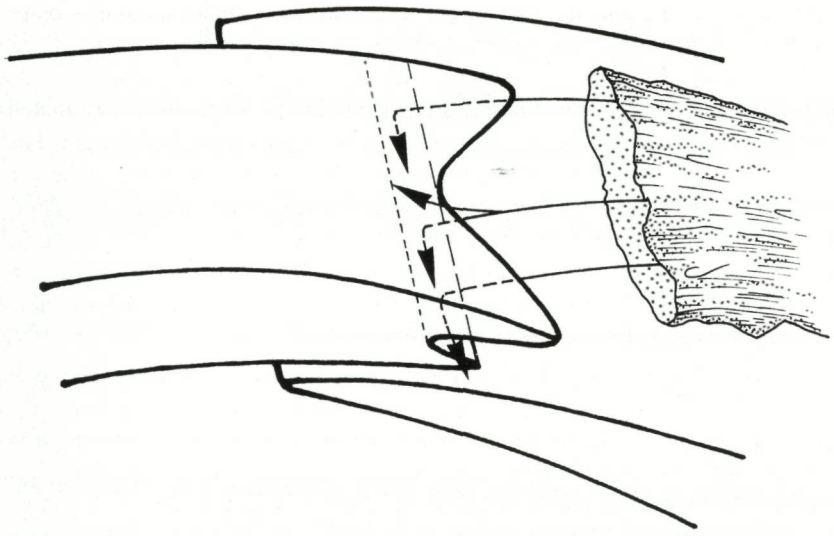

Fig. 32. – Schematic cross section and stereo-diagram of the premolar region of the left mandible and opposite part of the right mandible. The fragments of wood cut by the premolar tooth are pushed into the premolar hollow and, from there towards the molar region (dotted arrow), where they are further rasped. They can also be pushed between the 3d marginal tooth and the molar prominence towards the dorsal surface of the left mandible (continuous arrow) and, from there be moved back towards the molar region and the mouth by the movements of the labrum.

In most of the soil-feeder species that we observed, belonging to at least 4 phyletic clades, the premolar tooth has lost one or several functional features that characterise wood feeders. In particular it reaches more or less the same level as the marginal teeth, comes in alignment with them and becomes blunt. It becomes more similar to the marginal teeth and presumably plays a similar role. During the movements of the mandibles, together with the marginal teeth, it is suited to gather soil particles towards the midline and the rear part of the oral cavity where they are compressed between the mola of both mandibles before being ingested. These adaptive trends appeared at least four times in different groups of soil-feeder Termitidae.

In all cases the other mouthparts, especially the labrum and the hypopharynx, assist the mandibles in moving food items towards the mouth. Besides their key role in the processing of food the mandible teeth, including the premolar tooth, also certainly play a role in the processing of wood and soil material used by workers for building the nest.

CONCLUSIONS

In the left mandible of termite workers, the premolar tooth shows clear morphological adaptations to the species' diet.

In wood- and other plant matter-feeder species, the structure, position and functioning of the premolar tooth are analogous to those of the chisel of a carpenter's plane. It is suited for cutting superficial fragments out from the wood.

In many soil-feeder species, belonging to at least 4 clades, the premolar tooth has lost in a polyphyletic way one or several functional features that characterise wood feeders. Due to its structure and position it is suited to assist the marginal teeth in the gathering of soil particles towards the mouth before ingestion.

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THE ANATOMY AND ULTRASTRUCTURE OF THE PROBOSCIS IN *ZONORHYNCHUS*-SPECIES AND IMPLICATIONS FOR PHYLOGENETIC RELATIONSHIPS WITHIN THE EUKALYPTORHYNCHIA MEIXNER, 1928 (PLATYHELMINTHES, RHABDOCOELA)

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Abstract. The ultrastructural morphology of the proboscis in species of the genus *Zonorhynchus* Karling, 1952 is investigated. The proboscis epithelia are organized in four belts. The apical cone epithelium has intra-epithelial nuclei, and the distal belt of the sheath epithelium is formed by numerous cells lacking a distinct pattern. Multiciliary receptors are lacking. The diversity in ultrastructural data on the proboscis is used to elucidate the phylogenetic relationships within the Cicerinidae and Eukalyptorhynchia. A bipartite cone epithelium as found in *Zonorhynchus*-species is a common feature for all eukalyptorhynch species investigated. A syncytial basal cone epithelium is a synapomorphic character for all species except *Toia calceformis* Brunet, 1973 and *Nannorhynchides herdlaensis* Karling, 1956. A syncytial proximal belt of the sheath epithelium, and intra-epithelial nucleus(i) in the apical cone epithelium constitute apomorphic features for species of the genus *Zonorhynchus*. The absence of multiciliary receptors is regarded as the plesiomorphic state and distinguishes *Zonorhynchus*-species, *T. calceformis* and *N. herdlaensis* from other Eukalyptorhynchia investigated. *Cicerina remanei* Meixner, 1928, *Psammorhynchus tubulipenis* Meixner, 1938 and *Cytocystis clitellatus* Karling, 1953 have two insunk sensory organs with muticiliary receptors. The presence of four epithelial belts in *Zonorhynchus*-species as in *C. remanei*, *P. tubulipenis* and *C. clitellatus* is considered a shared plesiomorphic state within the Eukalyptorhynchia The presence of a third belt in the sheath epithelium is a synapomorphic character of all Koinocystididae, Cystiplanidae and Polycystididae.

Key words: Platyhelminthes, Eukalyptorhynchia, proboscis, phylogeny, ultrastructure.

INTRODUCTION

The family Cicerinidae Meixner, 1928 comprises ten genera. In the diagnosis of the family the presence of germovitellaria and of a glandular ring at the junction of the proboscis in most species are the main diagnostic features. However, germovitellaria are present in *Cytocystis clitellatus* Karling, 1953 as well (KARLING, 1964). Although less prominent, a ring of glandular secretion in the sheath epithelium just above the junction is

present in species of other families such as Psammorhynchidae, Gnathorhynchidae and Placorhynchidae (DE VOCHT, 1990; DE VOCHT, 1992). Because of this a distinct apomorphic character to define a monophyletic taxon Cicerinidae is lacking. After the establishment of the taxon Cicerinidae by MEIXNER (1928), KARLING (1952) proposed the name Zonorhynchidae with two subfamilies: Zonorhynchinae (including the genera *Zonorhynchus* Karling, 1952, *Cicerina* Giard, 1904, *Paracicerina* Meixner, 1928, *Ptyalorhynchus* Ax, 1951 and *Blennorhynchus* n.n.), and Ethmorhynchinae (for *Ethmorhynchus* Meixner, 1938). With the description of several new species and genera, as for instance by MARCUS (1952), KARLING (1956) and AX (1959), the family was named Cicerinidae again by KARLING (1964). A few years later, BRUNET (1965, 1973) described eight new species and two new genera and made a revision of the family based on light microscopic observations on twelve species. BRUNET (1973) distinguished two groups of genera within the Cicerinidae. One group contains the genera *Toia* Marcus, 1952, *Nannorhynchides* Karling, 1956 and *Pocillorhynchus* Brunet, 1973. The other group includes the genera *Cicerina*, *Paracicerina*, *Ptyalorhynchus*, *Ethmorhynchus*, *Zonorhynchus*, *Xenocicerina* Karling, 1956 and *Didiadema* Brunet, 1965. In this non-cladistic approach the distinction between the two groups was based on several criteria, and the first group is characterized by their small dimensions (0.2-0.7 mm), their oval body-shape and excellent swimming capabilities. Histologically these species are characterized by two frontal glands, pigmented eyes with lenses and a ciliated proboscis sheath epithelium. The second group is characterized by a body length exceeding 1 mm, in general paired vesiculae seminales and opposite or different character states of the former mentioned characters. EVDONIN (1977) distributed the genera into three subfamilies: the Cicerininae Meixner, 1928, Nannorhynchinae Evdonin, 1977 and Xenocicerininae Evdonin, 1977. More recently new species of Cicerinidae have been described by SCHOCKAERT (1982), KARLING (1989) and ARMONIES & HELLWIG (1987).

Species of the genus *Zonorhynchus* have been collected from salt marshes, lagunes and muddy sediments (KARLING, 1952; AX, 1959; ARMONIES & HELLWIG, 1987). Up to now four species of *Zonorhynchus* have been described (ARMONIES & HELLWIG 1987, KARLING 1952, 1956). All four species are light microscopically characterized by the position of the copulatory organ and the genital pore in the middle of the body, non-sclerotized bursal mouthpieces and a proboscis with long proboscis- and integument retractors.

MATERIAL AND METHODS

Specimens of *Zonorhynchus seminascatus* Karling, 1956 were collected from fine sediment of a shallow lagoon (0.4 m) near Blåbergsholmen in the Gullmar-fjord near Kristineberg (Sweden) in June 1988. An unidentified specimen of the genus was collected from fine sand from the bord of Etang de Lapalme at the Mediterranean coast near Banyuls-sur-Mer (France) in October 1987. *Zonorhynchus salinus* Karling, 1952 was collected from a salt marsh near Königshaven at the island of Sylt (Germany) in April 1988. Extraction was carried out by decantation with a $MgCl_2$-solution isotonic to seawater for the unknown species of *Zonorhynchus* and *Z. seminascatus*. *Z. salinus* was extracted by the «Übersand»-method described by ARMONIES & HELLWIG (1986).

Before primary fixation, animals were anaesthetized in a solution of $MgCl_2$ isotonic to the seawater. Primary fixation was carried out with 0.1 M cacodylate-buffered 2 % glutaraldehyde at 4°C for 2h. After double rinsing in the same buffer, specimens were postfixed in 1%-cacodylate-buffered osmium tetroxide at 4°C for 1h, pre-stained in 2% uranyl acetate solution (20 min) before dehydration in a graded acetone series and embedded in Araldite. Serial sectioning was carried out with Reichert OMU 3 and Reichert Ultracut microtomes. Thin sections were mounted on carbon-coated pioloform-covered grids and stained with 2 % aqueous uranyl acetate (5 min) and 1.2 % aqueous lead citrate (7 min), using an LKB-ultrostain. Fine structural examination was performed with a Philips EM 400 electron microscope.

Terminology and abbreviations used in the text and figures

Apex: Apical-most part of the cone.

Belts: Limited, circumferential parts of the epithelium, either the sheath (S_1 and S_2) or the cone epithelium (*A* and *B*). They can be formed by one cell, several cells or a syncytium.

Bulb: Muscular part of the proboscis separated from the surrounding parenchyma by a layer of extracellular matrix.

Cone epithelium: The part of the epithelium that covers the muscular part of the proboscis which protrudes into the cavity, constituted of an apical cone epithelium (*A*) and basal cone epithelium (*B*).

Dilators (D): Dilating muscles around the sheath.

Extracellular matrix (*ECM*): Intercellular components of connective tissue or the product of epithelia or muscles cells.

Gland necks (*g1-g2*): The apical parts of gland cells of which the secretory parts are located in the parenchyma. They usually store secretion granules.

Inner musculature: The circular (*icm*), central cylinder and peripheral blocks of longitudinal muscles (*cilm* and *pilm*) situated internally to the proboscis septum.

Insunk cell parts: Epithelial cell parts situated in the parenchym below the epithelial basal lamina.

Intra-epithelial: This notation is used in contrast with insunk or subepithelial in respect of the position of nuclei or receptors for instance.

Intrabulbar nuclei: Nuclei of the proboscis epithelia located internally to the bulbar septum in between the inner proboscis muscles.

Junction: The place where the apical part of the sheath epithelium makes contact with the apical part of the cone epithelium.

Motional muscles: The positioning muscles of the proboscis in the body. The motional muscles include dilators *(D)*, protractors (*P*), fixators, proboscis retractors (*PR*) and integument retractors.

Nodus (*n*): Posterior end of the bulb.

Nucleo-glandular girdle (ngg): Epithelial cell parts situated below the junction around the anterior part of the bulb, containing the nuclei of the epithelial cells or syncytia and gland necks.

Outer musculature: The muscles situated at the outside of the proboscis septum or underlying the sheath epithelium.

Proboscis: Frontal organ, mainly composed of epithelia and muscles with associated glands and sensory cells, used to capture prey.

Proboscis cavity: Cavity with terminal pore in which a part of the proboscis protrudes.

Proboscis cone: Terminal part of the muscular part of the proboscis, which protrudes into the proboscis cavity

Sensory receptors: Receptive terminal parts of nerve cells, which pierce the epithelia.

Septum: Layer of extracellular matrix surrounding the proboscis bulb.

Sheath epithelium: The part of the proboscis epithelium that lines the wall of the proboscis cavity, formed by a distal (S_1) and a proximal belt (S_2).

RESULTS

Zonorhynchus-species are large bulky animals compared to most Eukalyptorhynchia. The brownish animals measure 0.8-1.5 mm and possess a relatively small proboscis. Characteristic for the proboscis in species of the genus *Zonorhynchus* is the large amount of gland necks that pierce the epithelia of the sheath as well as the cone epithelium. The relatively long bulb shows a particular organization with internal circular and radial muscle fibres between the inner longitudinal muscles in the cone.

Epithelia. The proboscis epithelium in *Zonorhynchus*-species is formed by four belts (Fig. 1). The distal belt constitutes the main part of the epithelium lining the proboscis cavity and is formed by separate cells arranged without a distinct pattern (Figs 1, 2). A circumferential proximal belt of the sheath epithelium forms the junction between sheath and cone. The epithelia are devoid of cilia, and cells are interconnected by apical zonulae adhaerentes, septate junctions and dispersed desmosomes (Fig. 2). The cells of the distal belt are 5 µm high and bear slender microvilli, 900 nm long and about 10 per µm (Fig. 2). A fine fibrillar but not electron-dense layer, about 450 nm thick, is present in the apical parts of the cells just beneath the apical plasmalemma. All cells in the distal belt have intra-epithelial lobate nuclei (5-6 µm long). The cytoplasm contains only very few mitochondria, patches of endoplasmic reticulum and Golgi complexes. Anteriorly, differences in electron density of the cytoplasm of the cells can be seen. The lateral cell borders form many very narrow interdigitations with neighbouring cells. Underlying the epithelium is a uniform 140 nm-thick basal lamina. The proximal belt of the sheath epithelium is 5 to 0.5 µm high and is found at the junction (Fig. 7). The microvilli are distinctly shorter (350 nm long) than in the distal belt. In the specimen from Banyuls-sur-Mer, nine cytoplasmic strands are found in the nucleo-glandular girdle containing four nuclei (Fig. 8). In another species, *Z. seminascatus*, eight cell strands packed with swollen gland necks are found in the nucleo-glandular girdle (Fig. 9). Eight oblong and lobate nuclei (9-10 µm long) of the syncytium are found in a circle at the posterior end of this girdle (Fig. 10). The basal lamina underlying the sheath epithelium proceeds around the nucleo-glandular girdle but is not continuous with the bulbar septum.

In *Z. seminascatus* the cone epithelium is formed by a single apical cell and a basal syncytium that covers the flanks of the cone (Fig. 5). The epithelium is about 5 µm high. The stout microvilli are 350 nm long. The apical cell has no sunken cell parts, measures 8 µm in diameter and has a 6 µm long intra-epithelial nucleus. A basal lamina underlies the epithelium ; it is irregular in thickness and frequently pierced by the gland necks. A terminal web is not present but the cytoplasm has a fibrous appearance. The syncytial basal cone epithelium has five insunk nucleated cell parts. The microvilli of the basal belt do not possess significantly more dense tips than do the microvilli in the apical belt. Narrow strands of the syncytium are situated in between the eight or nine cell strands of the proximal belt of the sheath epithelium (Figs 8, 9). The nuclei of the syncytium are situated in five cytoplasmic strands around the median part of the bulb (Fig. 11).

Glands. The sheath epithelium in *Zonorhynchus*-species is not only characterized by its particular cellular organization but also by the many gland necks that pierce the epithelium from pore to junction. The most numerous type of gland necks (g_1) contains electron-

dense secretion granules that measure 800 to 900 nm (Figs 2, 4). Sometimes granules with a light centre or lighter granules are present. A second type of gland neck (g_2) piercing the distal belt of the sheath epithelium stows moderately electron-dense secretion granules of about 900 nm in diameter (Figs 2, 4). The gland necks of both types are constricted by the terminal web of the epithelium. These two types of gland necks (g_1 and g_2) are also found in the proximal belt at the junction but a third type (g_3) is present as well (Fig. 7). Type g3 gland necks are filled with secretion in the apical half of the epithelium and stand wide open at the surface. The moderately electron-dense secretion granules measure about 500 nm in diameter. Two different types of gland necks surface through the part of this belt covering the basal part of the cone. One type (g_4) is empty because the secretion granules are washed out during preparation, while the other type (g_5) is piled with closely-packed, moderately electron-dense granules of approximately 1000 nm diameter (Figs 8, 9). These granules sometimes possess a darker spot in the centre. Types g_4 and g_5 gland necks are grouped in eight (*Z. seminascatus*) or nine (*Zonorhynchus* spec. B-s-M) cytoplasmic strands of the junctional belt (S_2) beneath the junction.

The basal belt of the cone epithelium is pierced by four types of glands. Large gland necks (g_6) with 1400 nm wide, transparent secretion granules and lined by an up to 200 nm thick peripheral margin of cytoplasm appear in a regular pattern (Figs 4, 5, 7). In a first circle, four g6-type gland necks alternate with four gland necks of type g_7 (Fig. 6). The latter type of gland necks, which contain 700 nm wide, electron-dense secretion granules, are also found between the muscles in the cone. However, they are not present in the proximal part of the bulb and enter the proboscis at the same position where the epithelial cell strands of the basal cone epithelium sink in (Fig. 1). Dispersed between types g_6 and g_7 gland necks are two other types of gland necks. Narrow (700 nm) necks contain a closely-packed, moderately electron-dense secretion, 350 nm in diameter (g8) or spherical to ovoid, more electron-dense secretion granules 250 nm in diameter (g10) (Fig. 6). The apical cell is pierced by gland necks (g9) passing through the central part of the bulb (Fig. 3). Moderately electron-dense secretion granules (200 nm) are sparsely present near the terminal end of the gland necks.

Sensory receptors. Uniciliary receptors are spread throughout the sheath epithelium (Fig. 4). The apical part of the sensory cells is connected to the epithelium by zonulae adhaerentes and septate junctions. The cilia possess normal 9+2 axonemata, basal bodies and rootlets. Over 2 µm long primary rootlets are orientated perpendicularly to the epithelium and short slanting secondary rootlets radiate from the basal bodies towards the plasmalemma forming the zonulae adhaerentes. Multiciliary receptors are not present, either intra-epithelially or insunk. Only very few sensory cells were encountered in the basal cone epithelium.

Outer proboscis musculature. The sheath epithelium in *Zonorhynchus*-species is surrounded by circular and longitudinal muscles (Fig. 6). The outer circular muscles are present around the cavity from the region of the dilators of the sheath up to the nucleo-glandular girdle, more specifically up to the position of the nuclei of the junctional belt (S_2). Around the anterior part of the distal belt of the sheath epithelium, nine very thin longitudinal muscle fibres are found. More posteriorly a distinct layer of longitudinal and circular muscles appears under the epithelium. Longitudinal muscle fibres of the body

wall musculature deflect and continue around the sheath epithelium (Fig. 1). The connective parts that run through the parenchyma form the dilators of the sheath. Posteriorly of the dilators of the sheath, the outer longitudinal muscle layer is composed of eighteen or twenty fibres. Towards the junction the fibres bifurcate and insert on the basal lamina. In between these muscle fibres and between their bifurcations a new set of outer longitudinal muscle fibres appears below the junction and around the nucleo-glandular girdle. Their bifurcated anterior ends form eighteen or twenty muscle fibres as well. They are present around the bulb and the 2 µm thick insunk cytoplasmic cell strands. They finally insert on the postero-lateral sides of the bulb.

The motional muscles include three pairs of protractors, four pairs of proboscis retractors and one pair of integument retractors. This arrangement corresponds to the description of *Zonorhynchus tvaerminensis* (KARLING, 1952). Proboscis retractors adhere on the postero-lateral sides of the bulb behind the nucleated cell parts of the basal cone epithelium in between the six protractor muscles (Fig. 12).

Inner proboscis musculature. The inner circular muscles surround the internal cone retractors (inner longitudinal muscles) from the nodus nearly up to the junction (Figs 8-11). Behind the nucleo-glandular girdle this layer is about 1.7 µm thick. In the cone the inner longitudinal muscles can be divided into a central cylinder (*cilm*) and eight peripheral blocks (*pilm*). The muscles that form the central cylinder are found beneath the apical cell. About halfway down in the cone they are surrounded by thin, circular muscle fibres, which are continuous with eight radiating muscles fibres in between the peripheral blocks (Fig. 13). The radiating muscle fibres all bifurcate and include another eight blocks of longitudinal muscles at the periphery. These muscle fibres are probably continuous with the inner circular muscles that surround all longitudinal muscles in the bulb. The circular muscles are found when the radiating muscles disappear. Apically the longitudinal muscles show electron-dense condensations (Fig. 13). Sometimes they appear to be conical. All muscle fibres, including those in the outer proboscis musculature, possess a sarcoplasmic reticulum but cross striation is not observed. The circular muscles are present from the nodus up into the cone, below the basal belt of the cone epithelium. The apical-most fibres are connected to the radiating fibres in the cone that separate the eight blocks of peripheral longitudinal muscles and the one or few circular muscle fibres enclosing the central cylinder of longitudinal muscle fibres.

Surrounding the inner circular muscles is a distinct layer of extracellular matrix (ECM) or septum. Apically this ECM-layer continues with the inner circular muscles underneath the cytoplasmic epithelial cell parts forming the nucleo-glandular girdle, and forms the uniform basal lamina of the cone epithelium (Fig. 1).

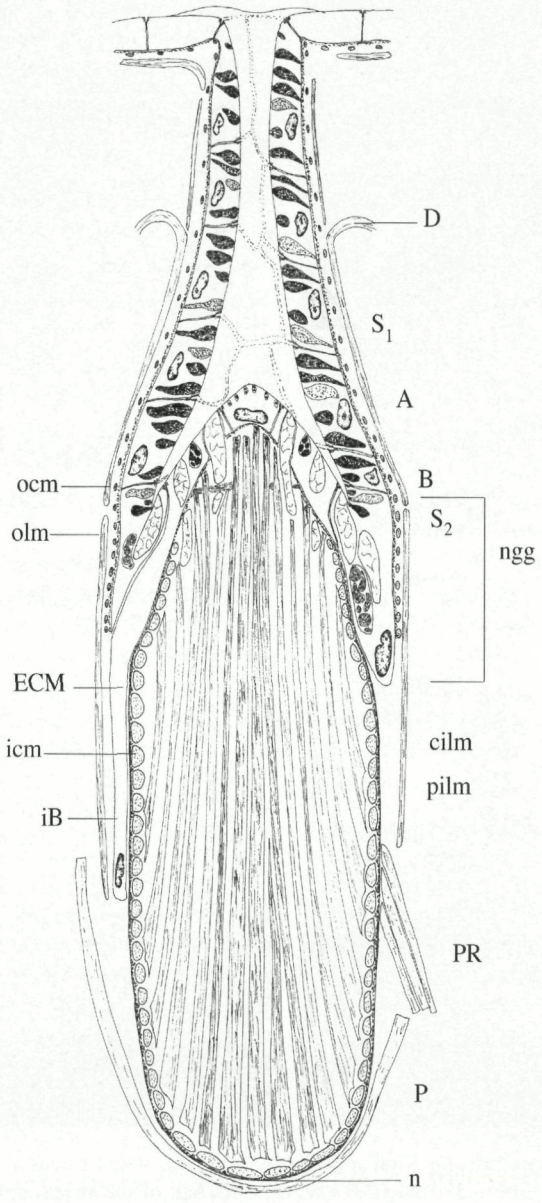
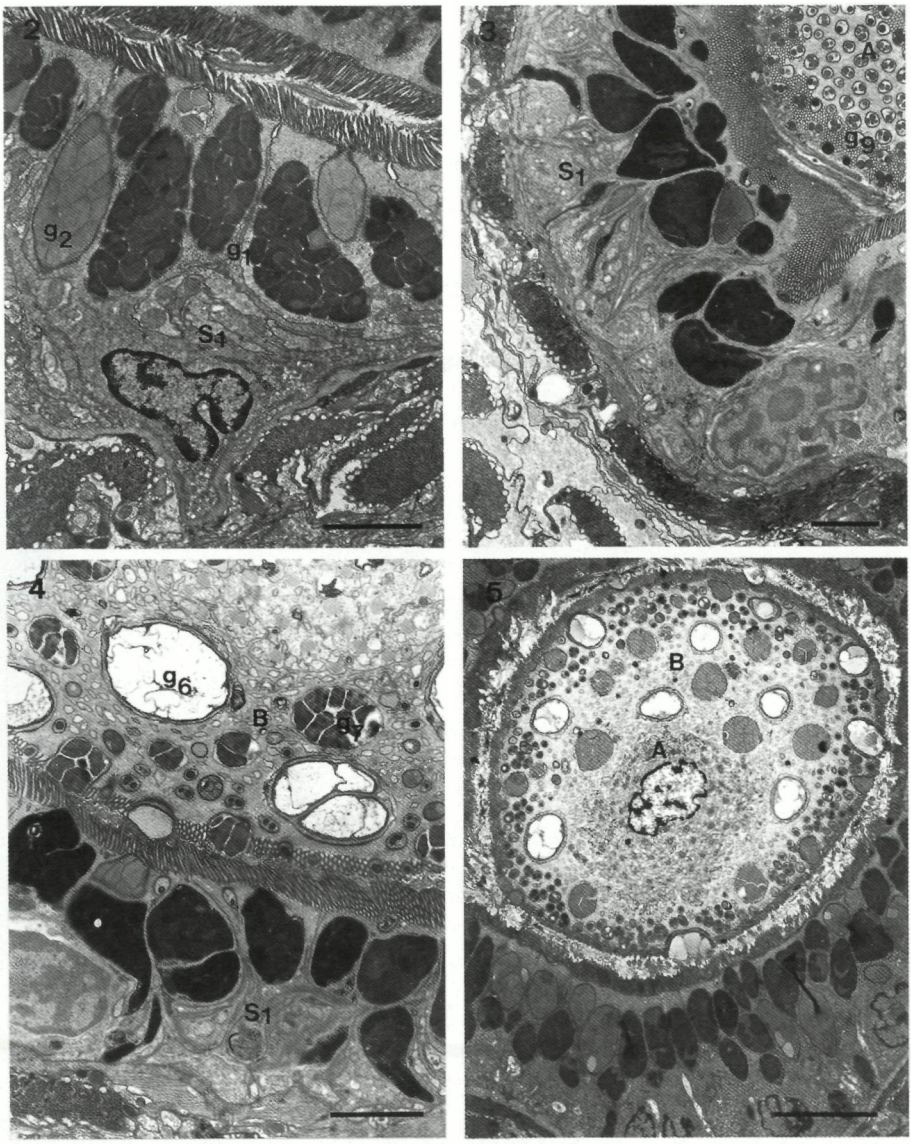
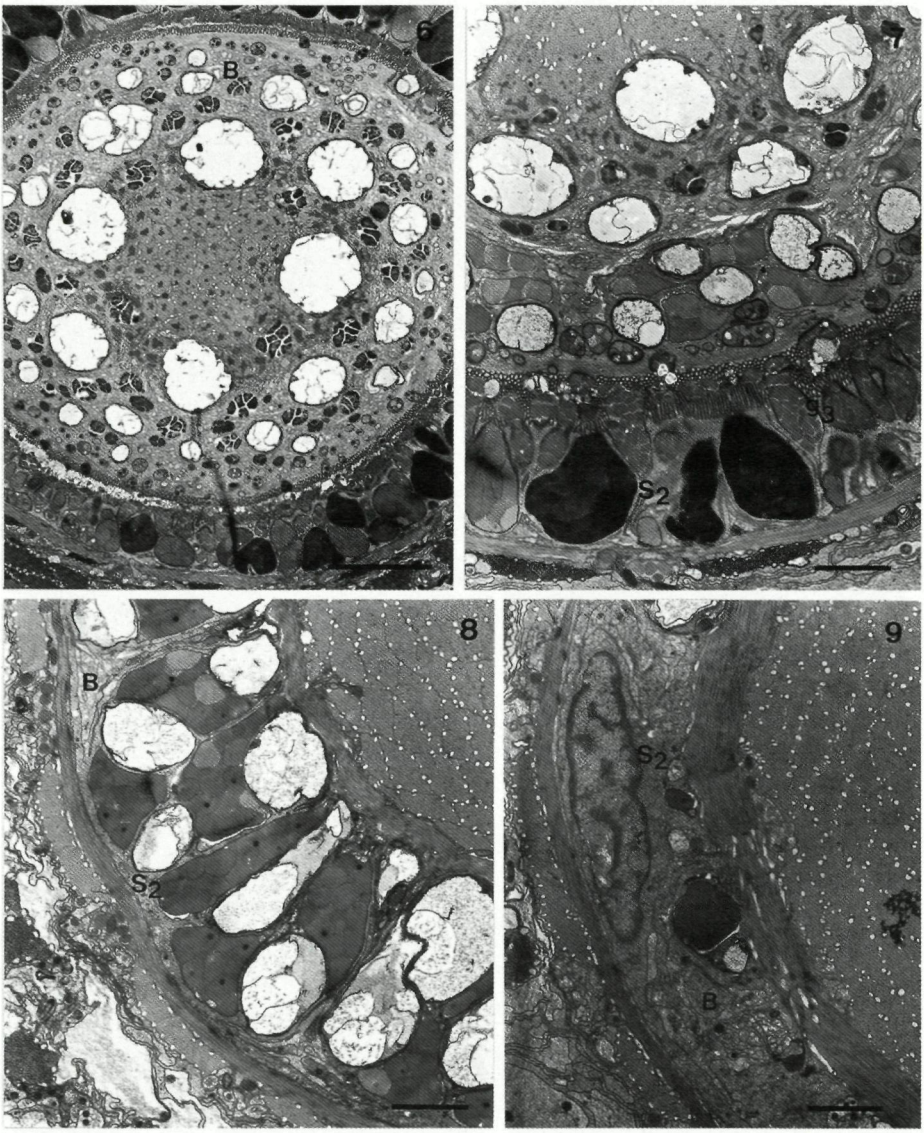


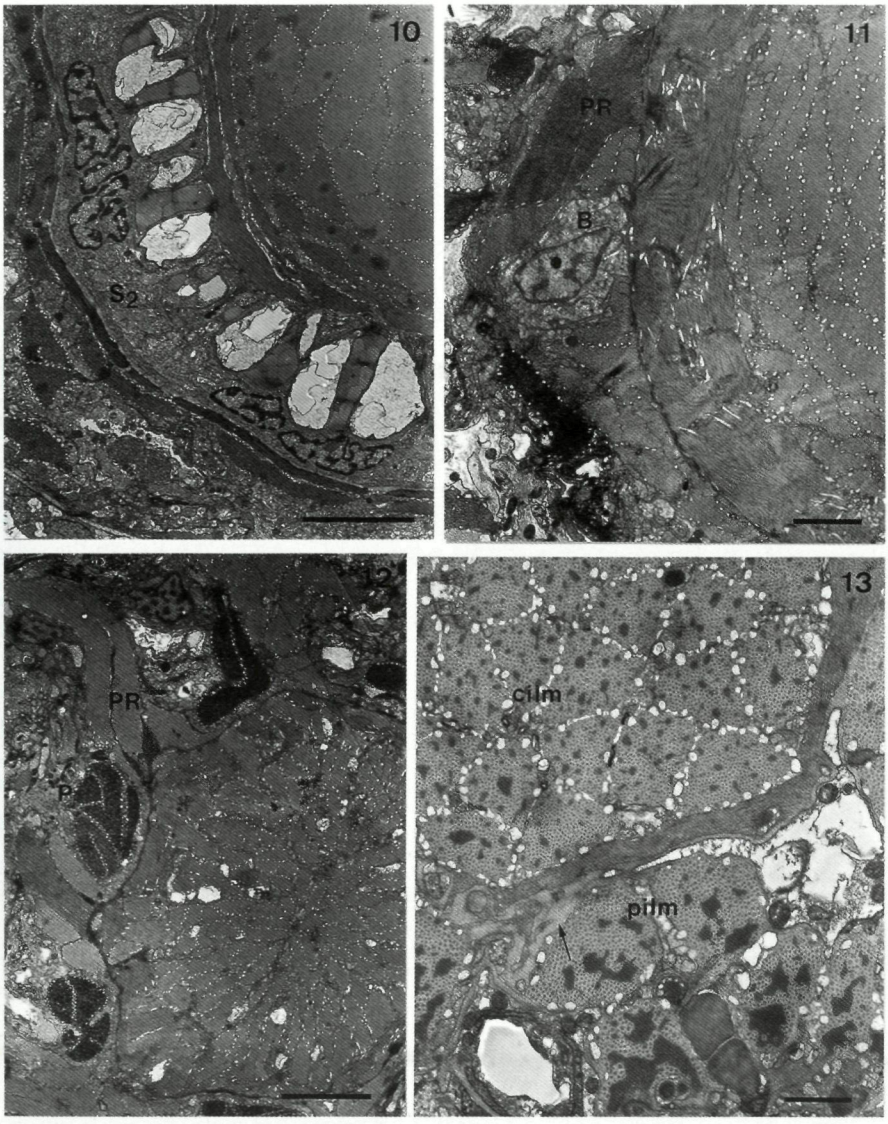
Fig. 1. – Reconstruction of the proboscis of *Zonorhynchus seminascatus*. – S_1 and S_2: resp. the distal and proximal belt of the sheath epithelium; cone epithelium formed by an apical cell (*A*) and a basal syncytial belt (*B*) with insunk nucleated cell parts (*iB*). Proboscis musculature with central (*cilm*) and peripheral (*pilm*) internal longitudinal muscle fibres, inner circular muscles (*icm*), outer circular (*ocm*) and longitudinal muscles (*olm*), proboscis retractors (*PR*) and proboscis protractors (*P*). The position of the nucleo-glandular girdle (*ngg*) is indicated.



Figs 2-5. — 2. *Z. seminascatus*. Cells of the distal belt (S_1) with nucleus and g_1 and g_2 gland necks. Scale bar: 2 µm. – 3. *Z. seminascatus*. Distal belt of the sheath epithelium (S_1) surrounded by circular and longitudinal muscles, and apical cone epithelium (*A*) with g_9 gland necks. Scale bar: 2 µm. – 4. *Zonorhynchus* sp. Distal belt of the sheath epithelium (S_1), and basal cone epithelium (*B*) with g_6 and g_7 gland necks. Scale bar: 2 µm. – 5. *Z. seminascatus*. Cross section of the cone with nucleus of the apical cone epithelium (*A*), and basal cone epithelium (*B*) with alternating g_6 and g_7 gland necks. Scale bar: 5 µm.



Figs 6-9. – *Zonorhynchus* sp. – 6. Cross section of the cone clearly showing alternating g_6 and g_7 gland necks with in between g_8 and g_{10} gland necks. Scale bar: 5 µm. – 7. Proximal belt of the sheath epithelium with g_1, g_2 and g_3 gland necks, basal cone epithelium with groups of g_4 and g_5 gland necks, and g_6 necks in the bulb. Scale bar: 2 µm. – 8. Cross section of the nucleo-glandular girdle showing type g_4 and g_5 gland necks and cytoplasmic cell parts of S_2 and B. Scale bar: 2 µm. – 9. Posterior portion of the nucleo-glandular girdle with nucleus of S_2 and cell strands of B. Scale bar: 2 µm.



Figs 10-13. – *Zonorhynchus seminascatus*.– 10. Nucleo-glandular girdle with groups of g_4 and g_3 gland necks and nuclei of S_2. Scale bar: 5 µm. – 11. Cross section of the median part of the bulb with insunk nucleated cell part of *B* in between proboscis retractors (*PR*). Scale bar: 2 µm. – 12. Cross section of the posterior part of the bulb with proboscis retractors (*PR*) and protractors (*P*). Scale bar: 5 µm. – 13. Cross section in the cone with central cylinder (*cilm*) and peripheral longitudinal muscles (*pilm*), separated by a circular muscle fibre. Note the ECM (*arrow*). Scale bar: 1 µm.

DISCUSSION

The epithelium of the proboscis in *Zonorhynchus*-species is divided into four belts as described for *Cicerina remanei* Meixner, 1928, *Psammorhynchus tubulipenis* Meixner, 1938 and *Cytocystis clitellatus* Karling, 1953 (De Vocht & Schockaert, 1988, De Vocht, 1990). In *Polycystis naegelii* K^lliker, 1845, *Cystiplana paradoxa* Karling, 1964 and *Cystiplex axi* Karling, 1964 and *Mesorhynchus terminostylis* Karling, 1956 five circumferential belts constitute the proboscis epithelium (Schockaert & Bedini, 1977; De Vocht, 1989; De Vocht, 1991). The cone epithelium in all investigated species is always formed by two belts while the number of belts in the sheath epithelium is two or three.

As in *C. remanei*, the distal belt of the sheath epithelium in *Zonorhynchus*-species lines the major part of the proboscis cavity. A distal belt formed by numerous cells, without distinct organization, is typical for all species of *Zonorhynchus,* and has not been recorded for any other species up till now.

A basal lamina under the cone epithelium, as in *C. remanei*, *P. naegelii* and Cystiplanidae is present in *Zonorhynchus*-species as well (Schockaert & Bedini, 1977; De Vocht & Schockaert, 1988; De Vocht, 1989). In *C. axi*, *P. tubulipenis* and *M. terminostylis* and Koinocystididae in general such a basal lamina is lacking (De Vocht, 1989; De Vocht, 1990; De Vocht, 1991; Karling, 1980). In all species of Eukalyptorhynchia investigated, with the exceptions of *Toia calceformis* Brunet, 1973 and *Nannorhynchides herdlaensis* Karling, 1956, the basal belt of the cone epithelium is syncytial (De Vocht, 1992). The organization of the nucleated parts of the cone epithelium is diverse and common tendencies are hard to discern. In all species of the genus *Zonorhynchus* the apical cone epithelium has intra-epithelial nuclei, one in *Z. seminascatus* and *Z. tvaerminensis,* and several in *Z. salinus* (Karling, 1952). Within the Eukalyptorhynchia, only species of the genus *Zonorhynchus* have intra-epithelial nuclei in the cone. In all other species the nuclei of the apical cone epithelium are situated beneath the junction in the nucleo-glandular girdle or inside the bulb. The basal cone epithelium in *Zonorhynchus*-species has insunk nucleated cell parts, not enclosed by a layer of ECM. These epithelial cell perikarya have already been noted but not characterized as such by Karling (1952).

The perikarya of the proboscis epithelia in *C. remanei* are fully enclosed by a layer of ECM and outer circular and longitudinal muscles (De Vocht & Schockaert, 1988). They are not considered insunk. In *Zonorhynchus*-species the nucleo-glandular girdle is not as such incorporated in the bulb as in *C. remanei*, and proximally not enclosed by a layer of ECM. As in *C. remanei*, but less pronounced, a grouping of g_4 and g_5 gland necks is present in this girdle in *Zonorhynchus*-species as well.

A delimited circumferential ring of gland necks above the junction is present in all species classified in the family Cicerinidae. However, such a ring is also present in Gnathorhynchidae and Placorhynchidae (De Vocht, 1992). In *Zonorhynchus*-species this glandular ring is less obvious because the sheath epithelium as a whole is pierced by numerous gland necks.

The conspicuous gland necks of type g_4 and g_5 are also found in *C. remanei*, but are not present in either *P. tubulipenis* or *C. clitellatus* (De Vocht & Schockaert, 1988; De

VOCHT, 1990). Type g_6 and g_7 gland necks in the basal cone epithelium show common features with gland necks found in *C. remanei, P. tubulipenis* and *C. clitellatus*. In *C. remanei* the basal cone epithelium is pierced by two similar types of gland necks ($g_6$and g_7) apically of the glandular ampullae as well (DE VOCHT & SCHOCKAERT, 1988). These gland necks pass through the bulb and enter it at the nodus. In *P. tubulipenis* and *C. clitellatus* homologous gland necks are also found in the basal cone epithelium (DE VOCHT, 1990). In *C. clitellatus*, type g_9 glands also form wide necks in the bulb but they do not form distinct groups.

The apical cone epithelium in *Zonorhynchus*-species is pierced by one type of gland necks that contain an electron-dense secretion packed in small granules (200 nm). This kind of secretion with relatively small granules is also found in *P. tubulipenis* (type g_8) and *C. clitellatus* (type g_{11}) (DE VOCHT, 1990). In *C. remanei* and Cystiplanidae two different types of secretions appear in this epithelium : small rod-like granules as in *P. naegelii* and larger spherical granules (SCHOCKAERT & BEDINI, 1977 ; DE VOCHT & SCHOCKAERT, 1988 ; DE VOCHT, 1989). Apparently gland necks with the small secretion granules in the apical cone epithelium are typical and a common feature for all species investigated.

As in all eukalyptorhynch species investigated, uniciliary receptors with blunt ciliary shafts, basal bodies and rootlets are also present in the cone epithelium of *Zonorhynchus*-species (REUTER, 1975 ; SCHOCKAERT & BEDINI, 1977 ; DE VOCHT & SCHOCKAERT, 1988 ; DE VOCHT, 1989 ; DE VOCHT 1990 ; DE VOCHT, 1991). Multiciliary receptors associated with the distal belt of the sheath epithelium, situated either intra-epithelially as in *C. paradoxa* and *C. axi,* or insunk as in *P. tubulipenis*, *C. clitellatus* and *C. remanei*, are not present in *Zonorhynchus*-species (DE VOCHT, 1989 ; DE VOCHT 1990 ; DE VOCHT 1992).

The motional muscles of the proboscis in *Zonorhynchus*-species include protractors, proboscis retractors and integument retractors. As in *C. remanei* fixators are lacking. In *Z. tvaerminensis* wide disk-shaped fibres are also present anteriorly in the inner circular muscle layer (KARLING, 1952). A clear distinction between groups of internal longitudinal muscles as encountered in *Zonorhynchus*-species is only found in *T. calceformis* and *N. herdlaensis* but in no other eukalyptorhynch species investigated thus far (DE VOCHT 1992).

Based on the ultrastructural data derived from investigations of the proboscis in different species of Eukalyptorhynchia, a tentative cladogram is proposed (Fig. 14). Within the Kalyptorhynchia the Eukalyptorhynchia forms a monophyletic taxon based on the presence of a conorhynch (proboscis with muscular bulb enclosed by a layer of ECM with a cone protruding in the proboscis cavity) (apomorphy 1). *T. calceformis* and *N. herdlaensis* are characterized by fully cellular proboscis epithelia. This character state is regarded as the plesiomorphic condition. Both *Toia*- and *Nannorhynchides*-species have pigmented eyes with lenses and a reduction of one or two sperm axoneme(s), which form synapomorphic features for both taxa (apomorphies 2 and 3) (BRUNET, 1973 ; DE VOCHT, 1992 ; WATSON, 1998). The presence of a syncytial basal belt on the cone epithelium forms a synapomorphy for *Zonorhynchus*-species and the other taxa investigated (apomorphy 4). A distal belt of the sheath epithelium with random, multicellular organization and an apical cone epithelium with intra-epithelial nuclei are apomorphic character states for *Zonorhynchus*-species (apomorphies 5 and 6). The presence of multiciliary receptors asso-

ciated with the distal belt of the sheath epithelium constitutes an apomorphic feature for the other taxa investigated (apomorphy 7). Two insunk sensory organs with multiciliary receptors are present in *C. remanei, P. tubulipenis* and *C. clitellatus* (apomorphy 8). The presence of a third belt in the sheath epithelium is a synapomorphic character for species of the families Koinocystididae, Cystiplanidae and Polycystididae (apomorphy 9) (De Vocht, 1989; De Vocht, 1991; De Vocht, 1992). *P. tubulipenis* and *C. clitellatus* are characterized by intrabulbar nucleated cell parts of the proximal belt of the sheath epithelium and the apical cone epithelium, as well as insunk nucleated cell parts of the basal belt of the cone epithelium (apomorphy 10) (De Vocht, 1990). *C. remanei* is characterized by the presence of four glandular ampullae, surrounded by circular muscles (apomorphy 11) (De Vocht & Schockaert, 1988). The presence of respectively one or three lens(es) in the pigmented eyes of species of the genus *Toia* and *Nannorhynchides* are autapomorphic characters for both taxa (apomorphies 12 and 13) (Brunet, 1973). The monospecific genera *Psammorhynchus* and *Cytocystis* are respectively typified by the appearance of multiciliary receptors which form stacks of flat sheet-like ciliary shafts in the sensory organs, and elongated insunk cell parts of the distal belt with 'normal'-shaped ciliary shaft (apomorphy 14 and 15) (De Vocht, 1990).

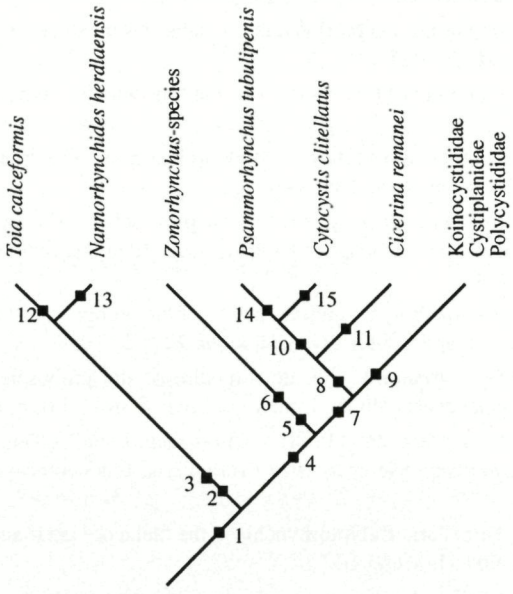


Fig. 14. – Tentative cladogram mainly based on characters derived from ultrastructural investigations on the proboscis of eukalyptorhynch species investigated. See text for the description of the apomorphic characters 1 to 15.

We conclude that the species or genera grouped in the family Cicerinidae lack a synapomorphic character. *Zonorhynchus*-species have an intermediate position in the group of species investigated thus far and have symplesiomorphic features in common with *T. calceformis* and *N. herdlaensis*. *Zonorhynchus* species are characterized by synapomorphic fea-

tures common with *P. tubulipenis*, *C. clitellatus* and *C. remanei*. *C. remanei* and the monospecific genera *Psammorhynchus* and *Cytocystis* can be regarded as monophyletic based on the presence of the sensory organs associated with the distal belt of the sheath epithelium.

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TWO NEW SPECIES OF THE GENUS *DUPLACRORHYNCHUS* SCHOCKAERT & KARLING, 1970, WITH REMARKS ON RELATIONSHIPS WITHIN THE GENUS AND ON THE DUPLACRORHYNCHINAE (PLATYHELMINTHES, POLYCYSTIDIDAE)

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Abstract. Two new species of Polycystididae are described : *Duplacrorhynchus megalophallus* sp. nov. from the bay of Marseille (France) and *D. heyleni* sp. nov. from Zanzibar (Tanzania). The female system of both is characterized by the presence of a muscular bulb at the proximal end of the bursal stalk, with three strong teeth in *D. megalophallus*, and in *D. heyleni* the muscle bulb is highly asymmetric. In *D. megalophallus* the copulatory bulb is divided by a septum, and the cirrus has small spines and a proximal umbrella-like expansion. In *D. heyleni* only the cirrus, which has strong teeth, is enclosed in the cirrus sack : the seminal and prostate vesicles are not. Relationships with the two known species of the genus (*D. minor* Schockaert & Karling, 1970 and *D. major* Schockaert & Karling, 1970) are discussed. The subfamily Duplacrorhynchinae Schockaert & Karling, 1970 may constitue a monophyletic taxon, characterised by the 3+2 configuration of the proboscis retractor system. Duplacrorhynchinae contains all genera of Polycystididae with an interposed prostate vesicle. The taxon *Duplacrorhynchus* may be the sister taxon of all these genera.

Key words : *Duplacrorhynchus megalophallus* sp. nov., *Duplacrorhynchus heyleni* sp. nov., Eukalyptorhynchia, systematics, phylogeny, Polycystidae, Duplacrorhynchinae.

INTRODUCTION

In 1970 SCHOCKAERT & KARLING introduced the subfamily Duplacrorhynchinae to contain « three new anatomically remarkable » species of Polycystididae : *Duplacrorhynchus minor*, *D. major* and *Yaquinaia microrhynchus*. The remarkable aspect of these species is the copulatory organ of the so-called conjuncta-duplex type (terminology of KARLING, 1956), *i.e.* with an interposed prostatic vesicle and an eversible cirrus enclosed in a muscular bulb or cirrus sack. Later, SCHOCKAERT (1971, 1974) added *Djeziraia pardii* Schockaert, 1971 to the subfamily (or at least suggested a close relationship) based on the fact that the prostate vesicle of that species is also interposed, and the stylet is single walled – a so-called « papillenstilet » (terminology of KARLING, 1956) – unlike the double-walled stylet of all other Polycystididae. However, the prostate vesicle is also interposed in the genera *Phonorhynchoides* Beklemischev, 1927 and *Annalisella* Karling, 1978 (both with a single-

walled stylet), and *Koinocystella* Karling, 1952 and *Paracrorhynchus* Karling, 1956 (both with a cirrus that is not enclosed in a cirrus sack). This is also the case in *Gemelliclinus flavidus* Evdonin, 1970, but the original description of this species does not provide enough data and the material in our possession is of inferior quality; hence we consider this species as *inquirenda* and it will not be considered further. Do these taxa all belong in the Duplacrorhynchinae? An extensive discussion on the matter is beyond the scope of this contribution, but we present some ideas in the discussion.

In this contribution we describe two new species, considered to be representatives of the genus *Duplacrorhynchus*: *D. heyleni* n.sp. from the African East Coast (Zanzibar) and *D. megalophallus* n.sp. from the Mediterranean. Both species show some characters in which they strongly deviate from each other and from the two previously described *Duplacrorhynchus* species, but we argue for their close relationship and consider that they are members of a single monophyletic taxon.

MATERIAL AND METHODS

Both species were found in marine environments and were extracted from the sediment by decantation, using an isotonic $MgCl_2$ solution (see Schockaert, 1996). Animals were studied alive and then mounted with lactophenol. Other specimens were fixed in Bouin's fluid and serially sectioned. Sections were stained with Heidehain's iron hematoxilin, using eosin as counterstain. The material of *Duplacrorhynchus megalophallus* was collected and studied alive by Dr. Brunet.

The size of organs is expressed as a percentage of the body length as is the position of the gonopore (distance from the anterior tip of the body). Figures without a scale are freehand.

Type material of the new species is deposited in the zoological collection of the Department SBG, Limburgs Universitair Centrum, Diepenbeek, Belgium. Type material of the species considered in the discussion are in the Swedish Museum of Natural History (Stockholm) where they were studied by T.A. during a visit in 1997. Some type material is also located in the Diepenbeek collection.

ABBREVIATIONS IN THE FIGURES

acg:	accessory glands	od:	oviduct
ag:	atrial glands	ov:	ovary
b:	bursa	p:	proboscis
bs:	bursal stalk	pb:	proboscisbulb
ci:	cirrus	ph:	pharynx
co:	copulatory organ	r1, r2, r3:	three different retractors
dir:	dorsal integument retractor	rs:	seminal receptacle
e:	eye	t:	testis
evs:	external seminal vesicle	ut:	uterus
fd:	female duct	vg:	prostate vesicle
g:	glands	vi:	vitellarium
ga:	common genital atrium	vir:	ventral integument retractor
gp:	gonopore	vs:	seminal vesicle
ivs:	internal seminal vesicle	x:	explanation in text
mb:	muscular bulb		

DESCRIPTIONS OF THE NEW SPECIES

Duplacrorhynchus megalophallus sp. nov.

Distribution. Bay of Marseilles, between the Chateau d' If and the Isle of Ratonneau, in *Amphioxus*-sand (14-16 m deep), 3/2/1966 (Type locality). Bay of Marseilles, between the Island of Jarre and the coast, on the «Plateau des Chèvres», in *Amphioxus*-sand (8-10 m deep), 3/2/1966.

Material. Drawings of living animals by Dr. Brunet. Several individuals sectioned, one of them designated as the holotype (sectioned horizontally, leg. Dr. Brunet).

Derivatio nominis. The species epithet refers to the very large copulatory organ; megalos (Gr.): big, phallos (Gr.): penis.

Description

The living animals are colourless, 0.8-1 mm long, with paired eyes. The epidermis is syncytial with flattened, lobate nuclei, 5 µm high, with cilia of 4 µm. Ovoid rhabdites are present, with a length of about 1/3 of the height of the epithelium. The pharynx is situated in the first body half and is of the same construction as in the other members of the genus (see SCHOCKAERT & KARLING, 1970).

The proboscis is 20% of the body length in living animals (160 µm according to Brunet's measurements) and about 30% in sectioned material. The proboscis cavity has an anucleated epithelium and is surrounded by internal circular and external longitudinal muscles. The apex of the conus is very small. There are no nuclei at the junction of the cone and the sheath epithelium. There are three pairs of proboscis retractors and two pairs of integument retractors.

The gonads are paired. Both testes are situated laterally and extend from about the anterior edge of the pharynx to the ovaries. The ovaries are rather small and situated ventro-laterally behind the pharynx. The vitellaria lie dorsally and extend from the level of the pharynx towards the caudal body end. The common gonopore is situated at about 85%. The genital atrium is divided into two parts by a slight constriction, one in which the copulatory organ opens while the uterus and the bursal stalk end in the other one. The atrium is lined with a nucleated epithelium and surrounded by weak longitudinal muscle fibers. In some sections the epithelium has a ruffled aspect (pseudociliation) or seems to have disappeared (pseudocuticula). Basophilic atrial glands are found where the copulatory organ connects to the atrium.

The copulatory organ has a caudal position, dorsally from the common genital pore. Its position may be variable: in living animals and in several sectioned animals its position is behind the genital pore, while in the holotype it is situated in front of it. The copulatory system consists of a large ovoid bulb that is surrounded by a spiral muscle sheath and is divided into two parts by a transverse septum. The distal part contains the cirrus, the proximal part the enlarged seminal duct (internal seminal vesicle) and the intracapsular parts of the prostate glands. The seminal duct narrows towards the septum and perforates it, to continue as the cirrus. The prostate glands enter the seminal duct here. The nucleated parts of the prostate glands are entirely extracapsular. The prostate secretion consists of coarse basophilic granules. There is also an extracapsular seminal vesicle.

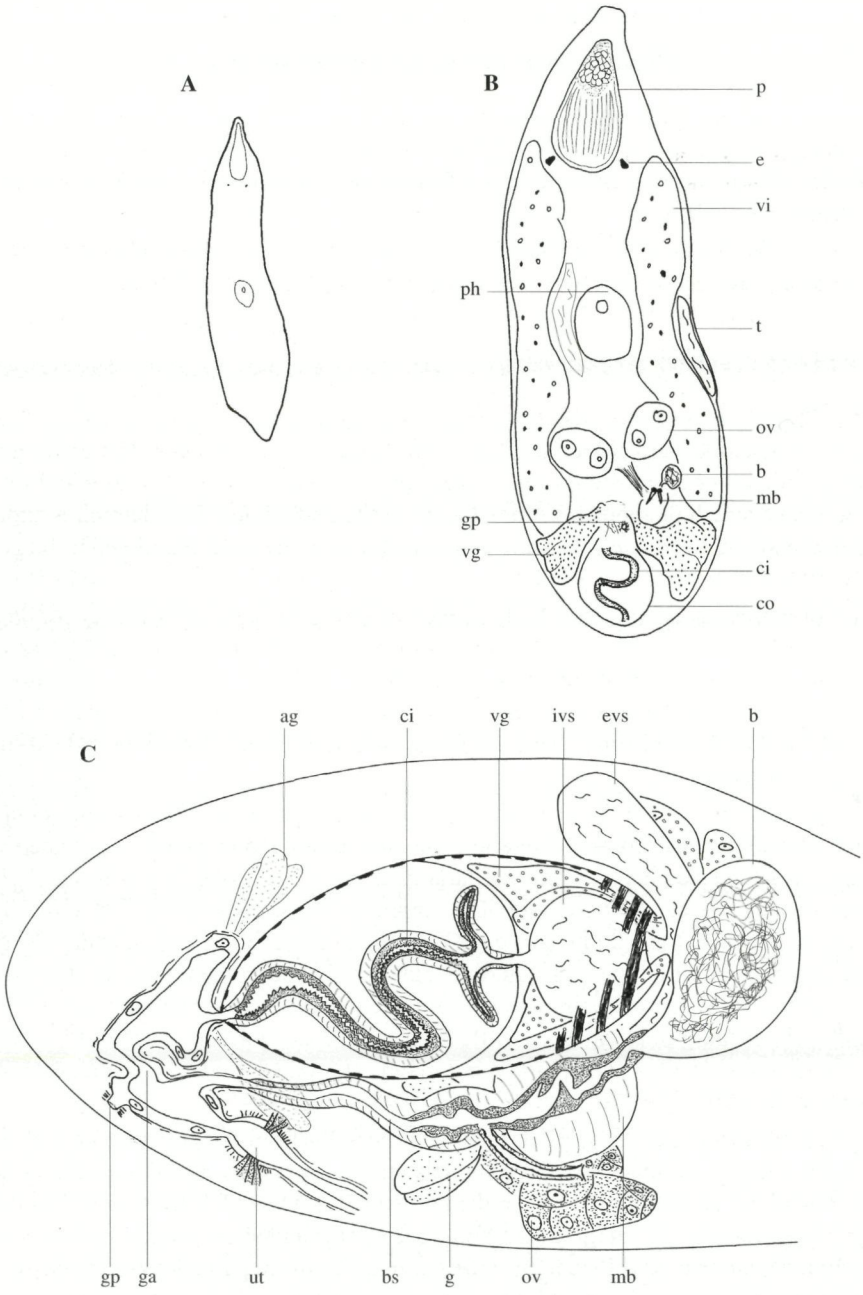

Fig. 1. – *Duplacrorhynchus megalophallus* sp. nov. – A. Habitus. – B. General organisation (from a living animal). – C. Horizontal reconstruction of the genital organs seen from the ventral aspect.

The most proximal part of the cirrus is narrow and moderately sclerotised. It widens almost immediately into an umbrella-shaped diverticulum of which the proximal wall is uniformly sclerotised; the distal wall bears tiny teeth. Such teeth occur over the whole length of the cirrus, and are all of equal size, slightly larger than those in the diverticulum. The cirrus is surrounded by a spiral muscle sheath over its whole length.

Bursal stalk and uterus (with the same structure as in most Polycystididae) end in the same diverticulum of the atrium. The bursal stalk runs straight in the cephalic direction, above the uterus, and ends in the large spherical to ovoid bursa. One sectioned individual had a nucleated epithelium in the bursa, while in all other specimens the bursa was surrounded by a thin membrane and was filled with sperm. The bursal stalk is long, surrounded by a thick circular muscle coat that becomes weaker towards the atrium. Its epithelium is anucleate distally, reduced to a pseudocuticula proximally. Close to the bursa the bursal stalk is surrounded by a very thick muscular bulb, and inside this bulb the pseudocuticula forms a ring of three strong teeth.

The female duct opens in the bursal stalk just distally of the muscular bulb. Some basophilous glands open here. From there on it also runs in a cephalic direction. It is lined by a thin pseudocuticula. The female duct then bifurcates into the two oviducts, which are very short and have anucleated epithelia. Both the oviducts and the female duct are surrounded by a thin circular muscle layer that disappears towards the ovaries.

***Duplacrorhynchus heyleni* sp.nov.**

Duplacrorhynchus spec. in WATSON, 1999

Distribution. Widely distributed in mangrove sand flats on Zanzibar Island (Tanzania): off Mahurubi Palace ruins, sand flat in the high mid-littoral with relatively clean coarse sand disturbed by crabs (5/8/1995) (type locality); same locality, in the sand of an exposed sea grass field, very rich in detritus (5/8/1995); beach behind the Mbweni Ruins Hotel, north of the creek, in a little pool with seagrass (*Thallasia* spec?) (11/8/1995), and in a tide pool with a broad leafed seagrass (17/8/1995); south of the creek, in a higher part ot the sand flat, with relatively coarse sand (11/8/1995); mangrove forest near Pete, in a tide pool with relatively fine sand and some algae (16/8/1995).

Material. Many animals studied alive, 7 whole mounts, 5 sectioned specimens, one of them designated as holotype (horizontal sections).

Derivatio nominis. Named after Wim Heylen, a friend of Artois'.

Description

Colourless and opaque animals of about 1 mm long, with paired eyes. The epidermis is syncytial with numerous optically empty vacuoles. The rhabdites are more or less oval, less numerous in the first body quarter. The position and construction of the pharynx is the same as in the other members of the genus.

The proboscis is about 20% of the body length, with a distinct apex. The epithelium of the proboscis cavity is relatively high with a ring of nuclei approximately in the middle of the cavity. The cavity is surrounded by an outer longitudinal and an inner circular muscle layer. The circular one is lacking in the distal 1/4 of the cavity. There are no nuclei at the

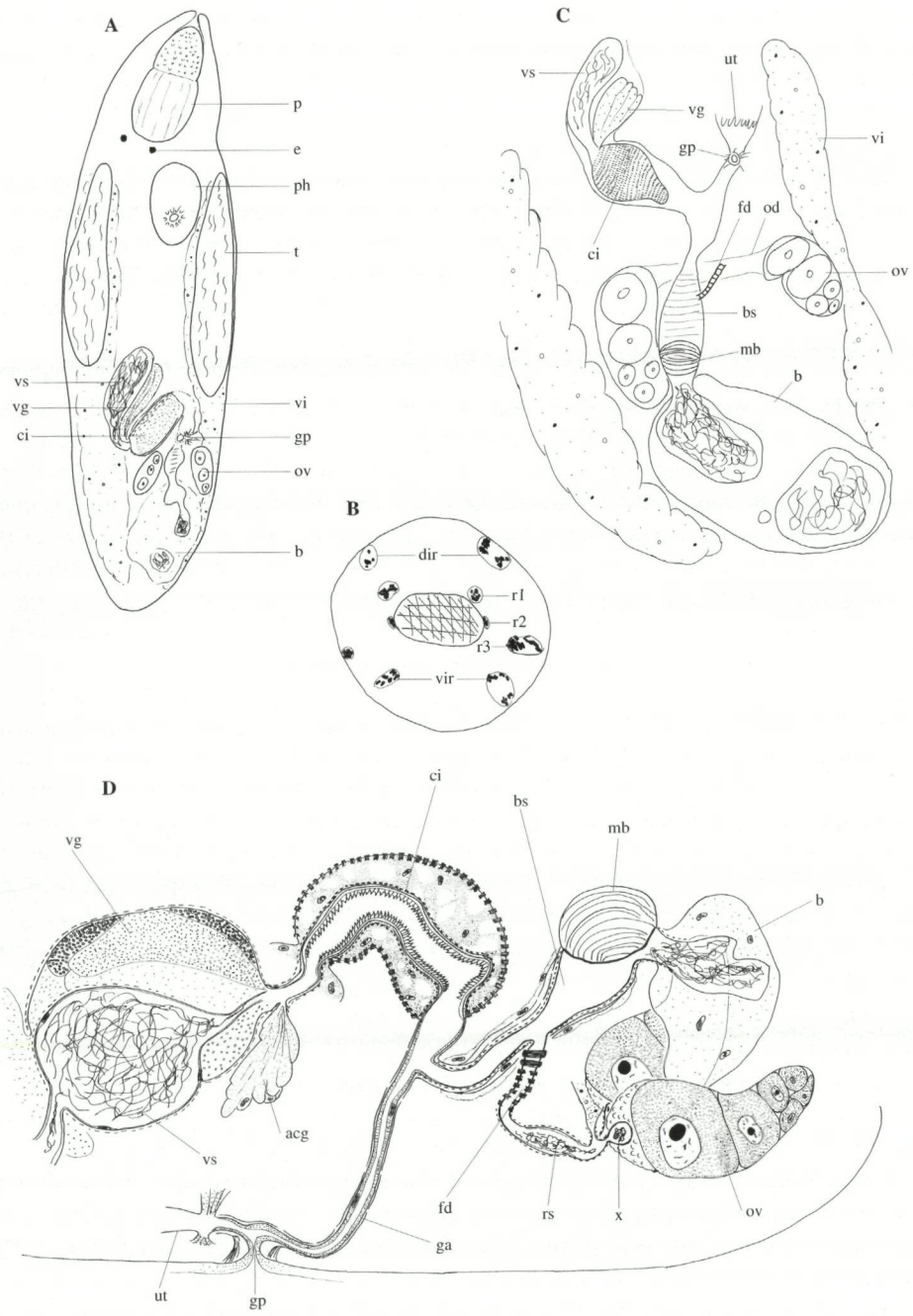

Fig. 2. – *Duplacrorhynchus heyleni* sp. nov. – A. General organisation (from a living animal). – B. Transverse section at the level of the distal end of the proboscis bulb. – C. Genital organs in a living animal. – D. Sagittal reconstruction of the genital organs seen from the left.

junction of the cavity and the cone epithelium. The basal cone epithelium is very glandular, with a strongly eosinophilic secretion. There are three pairs of proboscis retractors and two pairs of integument retractors.

The gonads are paired. The testes are situated dorsolaterally, and extend from the pharynx to the copulatory organ. The ovaries lie laterally, behind the copulatory organ. They are rather long, with the oocytes more or less arranged in rows. The vitellaria lie dorsally to the testes and extend from the level of the pharynx to the caudal body end. The gonopore is situated at about 70% and surrounded by a strong sphincter. The common genital atrium is unusually long, is lined by a nucleated epithelium and surrounded by a longitudinal muscle layer. Proximally it has a narrow diverticulum into which the copulatory organ intrudes.

The male copulatory system consists of a single seminal vesicle (lined with a low nucleated epithelium), narrowing distally into the seminal duct, which continues as a broader muscular ejaculatory duct towards the armed cirrus. The distal part of the seminal vesicle is asymmetrically surrounded by the large prostate vesicle. The seminal vesicle and the prostate vesicle are both enveloped in a common spiral (mainly circular) muscle layer. The prostate glands enter the seminal duct close to its connection with the ejaculatory duct. There are five different types of prostate glands: two with eosinophilic granules (light coarse granules, dark and very coarse granules) and three with basophilic granules (dark coarse granules, light and small granules, very light small granules). All nucleated parts of the prostate glands are outside the muscle coat. The ejaculatory duct and the cirrus are surrounded by an inner longitudinal and a strong outer circular muscle layer (both actually spiralling). The muscle layer surrounding the prostate vesicle is continuous with the circular layer. The cirrus is a broad duct, armed with numerous uniform, triangular teeth, with their sharp ends pointing proximally. It is enclosed by a highly muscular septum of which the muscles are mainly circular, and continuous with the circular muscle layer of the ejaculatory duct. A bundle of light basophilic accessory glands enters the ejaculatory duct at its initial part.

The bursal stalk enters the long genital atrium caudally, distally from the cirrus, and ends in the terminal bursa. It receives the female duct about mid-way. Close to the bursa there is a large, highly asymmetrical muscular bulb. Proximally from this bulb the bursal stalk has a relatively strong circular muscle layer, distally it has an inner circular layer and a double outer layer of longitudinal muscles that becomes single towards the muscular bulb, once the female duct has joined the stalk. The stalk is surrounded by a layer of cells, presumably muscle cells. The epithelium lining the bursal stalk and the female duct is membraneous; that covering the muscular bulb appears sclerotised (« pseudocuticula »). The female duct is surrounded by circular muscles, quite strong close to the entrance in the bursal stalk, becoming weaker proximally. The short oviducts, which enter the female duct at its proximal end, also have circular muscles. These oviducts each show a small globular widening that is embedded in the stromatic tissue at each ovary (see Fig. 2D, labelled x). Sperm is found in these widenings, both evidently functioning as seminal receptacles. In its proximal part the unpaired female duct is also widened, and some sperm can be found here as well. The uterus enters the common genital atrium through its anterior wall, close to the gonopore. It has a relatively low epithelium, but does not deviate from the usual polycystidid construction.

DISCUSSION

All four *Duplacrorhynchus* species have an interposed prostate vesicle and a cirrus enclosed in a bulb: the conjucta-duplex type. However, this type of copulatory organ can be found in many Platyhelminthes, in virtually all major taxa. So this is clearly a plesiomorphy and thus invalid to define the taxon, even though it is this characteristic that brought SCHOCKAERT & KARLING (1970) to define the subfamily Duplacrorhynchinae (but see below).

The female system of the four species, however, exhibits a number of features which can be considered unique for Polycystididae: a long and highly muscular bursal stalk, a single, long, muscular female duct that joins the bursal stalk mid-way, the oviducts being very short and entering the female duct at its proximal end. The combination of these characters can thus be put forward as a synapomorphy for the four species considered. (A rather similar situation is seen in the Koinocystididae, though differences can be found and there is probably no homology). The four species are, however, very different from each other in other characteristics, as well in the female as in the male system, even to the extent that erecting a new genus for each of them might be appropriate. We have chosen not to do so to avoid an «inflation» of names, and to keep the taxon *Duplacrorhynchus* on the genus «level».

The most striking distinction in the male system of *D. heyleni* is the very large prostate vesicle and the seminal vesicle both not enclosed in the bulb. Only the cirrus is enclosed. Moreover, the seminal vesicle enters the prostate vesicle eccentrically and the prostate vesicle contains the necks of five different glands. In *D. minor* the prostate vesicle appears «reduced» and the cirrus is devoid of spines. In *D. major* the cirrus has small spines only in its middle part, while in *D. megalophallus* the bulb is divided by a muscular septum in a proximal part (containing the prostate vesicle and the internal seminal vesicle) and a distal part with the cirrus that has an umbrella-like expansion). These are all autapomorphies for each of the species. Other autapomorphies are the elongated common genital atrium and the accessory glands at the beginning of the cirrus in *D. heyleni*, and the accessory glands in the male atrium where the cirrus enters it in *D. megalophallus*. No obvious synapomorphies can be found in the male system.

Both *D. major* and *D. minor* have a voluminous eccentric seminal receptacle attached to the female duct. Furthermore, the female duct is swollen into a spherical enlargment in which a «morula-shaped» appendage can be found (SCHOCKAERT & KARLING, 1970, p. 242). At least this peculiar structure can be considered a synapomorphy for both species. Also in *D. heyleni* the female canal is enlarged. It obviously functions as a seminal receptacle and the morula-like appendage is lacking. In *D. minor* the terminal bursa is lacking, an autapomorphy of this species.

In *D. heyleni* and in *D. megalophallus* the bursal stalk is enlarged and highly muscular close to the bursa: a synapomorphy for these two species. In *D. heyleni* the muscles form a unilaterally very thick mass, while in *D. megalophallus* it contains three highly sclerotised teeth.

Within the taxon (genus) *Duplacrorhynchus, D. minor* and *D. major* appear to be closely related and a close relationship of *D. heyleni* with *D. megallophallus* can be supported as well.

The main diagnostic feature given by SCHOCKAERT & KARLING (1970) for the Duplacrorhynchinae is the copulatory organ of the conjuncta-duplex type, however this is a plesiomorphy (see above). The copulatory organ as in *Djeziraia* (conjucta with a «papillen

stilet») can also be found in many taxa within and without the Kalyptorhynchia. The Duplacrorhynchinae thus seems to be paraphyletic. We have reconsidered all Polycystididae with a copulatory organ where the prostate vesicle is interposed (see enumeration in the introduction). It now appears that in all these species the proboscis can be retracted by a system of three pairs of retractors (one subdorsal, one lateral, one subventral) and that the body wall around the the proboscis opening can be retracted by two pairs of integument retractors. In all other Polycystididae and in most other Eukalyptorhynchia the retractor system consists of four pairs of proboscis retractors and, in the other Polycystididae, only one pair of integument retractors (except in the polycystidids *Acrorhynchides* Strand, 1928, *Macrorhynchus* Graff, 1882, *Opisthocystis* Sekera, 1912, *Papia* Karling, 1956, and except in the Cytocystididae Karling, 1964, where the 3+2 retractor system is most probably a convergence). Consequently, this 3+2 retractor system might be considered a synapomorphy for all Polycistididae with an interposed prostate vesicle (conjuncta type = plesiomorphy) which can now be included in the taxon Duplacrorhynchinae. The bulk of the other Polycystididae constitute the sister taxon with a copulatory organ of the divisa type, *i.e.* with the prostate vesicle entering the male atrium next to the sperm conducting system. Though a thourough discussion on the relationships of the genera within the Duplacrorhynchinae is beyond the scope of this contribution, we have reasons to believe that *Duplacrorhynchus* is the sister taxon of all other Duplacrorhynchinae. Other Duplacrorhynchinae do not have the combination of characters in the female system that is considered to be the apomorphy of the taxon *Duplacrorhynchus*, and they all have a copulatory organ that deviates from the typical duplex system.

AMENDED DIAGNOSES AND DIAGNOSES OF THE NEW TAXA

Duplacrorhynchinae Schockaert & Karling, 1970

Polycystididae with paired gonads, a copulatory organ with an unpaired seminal vesicle and an interposed prostate vesicle (conjuncta type). Three pairs of proboscis retractors and two pairs of integument retractors (apomorphy).

Type-genus : *Duplacrorhynchus* Schockaert & Karling, 1970

Genus *Duplacrohrynchus* Schockaert & Karling, 1970

Duplacrorhynchinae with a cirrus enclosed in a septum. Genital pore ventral, not terminal. Female system with a highly muscular bursal stalk, the unpaired muscular female duct entering the bursal stalk in its middle part (apomorphy).

Type species : *D. minor* Schockaert & Karling, 1970

D. minor + *D. major*

Duplacrorhynchus species with a compound copulatory organ, with seminal vesicle and prostate vesicle enclosed in the bulb. Female system with an eccentric seminal receptacle and the female duct with an enlargement containing a « morula-shaped » appendage.

***D. minor* Schockaert & Karling 1970**: *Duplacrorhynchus* species without a terminal bursa, small and entirely intracapsular prostate glands; cirrus unarmed.

***D. major* Schockaert & Karling 1970**: *Duplacrorhynchus* species with a terminal bursa, a large prostate vesicle with the nucleated parts of the prostate glands outside the septum; small spines in the middle of the cirrus.

D. megalophallus + *D. heyleni*

Duplacrorhynchus species with a muscular enlargement in the proximal part of the bursal stalk. Cirrus with spines over its entire length.

***Duplacrorhynchus megalophallus* sp.nov.**: *Duplacrorhynchus*-species with seminal vesicle and large parts of the prostate glands within the bulb. Copulatory organ very large, divided by a muscular septum in a proximal part (containing the seminal vesicle and prostate glands) and a distal part (with the cirrus). Cirrus with an umbrella-like expansion. Enlargement of the bursal stalk with three strong teeth. Genital atrium with a male and a female part.

***Duplacrorhynchus heyleni* sp.nov.**: *Duplacrorhynchus*-species with only the cirrus enclosed in the bulb. Seminal vesicle enters the prostate vesicle eccentrically. Strong triangular teeth in the cirrus. Common genital atrium very long. Muscular enlargement of the bursal stalk that is highly asymmetrical.

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METAZOAN PHYLOGENY AS A TOOL IN EVOLUTIONARY BIOLOGY: CURRENT PROBLEMS AND DISCREPANCIES IN APPLICATION

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« Everything is what it is because it got that way»
D'ARCY WENTWORTH THOMPSON, 1917

Abstract. An ever increasing number of comparative studies try to shed light on various aspects of animal evolution. Particularly studies in comparative ultrastructure and evolutionary developmental biology propose remarkable hypotheses about the history of animal life. These studies must logically depend on an accurate and comprehensive knowledge of recent developments in phylogenetic methodology and hypotheses. Unfortunately, this requirement is often not met. I discuss some important recent investigations from various fields in order to illustrate the many pitfalls involved, and emphasize the necessity for sound insight into current phylogenetics as an essential prerequisite to studies of animal evolution.

Key words: Metazoa, phylogeny, comparative biology, evolutionary developmental biology, Urbilateria, tree pruning

INTRODUCTION

The study of higher-level animal relationships has a long pedigree, going back in its most familiar form to Haeckel's artistic trees. Enormous numbers of phylogenies have been published since then, making it very difficult to orient oneself in this expansive and quickly exploding literature. This has led a significant number of zoologists (especially those not directly involved in phylogenetic research) to fail to see the forest for the trees.

A useful way to organize the literature is to focus on the studies that employ cladistic principles for phylogeny reconstruction. A characteristic of many pre-cladistic studies is the absence of a rigorous and well-defined methodology of analysis. Intuitive methods that grouped species on the basis of general similarity or even common ascent (JANVIER, 1996) prevailed in the past, and prevented any firm consensus about the phylogeny of the animal phyla. The advent of cladistics revolutionized the field, and a blossoming of higher-level

cladistic studies on the basis of both morphology and molecular sequence analyses has emerged in the last decade (*e.g.*, for molecular analyses see AGUINALDO *et al.*, 1997 ; FIELD *et al.*, 1988 ; LAKE, 1990 ; WINNEPENNINCKX *et al.*, 1995a ; for morphological analyses see AX, 1995 ; BRUSCA & BRUSCA, 1990 ; EERNISSE *et al.*, 1992 ; HASZPRUNAR, 1996 ; NIELSEN, 1995 ; NIELSEN, SCHARFF & EIBYE-JACOBSEN, 1996 ; SCHRAM, 1991). Some interesting results have arisen from this research. However, a detailed consensus is not yet apparent, both between and within the fields of molecular and morphological analyses. Upon examination of current cladistic analyses of metazoan morphology, we found that the lack of consensus is due to differences in fundamental methodology underlying the various cladistic analyses (JENNER & SCHRAM, in press). This study was a comprehensive attempt to explicitly introduce theoretical issues of cladistic methodology to explain the diversity of results of higher-level animal phylogenies. In order to construct a morphological reference framework, a more experimental approach toward higher-level animal phylogeny is needed. Increased attention to issues of character and taxon selection, character coding, scoring, weighting, and ground pattern reconstruction is of crucial importance (JENNER & SCHRAM, in press). Unfortunately, current authors frequently seem falsely convinced of the robustness of their phylogenies. The diversity of recently proposed phylogenetic schemes belies this misplaced confidence. It is time for a more constructive assessment of current conflicting hypotheses.

Apart from difficulties associated with the construction of a robust metazoan phylogeny in itself, there are also problems relating to the proper use of phylogenetic information in comparative biology. In this paper, I want to focus attention on how phylogenetic information should be used when studying animal evolution on the basis of comparative studies. Increasing numbers of researchers are trying to illuminate animal evolution by in-depth analysis of a small number of species, in particular by employing the model system organisms used in molecular and developmental biology. I suggest that conclusions drawn from the study of only a few model system organisms are likely to be meaningless when insufficient attention is paid to overall invertebrate phylogeny and modern phylogenetic methods.

USE AND MISUSE OF PHYLOGENIES : CRITICAL REMARKS ON THE RECONSTRUCTION OF THE « BILATERIAN ANCESTOR »

The need for a solid and well-resolved phylogeny of the Metazoa is now greater than ever. For a detailed understanding of metazoan evolution we need to compare and integrate the evidence from diverse fields, such as morphology, molecules, paleontology, and evolutionary developmental biology. Paraphrasing DOBZHANSKY, we could state, « Nothing in evolutionary biology makes sense, except in the light of phylogeny ». A phylogenetic framework is necessary for studying the evolution of any organismal feature (*e.g.*, a phenotypic trait, behavioral trait, life-history characteristic), and for reconstructing the ancestral features of a taxon or group of taxa. Researchers often rely, however, on « plausibility » or « common sense » approaches to argue for a particular evolutionary transformation. Unfortunately, such *ad hoc*, intuitive approaches lack any methodological

rigor and often lead to spurious results, as shown by various authors (*e.g.*, McHugh & Rouse, 1998 ; Packer, 1997 ; Sturmbauer *et al.*, 1996 ; Hart *et al.*, 1997).

Recent advances in diverse fields of research (ultrastructure, molecular developmental biology, paleontology) have invited increased interest in the *Big Questions* about animal evolution. These include the origin, diversification, and stability of animal body plans, and trends of metazoan evolution such as changes in organismal complexity. A solid phylogenetic framework is the only valid background for such discussions. The choice of a particular phylogenetic framework is therefore a most crucial step during any study. An uncritical choice can easily render resultant hypotheses of evolutionary scenarios meaningless.

One problem that remains elusive to this day is the nature of the bilaterian ancestor. Striking similarities in the molecular developmental biology of insects and chordates (*e.g.*, formation of the dorso-ventral axis, development of « segments ») have particularly stimulated a resurgence of interest into the characteristics of their common ancestor (Arendt & Nübler-Jung, 1995 ; Holland *et al.*, 1997 ; Holley & Ferguson, 1997 ; Müller *et al.*, 1996). I will illustrate some recent approaches to the use of phylogenetic information in the reconstruction of the ancestor of the Bilateria. The first two studies I will discuss deal with evolutionary developmental biology. A common problem of these studies is that they routinely employ *pruned* phylogenies to depict the relationships of only a few model system species. These pruned phylogenies can either represent incomplete phylogenies due to paucity of data, or phylogenies from which taxa are deliberately removed. They are then used to reconstruct ancestral ground patterns and thus function as the foundation for evolutionary scenarios. I will first illustrate the dangers of this approach.

Pruned phylogenies : handle with care !

Arthur (1997) rightly argued for the importance of phylogeny for studying the origin and evolution of animal body plans. Some fundamental flaws in logic, however, underlie his discussion. Arthur argued that pruning a phylogeny to only those taxa of interest reduces the information content of the cladogram, but also reduces the probability of it being wrong. I agree with the first conclusion, but I strongly disagree with the second.

Why is pruning a problem? Let us examine a hypothetical phylogeny, and its pruned version (Fig. 1). Fig. 1a depicts the « real » evolutionary relationships of the taxa W, X, Y, and Z as inferred by a comprehensive phylogenetic analysis. « A » represents the last common ancestor of taxa W, X, Y, and Z. Characters 1 to 4 represent morphological synapomorphies at different levels in the tree. Character 5 evolved independently in taxa Y and Z, but appears very similar. Fig. 1b depicts a pruned version of this tree.

The first problem arises with the reconstruction of the ground pattern of the ancestor A. The pruning of the original tree removed the basal branches of the larger clade that includes Y and Z. Such basal taxa are essential, however, for a proper reconstruction of the ground pattern of ancestor A (Yeates, 1995). The anatomical variation present in the stripped taxa is not represented in the pruned tree, and will therefore not contribute to the reconstruction of ancestor A. The improper reconstruction of a segmented common ancestor of protostomes and deuterostomes by Holland *et al.* (1997) and De Robertis (1997) can be directly attributed to such a methodological oversight.

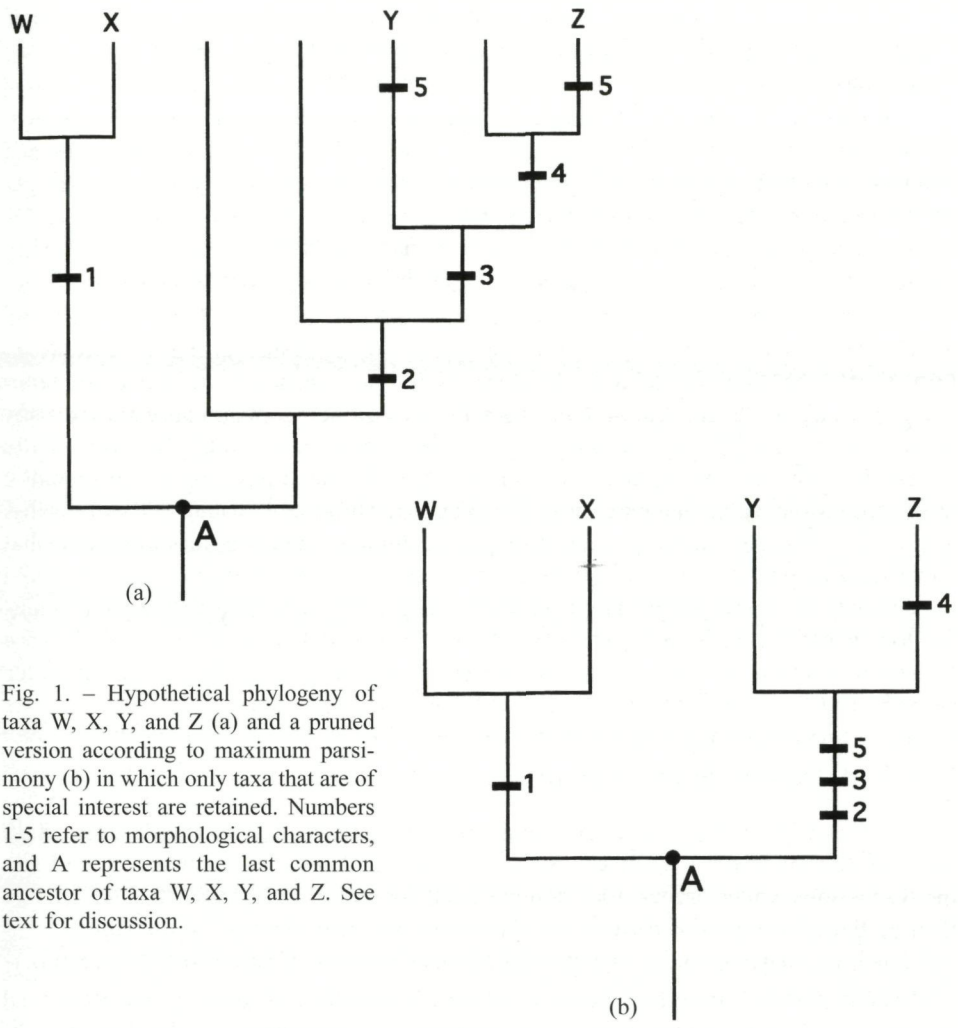

Fig. 1. – Hypothetical phylogeny of taxa W, X, Y, and Z (a) and a pruned version according to maximum parsimony (b) in which only taxa that are of special interest are retained. Numbers 1-5 refer to morphological characters, and A represents the last common ancestor of taxa W, X, Y, and Z. See text for discussion.

Secondly, the pruned tree misrepresents the topology of the original tree. The apparent symmetry of the pruned tree masks the asymmetry of the original cladogram. The exclusion of essential anatomical variation, and the misrepresentation of topology in the pruned tree do not allow the reconstruction of either the nature or the sequence of evolutionary changes on the tree. Moreover, ambiguity is introduced about the interpretation of the characters. Character 1 is a true synapomorphy of taxa W and X in both the original and pruned trees, but characters 2, 3, 4, and 5 introduce problems. Characters 2 and 3 are not synapomorphies of Y and Z as is implied by the pruned tree. Character 2 however, actually is a symplesiomorphy at the level of the last common ancestor of Y and Z, while character 3 does arise as an evolutionary novelty in this ancestor. The pruned topology also does not allow one to determine that character 2 evolved earlier than character 3.

Moreover, the pruned tree falsely suggests that character 5 was acquired by taxon Z earlier than character 4, where in fact the reverse is true. The pruned tree would also suggest that character 4 is an autapomorphy of Z, but in reality it arose in the last common ancestor of Z and its sister taxon. The pruned tree does not permit this resolution. Finally, character 5 evolved independently in taxa Y and Z. The pruned tree, however, would suggest it was a synapomorphy of these taxa.

Summarizing, it should be clear that in this case the pruned tree does not allow a proper reconstruction of ancestors. It does not allow one to distinguish between homoplasies, autapomorphies, synapomorphies or symplesiomorphies. The incorrect representation of phylogenetic information does not allow one to retrieve the true nature and sequence of evolutionary changes. All these problems contribute to the speculative nature of ground pattern reconstructions and evolutionary transformations in a significant number of studies in various fields of comparative research, notably evolutionary developmental biology (*e.g.*, DE ROBERTIS, 1997; GERHART & KIRSCHNER, 1997; HOLLAND *et al.*, 1997). A number of these important problems can also be recognized in phylogenetic analyses that deal with only a subset of the animal phyla (JENNER & SCHRAM, in press).

The roundish flatworm hypothesis

In their recent book on evolutionary developmental biology, GERHART & KIRSCHNER (1997) provided a hypothesis for the evolutionary origin of metazoan body plans. They focused on the diversification of the Nematoda, Arthropoda, Chordata, Mollusca, and Annelida from a common ancestor named the *roundish flatworm.* The roundish flatworm was first proposed as an appropriate ancestor for the protostomes and deuterostomes by VALENTINE (1994) on the basis of trace fossils, although his reconstruction differs from that of GERHART & KIRSCHNER. GERHART & KIRSCHNER reconstructed the body plan of the roundish flatworm and then proposed an evolutionary scenario deriving the body plans of the five modern phyla from this ancestor. There are, however, some fundamental flaws in their methodology that seriously undermine their hypothesis.

The first problem is the body plan reconstruction of the roundish flatworm. GERHART & KIRSCHNER (1997) derived this body plan by intuitively assembling some anatomical characters present in modern invertebrates, however no phylogenetic context was provided. Among the morphological features thought to be part of the roundish flatworm body plan were spiral cleavage, 4d-mesentoblast, blastopore becoming the mouth, pseudocoelom, and a complete gut. This assemblage of features is hardly more than speculation. A more rigorous method for reconstructing ancestral characters would have been to employ phylogenetic systematics with a maximum parsimony algorithm or maximum likelihood methods (CUNNINGHAM *et al.*, 1998; SWOFFORD & MADDISON, 1987). It then becomes clear that the phylogenetic distribution of anatomical features in Fig. 2 (fig. 7-28 in GERHART & KIRSCHNER) in fact does not support this body plan reconstruction! For example, spiral cleavage and a 4d-mesentoblast have only been convincingly demonstrated in molluscs and annelids. They are absent in chordates and nematodes and very debatable in arthropods. Blastopore fate has been overemphasized in traditional phylogenetic analyses, and the variation in blastopore fate in annelids, nematodes, arthropods, and

chordates should be carefully assessed. Furthermore, possession of a pseudocoelom in the roundish flatworm, based as it is on nematode morphology, is highly questionable considering the range of anatomical variation present in nematodes, and the inappropriateness of a pseudocoelom as a well-defined anatomical feature (*e.g.*, see RUPPERT, 1991). A complete gut is the only character likely to be present in the roundish flatworm ancestor. Note that, even if they were used at all, the out-groups are singularly unhelpful for establishing the body plan of the roundish flatworm.

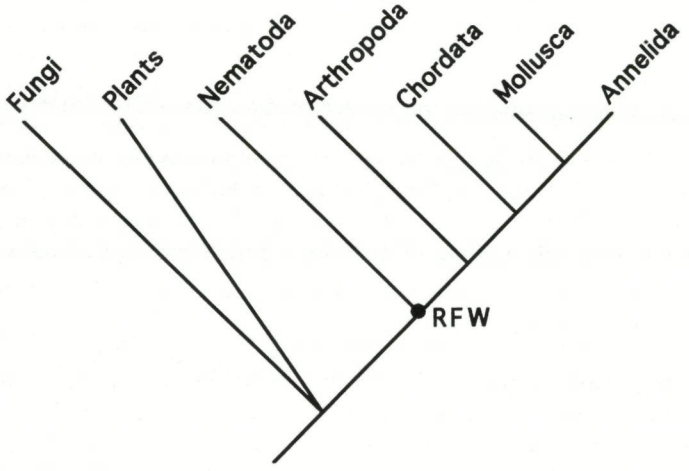

Fig. 2. – Phylogenetic relationships of Nematoda, Arthropoda, Mollusca, Annelida, and Chordata as depicted in GERHART & KIRSCHNER (1997) (see their figs 7-28). RFW represents the roundish flatworm proposed by GERHART & KIRSCHNER (1997) as the last common ancestor of these five phyla.

The second problem is the supposed phylogeny on which GERHART & KIRSCHNER (1997) based their scenario. They quote a number of phylogenetic studies primarily based on molecular data, to provide a branching sequence for the nematodes, arthropods, chordates, annelids, and molluscs. Although the data allowed for a number of different branching sequences, they exhibited one consistent feature : the chordates are derived from within the protostomes, making the protostomes paraphyletic. This particular hypothesis, however, is likely to be the result of undersampling of the chordates, and works published both before (TELFORD & HOLLAND, 1993 ; TURBEVILLE *et al.*, 1992 ; WINNEPENNINCKX *et al.*, 1996) and after (AGUINALDO *et al.*, 1997 ; GIRIBET & RIBERA, 1998) the publication of GERHART & KIRSCHNER's book indicate a monophyletic Protostomia and Deuterostomia (excluding the lophophorate phyla). Another consistent feature of GERHART & KIRSCHNER's phylogeny is the position of the nematodes, basal to the molluscs, annelids, arthropods, and chordates. This is likely to be an artifact that results from the fast rates of molecular evolution of the sampled nematodes. Substitution rates are known to be 2-3 times greater for some nematodes (such as the widely studied *Caenorhabditis elegans*) than for most other Metazoa (AGUINALDO *et al.*, 1997). This may result in long-branch attraction and forcing the nema-

todes to a basal position in the metazoan tree (*e.g.*, WINNEPENNINCKX *et al.*, 1995b). It would seem that these critical problems of GERHART & KIRSCHNER's phylogeny are sufficient to raise serious doubts about the value of their evolutionary scenario.

A third problem concerns the re-introduction of morphology into the molecular phylogeny to provide an evolutionary scenario of body plan changes. The principal difficulty arises with the introduction of morphology when the phylogeny is pruned down to only those few phyla under consideration. As discussed above, the use of such pruned trees to visualize relationships is methodologically flawed, and is likely to lead to untrustworthy results. For example, GERHART & KIRSCHNER hypothesize teloblastic segmentation to have arisen somewhere before the split of the chordates from the protostomes. The segmented mesoderm in chordates, and the segments of arthropods and annelids, may thus have been derived from a common ancestor as suggested by GERHART & KIRSCHNER. However, when one considers the diversity present in other phyla not included in their phylogeny, the picture changes drastically (*e.g.*, AGUINALDO *et al.*, 1997 ; NIELSEN *et al.*, 1996). These more comprehensive morphological and molecular phylogenetic studies indicate that the most parsimonious solution is the independent evolution of segmentation in chordates and protostomes. If segmentation is derived from a common ancestor, it must have been lost several times independently in a large number of phyla. I believe that if GERHART & KIRSCHNER would have paid more attention to the comparative anatomy of the phyla within a rigorous phylogenetic context, the improbability of deriving the chordates from deep within the protostome clade would have been apparent (*e.g.*, SCHAEFFER, 1987 ; NIELSEN, 1995).

We can thus identify some very serious shortcomings of GERHART & KIRSCHNER's roundish flatworm hypothesis. Explicit incorporation of animal phylogeny, rigorous use of phylogenetic systematics, and increased attention to comparative anatomy is needed for a more robust hypothesis to emerge that transcends the anecdotal realm (LEROI, 1998). Although G & K admit that theirs is « but a hypothesis », it is not supported by available data.

Reconstructing Urbilateria : insects, chordates, and segmentation

Recently, there have been tantalizing claims in the literature for the existence of a common segmented ancestor for the protostomes and deuterostomes (HOLLAND *et al.*, 1997 ; KIMMEL, 1996). This hypothesis is based on exciting new discoveries of the molecular developmental biology underlying segment formation in insects and chordates. Unfortunately, the authors paid insufficient attention to the comparative context of their work, which resulted in their advancing a hypothesis prematurely. The early introduction of an explicit phylogenetic framework is a necessary but missing step in these analyses. A comprehensive and detailed discussion of the nature, developmental control, and evolution of segmentation in the Metazoa is not my purpose here. In 1996, a European symposium was largely devoted to this subject (MINELLI, 1998). Instead, my goal is to point out how a phylogenetic framework is a necessary and powerful tool for understanding the true evolutionary meaning of these findings from molecular developmental biology.

MÜLLER *et al.* (1996) discovered that the zebrafish expression pattern of *her-1*, a vertebrate homolog of the insect pair-rule gene *hairy*, was strikingly similar to that of its insect homolog in developing short germ band insects. This led KIMMEL (1996) to suggest the possibility of a common segmented ancestor of protostomes and deuterostomes, named Urbilateria by DE ROBERTIS & SASAI (1996). Subsequently, HOLLAND *et al.* (1997) found a surprising resemblance in the expression pattern of the *Drosophila* segment-polarity gene *engrailed* and its chordate homolog *AmphiEn* in *Branchiostoma* (amphioxus). Both *engrailed* and *AmphiEn* are expressed in the developing segments before these become morphologically distinct. This suggests that these genes may play homologous roles in segment development in protostomes and deuterostomes.

To assess the evolutionary meaning of these findings, two steps need to be undertaken. First, we have to establish whether the gene expression patterns under investigation show detailed similarities that would allow a primary hypothesis of homology to be proposed. Second, this information should be assessed in a phylogenetic context, either by performing a character congruence study that allows the findings to be evaluated against all other informative characters, or by mapping the character onto a phylogeny in order to assess the initial homology determination. Unfortunately, it is all too common in recent studies in evolutionary developmental biology to completely neglect the second step in the procedure.

In this example, the expression patterns of the insect pair-rule gene *hairy* and its vertebrate homolog *her-1* do indeed show remarkable resemblances in both the pattern and dynamics of gene expression (MÜLLER *et al.*, 1996 ; KIMMEL, 1996). Likewise, the expression patterns of the insect segment-polarity gene *engrailed* and its homolog *AmphiEn* in amphioxus appear similar (HOLLAND *et al.*, 1997 ; DE ROBERTIS, 1997). These similarities can serve as a basis to suggest the homology of the expression patterns and, by extrapolation, the resultant phenotypes. However, this is precisely the point where the studies terminate the analytical process. DE ROBERTIS (1997, p.25) simply stated that « The fact that *engrailed* is expressed in both *Drosophila* and chordate metameres tells us that segmentation was present in the common ancestor from which the insect and chordate lineages diverged 500 million years ago, the Urbilateria ». HOLLAND *et al.* (1997, p.1731) go so far as to suggest that this information « favors phylogenetic scenarios deriving vertebrates from annelid-like or arthropod-like body plans ». However, these are extremely premature conclusions not supported by available information.

Consideration of the phylogenetic relationships of insects and chordates in the context of all associated phyla would provide a powerful opportunity to test the proposed homology, and to uncover any alternative perspectives. When the phylogenetic relationships of insects and chordates are considered on the basis of presently available molecular or morphological information from all phyla, it becomes clear that segmentation has evolved independently in these lineages. Indeed, this alternative is currently by far the most parsimonious interpretation (AGUINALDO *et al.*, 1997 ; NIELSEN *et al.*, 1996). If insects and chordates really did diverge from a common segmented ancestor, then the multiple independent losses of segmentation in other protostomes and deuterostomes have to be explained. HOLLAND *et al.* (1997) only briefly mention the possible loss of segmentation in echinoderms, hemichordates, and urochordates, but they never even take up the pro-

blem of explaining the supposed loss of segmentation in various protostome phyla (possibly including various acoelomate and pseudocoelomate groups). With the possible exception of the molluscs, there is precious little evidence for this scenario of multiple losses of body segmentation. Explicit attention to a total invertebrate phylogeny might suggest a different interpretation of the data, one connected to the hierarchical nature of homology (*e.g.*, ABOUHEIF, 1997 ; BOLKER & RAFF, 1996).

It can be dangerous to use shared patterns of regulatory gene expression to determine morphological homology. There is mounting evidence that the genotype-phenotype map may be very fluid. This means that developmental regulatory genes can function in very different contexts within a single organism and between different organisms (*e.g.*, LOWE & WRAY, 1997 ; PANGANIBAN *et al.*, 1997 ; RAFF, 1996 ; WU & ANDERSON, 1997). These genes are not rigidly restricted to the development of a certain morphological character. Therefore, the determination of morphological homologies as indicated by regulatory gene expression patterns may in fact not be straightforward (*e.g.*, ABOUHEIF, 1997 ; DICKINSON, 1995 ; MÜLLER & WAGNER, 1996). This is clearly indicated by current debates on the supposed homology of insect and vertebrate eyes as revealed by expression of insect *eyeless* and vertebrate *Pax-6* genes, and the evolution of animal appendages as revealed by expression of *Distal-less* in body wall outgrowths of various animal phyla (PANGANIBAN *et al.*, 1997). In such cases, the use of phylogenetic information will prove to be especially valuable.

The actual distribution of segmentation among all the phyla, and the lack of detailed anatomical correspondence between insect and chordate segmentation do not support real homology. The information does indicate, however, that the last common ancestor of insects and chordates possessed homologs of the pair-rule gene *hairy* and the segment-polarity gene *engrailed*. This indicates the possibility of a deeper homology. For example, we might suspect that *engrailed* may have originally functioned in regional patterning in general, and later became independently co-opted into the formation of insect and chordate metameres. However, HOLLAND & HOLLAND (1998) do not explore this alternative perspective. They simply state (p.656) that « it is important to stress that we are comparing body parts and not deeper homologies ». However, consideration of a real phylogeny would immediately suggest the value of this different, and useful hypothesis. Various authors have pointed out the value of explicitly incorporating phylogenetic information into the study of the evolution of developmental processes in more or less closely related species (*e.g.*, ABOUHEIF, 1997 ; MEYER, 1996 ; RAFF & POPODI, 1996). Furthermore, a phylogenetic framework is also absolutely necessary when one wants to compare distantly related organisms, such as the widely used model systems of molecular and developmental biology.

Evolving bilateral symmetry : insights from the scleractinians?

EZAKI (1998) argued that scleractinian corals may have evolved as early as the Paleozoic and could thus constitute an early anthozoan radiation. EZAKI argued that understanding the evolution of the scleractinian body plan may help to understand the evolutionary origin of the Bilateria, and in particular the evolution of a bilaterally symmetrical

body plan. EZAKI'S argument is as follows. Anthozoa (including scleractinians) share a bilaterally symmetrical body plan that is traditionally considered as derived from other radially symmetrical cnidarians. Traditionally, the Bilateria are thought to have been derived from the Radiata, and the bilaterally symmetrical Anthozoa are the most likely candidates. The early origin of the scleractinian body plan may thus help to elucidate the anthozoan radiation within the Bilateria and so shed light on the early evolution of body plans within the Bilateria. He presented a phylogeny of the Cnidaria (Fig. 3a; Fig. 5 in EZAKI, 1998) to support his arguments. Unfortunately, insufficient attention to the phylogenetic basis of his arguments resulted in a conclusion that is virtually devoid of evolutionary significance.

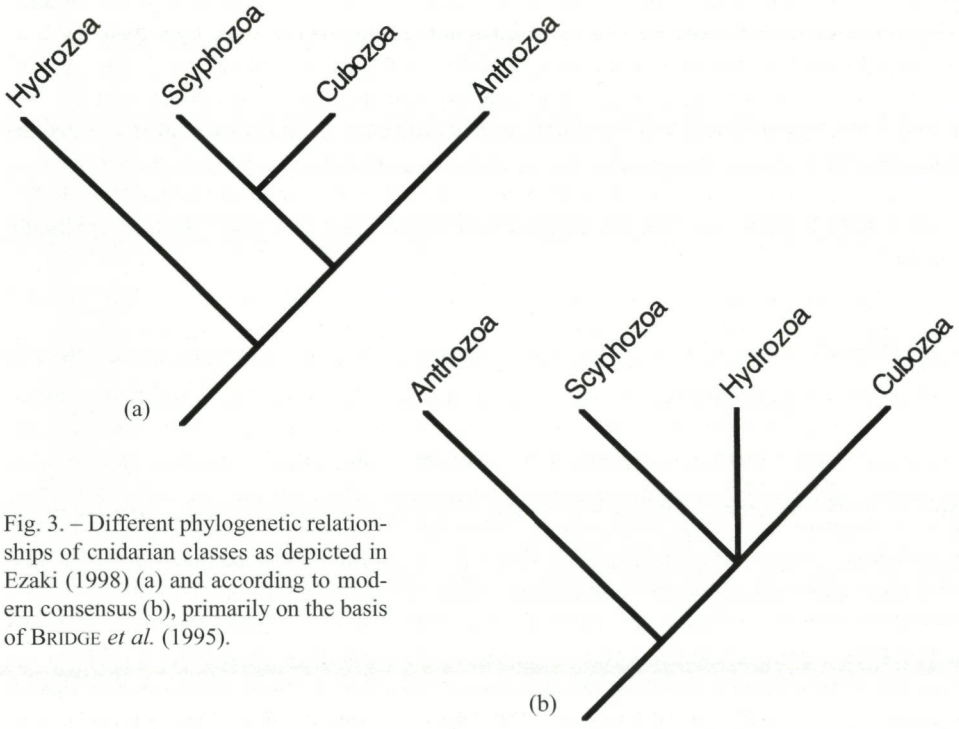

Fig. 3. – Different phylogenetic relationships of cnidarian classes as depicted in Ezaki (1998) (a) and according to modern consensus (b), primarily on the basis of BRIDGE *et al.* (1995).

EZAKI presented a « traditional » phylogeny of the Cnidaria in which the Hydrozoa is a sister group to the other cnidarians. Anthozoa is the sister group to the Scyphozoa + Cubozoa. This phylogeny is hardly a reflection of our current understanding of cnidarian phylogeny. In fact, EZAKI did not mention any source for this phylogeny, nor did he specify what kinds of data it is based on (molecular or morphological), nor what the supportive characters for this phylogeny are. The close relationship between Scyphozoa and Anthozoa has traditionally been based upon the shared possession of a cellular mesoglea, gastrodermal gonads, and gastrodermal nematocytes (*e.g.*, BARNES & HARRISON, 1991; MEGLITSCH & SCHRAM, 1991; RUPPERT & BARNES, 1994). It was not until recently, how-

ever, that this hypothesis was tested by a comprehensive character congruence study. BRIDGE *et al.* (1995) showed that these characters do not effectively support a close relationship between Anthozoa and Scyphozoa (and Cubozoa) (Fig. 3b). This clearly shows the danger of proposing sister group relationships on the basis of single characters outside the context of all pertinent information. The current consensus on cnidarian relationships (based on morphology and 18S rDNA data) now indicates a basal position of the Anthozoa and the existence of a clade of cnidarians with medusae (Scyphozoa, Cubozoa, Hydrozoa) (AX, 1995; BRIDGE *et al.*, 1995; NIELSEN, 1995; SCHUCHERT, 1993). Furthermore, there is no firm evidence for a phylogenetic link between Anthozoa and Bilateria. In this scheme, it is likely that the supposedly bilateral symmetry of anthozoans and Bilateria is convergent, and the evolution of the scleractinian body plan within the Anthozoa is unrelated to the origin of the Bilateria. Consequently, the anthozoan condition may be more accurately described as biradial. EZAKI's study clearly indicates the danger of relying on outdated, and weakly supported textbook trees.

Evolution of muscle and body cavities: reconstructing the bilaterian ground pattern

Based on ultrastructural studies of muscle systems and body cavities in various invertebrate groups, RIEGER and BARTOLOMAEUS advanced opposing hypotheses of the nature of the bilaterian ancestor (BARTOLOMAEUS, 1994; RIEGER, 1986; 1988; RIEGER & LOMBARDI, 1987). RIEGER suggested a myoepithelial organization of the coelomic lining as the ground pattern of the Bilateria. The acoelomates and pseudocoelomates would have been derived from this bilaterian stem species by repeated events of progenesis. In contrast, BARTOLOMAEUS (1994) argued for a compact bilaterian ancestor without internal body cavities. The pseudocoelomate and coelomate organizations would have been derived from this ancestor. The detailed arguments these authors use are not important here. What is important is that both authors make only minimal use of both phylogenetic methods as well as currently available information about invertebrate phylogeny.

RIEGER only inserts various intuitive phylogenetic arguments into his work. His conclusions are based chiefly upon extrapolation from echinoderms and annelids to the whole of the coelomate Bilateria. This implies that the coelomate Bilateria (protostomes and deuterostomes) are monophyletic. In this regard, however, it is crucial to understand the phylogenetic position of the pseudocoelomate and acoelomate phyla. While recent phylogenetic analyses of the animal phyla do not agree in detail, both molecular (AGUINALDO *et al.*, 1997; WINNEPENNINCKX, 1995a, b), and morphological analyses (EERNISSE *et al.*, 1992; NIELSEN *et al.*, 1996) suggest that the pseudocoelomates and acoelomates may be distributed among coelomate bilaterians. Irrespective of whether or not they form coherent clades, this possibility indicates that a coelomate bilaterian common ancestor of all coelomate phyla might not even have existed. Even if the coelomate bilaterians form a coherent clade with the acoelomates and pseudocoelomates outside it, RIEGER's extrapolations at the very best are only able to reconstruct the ground pattern of the coelomate Bilateria. Bilateria and coelomate Bilateria are, however, used interchangeably in RIEGER & LOMBARDI (1987). Only explicit consideration of phylogenetic relationships of all the invertebrate phyla will resolve this ambiguity.

BARTOLOMAEUS' (1994) scenario suffers from similar problems. He reconstructed the bilaterian ground pattern by reference to Ctenophora and acoelomate Bilateria (*e.g.*, Platyhelminthes, Nemertinea, Entoprocta, Gastrotricha). This only makes sense, however, if these phyla are in fact primitive branches within the Bilateria. The basal branches of a clade are the ones most likely to provide relevant information concerning the ground pattern of the clade (YEATES, 1995). Here again, a comprehensive phylogenetic framework is needed for an accurate re-evaluation of this hypothesis.

Although RIEGER's and BARTOLOMAEUS' hypotheses about the bilaterian stem-forms are presented as alternative reconstructions, a more rigorous use of phylogenetic methods would have alerted them to the pointlessness of the debate. In fact, RIEGER and BARTOLOMAEUS reconstructed different ancestors. Fig. 4 illustrates this. By extrapolating from studies on echinoderms and annelids to the whole coelomate Bilateria, RIEGER reconstructed an ancestral ground pattern at the in-group node (node I) of the phylogeny. In contrast, by focusing on the presumptive sister group of the Bilateria (Ctenophora), Bartolomaeus reconstructed the ground pattern of the out-group node (node O) of the phylogeny. This means that in principle both hypotheses could be vindicated by the data, because character transformations may occur on the internode connecting the in-group and out-group nodes. The relative merits of the contrasting hypotheses proposed by RIEGER and by BARTOLOMAEUS need to be re-assessed, but that can only be done with reference to cladistically-framed hypotheses about the phylogenetic relationships of all the invertebrate phyla, and by employing parsimony algorithms for ground pattern reconstructions.

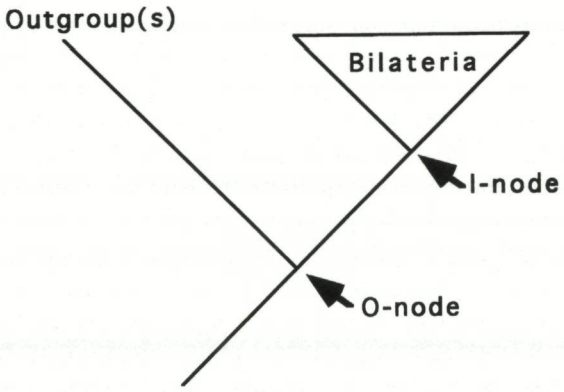

Fig. 4. – Locations of the in-group node (I-node) and out-group node (O-node) with regard to the different hypotheses for the bilaterian ancestor proposed by BARTOLOMAEUS (1994) and RIEGER & LOMBARDI (1987). See text for discussion.

CONCLUSION AND RECOMMENDATION

This is a time in which many new research programs are established in a variety of biological disciplines. These developments are associated with the transformation of com-

parative biology by phylogenetic systematics. Although the comparative method has existed at least since CUVIER, and with a particular focus on phylogenetic relationships since HAECKEL, the elaboration of explicit phylogenetic methods greatly facilitated the rigorous use of phylogenetic information in comparative studies. The modern meaning of a comparative, historical, or evolutionary perspective therefore refers to the phylogenetic relationships of organisms. This phylogenetic perspective was a major force for the establishment of, for example, historical ecology and ethology, various parts of evolutionary paleobiology, and evolutionary developmental biology (*e.g.*, ARTHUR, 1997; BROOKS & MCLENNAN, 1991; HARVEY & PAGEL, 1991; JABLONSKI *et al.*, 1996).

Evolutionary biology principally derives its strength and merit from extrapolation from case studies to more comprehensive contexts (GRANDCOLAS *et al.*, 1997). Phylogenetic methods and information provide a robust and testable means for such extrapolation. It should be clear, however, that posing a question in an evolutionary context is not the same as incorporating evolutionary information into the answer. This pinpoints the problem with a variety of modern comparative studies, especially in the field of evolutionary developmental biology. Frequently, the central importance of primary homology assessments is over-emphasized, while the assimilation of phylogenetic information is ignored (*e.g.*, GILBERT *et al.*, 1996; HOLLAND & HOLLAND, 1998; HOLLAND *et al.*, 1996). In addition, studies of this kind often put disproportionate emphasis on only one or very few characters. Such a monothetic approach fundamentally violates the principles of phylogenetic systematics, which I believe is the only rigorous method currently available for reconstructing phylogeny. Taking up only one or a few characters is often misleading and very unlikely to increase our understanding of evolution. In other cases, phylogenetic data are only used in an intuitive fashion, with little attention to selection of a particular phylogeny (*e.g.*, EZAKI, 1998) or proper methods of phylogenetic inference (*e.g.*, BARTOLOMAEUS, 1994; GERHART & KIRSCHNER, 1997; RIEGER & LOMBARDI, 1987). We are on the right track, however. A more intense dialogue between phylogeneticists and other biologists is necessary for a proper understanding of macroevolutionary change, and for the development of a more robust and unified evolutionary theory (GILBERT *et al.*, 1996; GRANDCOLAS *et al.*, 1997; LARSEN *et al.*, 1997).

The literature on all aspects of animal evolution is expanding at an ever increasing rate. Indeed, a high rate of research may lead to rapid turnover of phylogenies. Continual re-evaluation and additions to the character sets, however, should result in increasingly robust phylogenetic hypotheses. Nevertheless, the dynamics of the field cannot be used as an argument for ignoring phylogenetic information in comparative studies. Clearly, there are phylogenies (both molecular and morphological) available, and despite differences in their topology there is much to be gained from incorporating them into comparative studies.

If we do not want to be swamped by the growing forest of trees and evolutionary scenarios we need to be conscious of our methods of analysis (JENNER & SCHRAM, in press). Our own future efforts will concern a comprehensive cladistic analysis of comparative anatomy, embryology, and developmental genetics to shed light on the higher-level phylogeny of invertebrates.

ACKNOWLEDGMENTS

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ELECTRORECEPTION IN CATFISH: PATTERNS FROM MOTION

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Abstract. An electrosensitive catfish, *Ictalurus melas*, was trained in a two-alternatives forced-choice conditioning paradigm to discriminate between the electric fields of two direct-current (dc) dipoles, spaced 12 cm apart, the dipole axes parallel to the swimming path of the subject. The dipole size could be varied between 1 and 10 cm. The dipole current was about 5 μA. When two dipoles of different sizes were presented simultaneously, the subject's electrodiscrimination performance exceeded the 85% correct choices level provided the dipole of 1 cm was tested against a dipole with a span of 1.5 cm or more. The average stimulus strength at 1 cm distance from the dipole axis ranged from 1 to 10 mV/cm. The swimming speed of the subject was 7 ± 3 cm/s. The potential swing over the skin caused by the subject passing the dipole, matched the frequency band of the ampullary electroreceptor organs. Apparently motion of the fish with respect to a stationary direct-current stimulus source, or vice versa, generates a biologically adequate form of reafferent stimulation. Without relative motion an electrical dc-source would remain unnoticed.

Key words : Conditioning, direct-current, electric flow, dipole discrimination, two-alternative forced-choice (2AFC), electrolocation, electro-orientation, sensory-motor integration, exafferent, reafferent.

INTRODUCTION

Among the electroreceptive fish there are many species that do not have electric organs. Such fish are said to have « passive » electroreception. Cartilaginous fish, sharks, rays, and bony fish such as species of Siluriformes, belong to this category (MOLLER, 1995). Electrophysiological studies in such « passive electroreceptive » fish have revealed the receptive characteristics of the various electrosensory organs (DIJKGRAAF & KALMIJ , 1966; MOLLER, 1995; MURRAY, 1962; PETERS & BUWALDA, 1972; ROTH, 1968, 1972; ROTH & SCHLEGEL, 1988) and some principles of central processing (ANDRIANOV *et al.*, 1974; KNUDSEN, 1976a,b; MCCREERY, 1977; MONTGOMERY, 1984; WEILLE DE, 1983). Ecophysical studies have shown what kind of electrical stimuli are present in the habitat (BUTSUK & BESSONOV, 1981; KALMIJN, 1972; PETERS & BRETSCHNEIDER, 1972; ROTH, 1972). In addition, behavioural experiments have revealed that « passive electroreceptive » fish are capable of prey detection (GUSEV *et al.*, 1986; KALMIJN, 1971; PETERS & MEEK,

1973 ; ROTH, 1972), prey localization, and spatial orientation with respect to electric fields that occur everywhere in nature (DIJKGRAAF, 1968 ; KALMIJN *et al.*, 1976a,b ; PETERS & VAN WIJLAND, 1974, 1993 ; ROTH, 1972 ; SISNEROS *et al.*, 1998 ; WILKENS *et al.*, 1997). In spite of this rather complete general impression of the role of passive electroreception in everyday fish life, questions concerning feature detection of external fields remain largely unexplored. Particularly the paradoxical mismatch between the tuning curve of the electroreceptor organs (band pass filter) and the predominant direct-current (dc) nature of the natural stimuli remains enigmatic (PETERS *et al.*, 1988, 1995). Has the fish to move with respect to a dc source – or vice versa – in order to detect it?

In addition to our earlier frequency discrimination studies (PETERS & BARETTA, 1998), where two exafferent electric alternating current sources were used as exafferent stimulus sources, we present here the first results of a frequency discrimination study where motion with respect to a stationary dc source generates a reafferent electrical stimulus that does match the bandwidth of the electroreceptor organs. The results presented hereafter tell us that passive electroreceptive fish do indeed recognize patterns from motion. A fish that passes an electric dc-current source generates in this way its own reafferent electric stimulus.

MATERIAL AND METHODS

The experiments were performed on a single male specimen of *Ictalurus melas* of 22 cm total length and a weight of about 160 g. The subject was kept in a rectangular all-glass tank of 110 by 30 cm with a water depth of 10 cm, at 18 °C. The water resistivity was kept between 300 and 340 µS/cm. Between tests the subject was allowed to swim freely either in full daylight or in the dark. During the tests, which were performed at night, there was complete darkness, except for light emitted by indicator LEDs of the equipment.

Each test began with an intertrial interval period (ITI) of 30 s, marked by switching on a light above the tank. This induced the subject to hide under an opaque shelter. At the end of the ITI the light was switched off, upon which the subject started to swim in the direction of the other end of the tank, where two parallel electrical dipoles were presented simultaneously. The dipoles were of different sizes. One dipole had the electrodes spaced at a fixed distance of 1 cm. The electrode spacing of the other dipole could be varied between 1 and 10 cm. The dipoles were 12 cm apart, and parallel to the swimming direction of the subject. The subject was trained to receive food upon turning in the direction of the smaller of the two dipoles (Fig. 1). The stimulus current was about 5 µA, which produced voltage gradients of 1 to 10 mV/cm at about 1 cm from the dipole axis. Upon choosing correctly, the subject received some food delivered via a peristaltic dispenser, and an additional 60 s of darkness. Upon the subject choosing falsely, the lights were switched on immediately, which urged the subject to return to its shelter again, waiting for the next trial. In this way 4 sessions of 50 trials per night were done. The experiment began with testing the discrimination between a 1cm and a 10 cm dipole. These sessions were repeated 20 times. Then, following the same schedule, 7, 4, 3, 2, 1.5, and 1.2 cm dipoles were tested against the 1 cm reference dipole. After completion of the series, the discrimination between a 1 and 3 cm dipole was tested again, while the amplitudes of the dipole fields were randomized, and after a silk screen had been placed that kept the subject at

1 cm or more from the electrodes. A more detailed description of the general setup is given in PETERS *et al.* 1995.

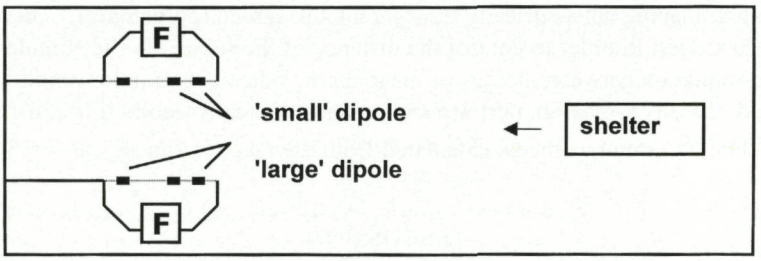

Fig. 1. – Overview of the tank with stimulus electrodes and feeding compartment. During the intertrial interval the fish hides at right under an opaque shelter. At « lights out », it starts to swim into the feeding compartment (direction of arrow), passing the two direct current sources of unequal size. Turning towards the dipoles at right or at left causes the lights to be switched on again, or food to be delivered, depending on the position of the positive discriminant, i.e. the 1cm dipole. The dotted line represents an infrared beam that monitors the subject's position. The silk screen, not shown here, was put horizontally one cm above the dipole electrodes, between the two feeding compartments. F : food dispenser in compartment with electrodes. Black blocks on dotted line are electrodes delivering the stimulus.

RESULTS

The outcome of this experiment was that the subject could recognize the different stimulus situations. The subject mastered discrimination between a 1 cm and a 10 cm

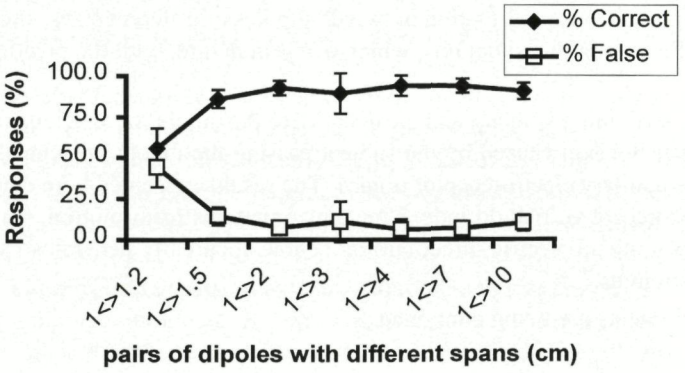

Fig. 2. – Direct-current dipole discrimination performance of *Ictalurus melas*. The « rewarded dipole » had always a span of 1 cm. The non-rewarded dipole size was varied between the experiments from 10 cm to 1.2 cm. Error bars are standard deviations of the different sessions (n=20). Each point in the graph represents the average score of 20 sessions of 50 trials each.

dipole within 30 sessions, without specific shaping. Dipoles of smaller sizes could be discriminated equally well from the reference dipole. At dipoles of 3 cm the number of mistakes began to increase (Fig. 2). Dipoles of 1 cm length and 1.2 cm could not be discriminated. Placing an electrically transparent silk screen (polyamide) 1 cm above the stimulus electrodes, in order to control the distance of the subject to the stimulus, did not affect discrimination between dipoles of 1 and 3 cm. When the stimulus amplitudes were randomized, the discrimination performance remained equally good.

The average swimming speed, calculated from the reaction times, was 7 ± 3 cm/s.

DISCUSSION

The relative ease with which the subject could be conditioned to the dipole-size discrimination task betrays its familiarity with such a kind of stimulus presentation. Indeed, electrical stimulus situations as presented above occur in nature abundantly. Also earlier laboratory observations revealed that *I. melas* turns toward an electrically simulated dc-prey when it has already passed it by several cm, and that it is very well able to follow a trail of small dc-dipoles.

The two simultaneously presented electrical fields are detected as a single field, being the sum of both dipole fields. This field is sampled by the ampullary electroreceptor organs dispersed over the skin. Apparently the catfish is able to transpose the stimulation patterns to « food at right » or « food at left ». The present experiment does not reveal which specific feature of the electric field is used for making the decision to turn right or left, but even a very simple neural mechanism, like a bisensor network (*cf.* HOPKINS *et al.*, 1997 ; SCHÖNE, 1984), could achieve this. Any imbalance between the two halves of such a neural network could steer the subject in either of the two directions. It is not clear from this first experiment how we should describe the internal representation of the electric field. What is clear is that the subject is capable of responding in a biologically adequate way to the simultaneous presentation of two dc-dipoles of different sizes, and that it is able to associate these stimulus situations, which occur in nature, with the position of a potential prey.

From the swimming velocity and the distance to the dipole we conclude that the potential swing over the skin caused by the subject passing the dipole, matches the frequency band of the ampullary electroreceptor organs. The results presented here demonstrate that passive electroreceptive fish do indeed recognize patterns from motion. An electrosensitive catfish passing an electric direct-current source apparently generates its own reafferent electric stimulus.

The experiments are being continued.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help of Gerard Stooker, Ed H.P. van der Veen and Rob J.Loots, and Rob van Weerden during the various phases of design and construction of the setup.

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BIOAVAILABILITY OF CADMIUM AND ZINC TO MIDGE LARVAE UNDER NATURAL AND EXPERIMENTAL CONDITIONS: EFFECTS OF SOME ENVIRONMENTAL FACTORS

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Abstract. In this paper the effects of environmental factors on cadmium and zinc uptake by larvae of chironomids are discussed. The results of several laboratory experiments and field studies were pooled and analysed using uptake and accumulation models. In the field studies, the relationship between metal concentrations in larvae and sediment was studied on samples from several watercourses. The effect of different sediment characteristics on these relationships was investigated. In the laboratory experiments, larvae of *Chironomus riparius* were exposed to metals via the water, and the effects evaluated of three changing environmental factors, *i.e.* salinity, temperature, and pH. Non-linear regression models were constructed to determine the relative importance of the different environmental factors contributing to the variation in metal uptake or accumulation. For the field data, the amount of variation that could be explained by these models was limited. Only for zinc was a significant amount of variation (up to 66%) explained relating accumulated zinc to easily extractable zinc and considering total organic carbon (TOC) in the model. For the laboratory data, relating uptake levels to the metal ion activities explained no more than 6% and 24% of the total variation in respectively cadmium and zinc uptake. The integration of the different effects of the environmental factors in the models explained 67% of the total variation in cadmium uptake and 56% of the total variation in zinc uptake. Factors contributing most significantly to the explained variation were temperature, pH, and salinity of exposure, calcium ion activity and salinity of acclimation. The high, unexplained variation under field conditions is probably due to the large variation in exposure conditions in natural environments and a lack of knowledge concerning the relative importance of the different exposure routes under these circumstances.

Key words: metal uptake, environmental factors, Chironomidae, modelling.

INTRODUCTION

When trace metals are added to natural waters they are distributed over different compartments of the ecosystem *i.e.* the water column, the suspended matter, the sediment and the interstitial water (TESSIER & CAMPBELL, 1987; LUOMA, 1989; AMYOT *et al.*, 1994). Within each phase, metals will be partitioned among specific ligands, the so-called chemical speciation. In the sediment and the suspended solids, metals can interact with iron- or manganese oxides, adsorb to clay particles, bind to organic ligands, etc. In the water column and in the

interstitial water, the trace metals will be present as hydrated ions, associated with dissolved inorganic or organic ligands, or adsorbed to inorganic and organic colloids. The distribution of a metal among the different components depends not only on the concentration but also on the nature of the compartments. Changes in the physical or chemical conditions such as pH, salinity, and alkalinity, influence the distribution between the phases (BORGMANN, 1983; SALOMONS & FÖRSTNER, 1984; LUOMA, 1989). The bioavailability of trace metals will largely depend on the chemical speciation. Thus, the total trace metal concentration in sediment or water should not be used as the only measure of metal contamination. Several studies have verified that metal availability from solutions is a function of the free metal ion activity (ALLEN *et al.*, 1980; DE LISLE & ROBERTS, 1988; BLUST *et al.*, 1992). Besides chemical processes, biological processes will influence the uptake of trace metals by organisms. Two major uptake routes have been identified, *i.e.* direct absorption of dissolved metal species from solutions, and via ingestion of particulate matter (including food) (LUOMA, 1989; HARE, 1992; RAINBOW & DALLINGER, 1993). Although mostly present at higher concentrations in food than in solution, trace metals associated with particles tend to be less available than are those in solution. Uptake mechanisms may be controlled physiologically by the organism. Control that could be exerted by the invertebrate with respect to these uptake mechanisms would be via some alteration to the number, or affinity of the membrane transport ligands (SIMKISS & TAYLOR, 1995).

In this paper the data of several studies that assessed the influence of environmental factors on the net uptake (in the laboratory) and accumulated levels (in the field) of trace metals by chironomid larvae, are pooled and analysed. In the field studies, the effect was assessed of the geochemical characteristics of sediments on the levels of trace metals accumulated by larvae of the midge *Chironomus riparius* (Meigen, 1804) (Diptera, Chironomidae). To relate metal levels in organisms with levels in sediments, trace metal partitioning among various sediment phases was determined and geochemical sediment characteristics were analysed (BERVOETS *et al.*, 1994, 1997, and 1998). In this study we analysed the pooled data of the three field studies for zinc and cadmium using accumulation models. In the laboratory experiments, the effects of some environmental factors on the net uptake of two trace metals, cadmium and zinc, by larvae of *Chironomus riparius* were studied (BERVOETS *et al.*, 1995, 1996a, 1996b; BERVOETS & BLUST, unpublished). Among the factors influencing uptake and accumulation of metals by aquatic organisms, salinity, temperature, pH, hardness, and the presence of organic ligands are the most important (PHILIPS, 1976; SIMKISS & TAYLOR, 1995). The separate and combined effects of the different components of these environmental factors were assessed by analysing the pooled data. The effects of changing environmental factors on the net metal uptake by fourth instar larvae were studied, in relation to the effects of the factors on the chemical speciation and on the physiological condition of the organism.

MATERIALS AND METHODS

Test organism

Midge larvae (Diptera, Chironomidae) are the central organisms in this study. Chironomid larvae were chosen because of their important position in freshwater ecosys-

tems. Larval members of this family are found in almost every kind of fresh- and brackish water. Under certain conditions such as low levels of dissolved oxygen, larval chironomids may be the only insects present in benthic sediments (PINDER, 1986; WARWICK, 1990; CRANSTON, 1995). All larvae collected from natural waters for metal analysis in the field studies were identified as *Chironomus* gr. *thummi*. This species group contains at least 13 different species, which are morphologically very similar (WEBB & SCHOLL, 1985). Larvae used in the laboratory experiments were obtained from a controlled laboratory culture at the Royal Belgian Institute for Natural Sciences (KBIN, Brussels, Belgium). Larvae were cultured in 10 L plastic aquaria according to the methods of VERMEULEN *et al.* (1997). When the fourth larval stage (instar 4) was reached, the larvae were placed at 15°C in the dark and held in aquaria at high densities (1 larva per cm^2) to retard pupation while maintaining them in normal physiological state (INEICHEN *et al.*, 1979; BANGENTER & FISCHER, 1989).

Field studies

In the field studies midge larvae were collected at different sampling sites. Concentrations of Cu, Zn, Cd, Pb, Cr and Ni were measured in organisms and sediments. To identify trace metal partitioning among various geochemical phases, sediments were subjected to simultaneous extraction schemes. Four metal fractions were determined: (1) easily reducible (ER) metal (trace metals associated with Mn oxides); (2) reducible (R) metal (trace metals associated with Fe oxides); (3) metals bound to organic matter (ORG); (4) total metal (TOT), metals extracted using a mixture of HNO_3 and $HOCl_4$. In addition, several geochemical characteristics of the sediments were analysed; total organic carbon (TOC), Fe-oxides, Mn-oxides, and particle size distribution. For a detailed description of sampling, sediment handling, and analytical procedures, we refer to BERVOETS *et al.* (1994, 1997, and 1998). In this paper the analysis of the pooled data of cadmium and zinc is presented.

Laboratory experiments

In the laboratory experiments the effects of three environmental factors, *i.e.* salinity (0.24 to 10 ppt), temperature (5 to 25°C) and pH (5.5 to 10.0) on the net uptake of cadmium and zinc by larvae of the midge *Chironomus riparius* were studied using artificial river water. The composition of 1 L of this chemically-defined freshwater was 0.096 g $NaHCO_3$, 0.004 g KCl, 0.123 g $MgSO_4.7H_2O$ and 0.06 g $CaSO_4.2H_2O$, resulting in a salinity of 0.2 ppt and a pH of 7.8. The medium was prepared by dissolving the analytical grade products (Merck p.a.) in deionized water. Ionic stocks of cadmium and zinc, containing 100 µM Cd and 1,000 µM Zn respectively, were prepared. The radioisotopes ^{109}Cd and ^{65}Zn (Amersham International, UK) were used as tracers, 460 KBq/L of each tracer being added to the metal stock solutions. In all experimental exposure solutions, the resulting metal concentrations were 0.1 µM Cd and 1.3 µM Zn. These concentrations were chosen for their environmental relevance. The resulting radioactivity of both tracers was 0.46 KBq/L. For all experiments, 50 midge larvae of comparable size were placed in a series of plastic vessels containing 50 ml solution. For each experimental condition we used 6 to 8 repli-

cates. These vessels were placed in a thermostatic water bath at the required temperature. Net uptakes of both cadmium and zinc were linear over time for at least 8 hours during exposure to a total concentration of 0.1 µM cadmium and 1.3µM zinc. Therefore accumulation was measured after 6 h of exposure. After exposure, the 50 individuals were collected on a 250 µm sieve and rinsed with 50 ml of deionized water. The influence of prior acclimation at the different environmental conditions on metal uptake was assessed. In addition the effect of the environmental factor on the chemical behaviour was added to uptake models. For a detailed description of the experimental set-up we refer to BERVOETS *et al.* (1995, 1996a,b).

The equilibrium concentrations of the chemical species considered were calculated using the computer program SOLUTION (BLUST *et al.*, unpublished), an adaptation of the program COMPLEX (GINZBURG, 1976). This speciation model allows the calculation of the composition of solutions in equilibrium with the atmosphere. Activity coefficients were calculated using the estimation method of Helgeson (BIRKETT *et al.*,1988). Results of the chemical speciation calculations were expressed on the molar concentration scale and multiplied by the appropriate activity coefficients to obtain species activities.

Modelling net uptake or concentration

To determine the relative importance of the different factors contributing to the variation in the net uptake of metals (in the laboratory experiments) or concentration (in the field studies), non-linear regression models were constructed (BLUST *et al.*, 1994, 1995; BERVOETS *et al.*, 1997). For the pooled data of the laboratory experiments, the net uptake was related to the terms that describe the change in the free metal ion activity, and different components of the environmental factors (*i.e.* salinity, temperature, and pH of exposure and acclimation) and the free calcium ion activity. In the field studies the metal concentration in the organisms was related to a term describing the variation in the metal concentration in the different sediment fractions (*i.e.* total, reducible, easily reducible metal concentrations or metals bound to organic matter) and to the different sediment characteristics (iron oxides, manganese oxides, TOC and particle size distribution). The relative importance of the different terms was determined by a forward selection procedure. This was done by starting with a single factor, the metal activity or concentration, and stepwise adding the other terms and evaluating their contribution to the total variation. A coefficient of proportionality (C_t) was introduced to relate the concentration in the environment to the metal concentration in the organisms.

RESULTS

Field studies

Total metal levels were always higher than reducible metal levels but important differences were observed between essential and non-essential metals. In this paper we discuss only the results for cadmium and zinc. For cadmium, total levels were only slightly higher than reducible levels. For zinc, however, the differences were much more pro-

nounced (up to 5 times more total zinc compared to reducible zinc). This is illustrated in Fig. 1 for three different rivers.

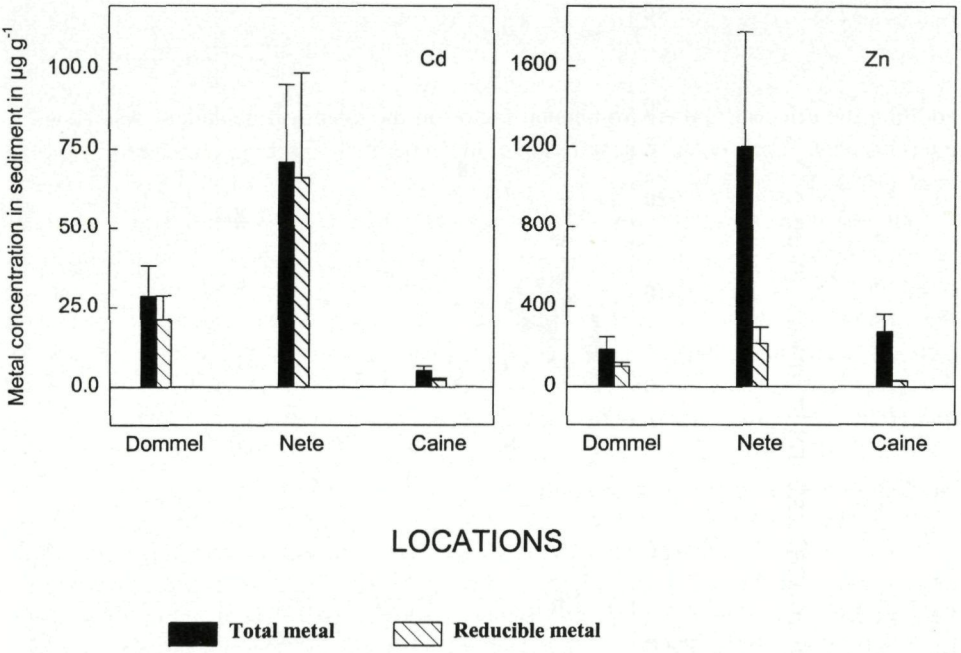

Fig. 1. – Results of metal partitioning in sediments from three rivers. Mean values with standard deviation are presented in µg g^{-1} dry weight.

The relationships between total metal concentration in sediments and midge larvae for the pooled data are shown in Fig. 2. For both metals only poor relationships were found. To determine the relative importance of the different sediment factors we used non-linear regression models. The metal concentration in the organisms was related to a term describing the variation in the metal concentration in the different fractions (ER, R, ORG, TOT) and a term that described the variation in metal concentration in the pore water. The different sediment factors considered in the models were the manganese (Mn), the iron (Fe) and the organic matter (TOC) content, and grain fraction < 63 µm. However, it was not possible to model the accumulated metal levels using the pooled data because we did not measure the same characteristics in the three studies. In the separate studies, only for zinc was it possible to explain 48 and 66% respectively of the variation, when organic matter was included in the accumulation model for zinc. The models that explained most of the variation were: $Zn_{CHI} = F*Zn_{TOT}*TOC^k$, in a first study (BERVOETS *et al.* 1997) and $Zn_{CHI} = F*ZnR*TOC^k$ in a second study (BERVOETS *et al.*, 1998).

For cadmium it was not possible to construct a model that explained a significant amount of variation in levels accumulated by the larvae.

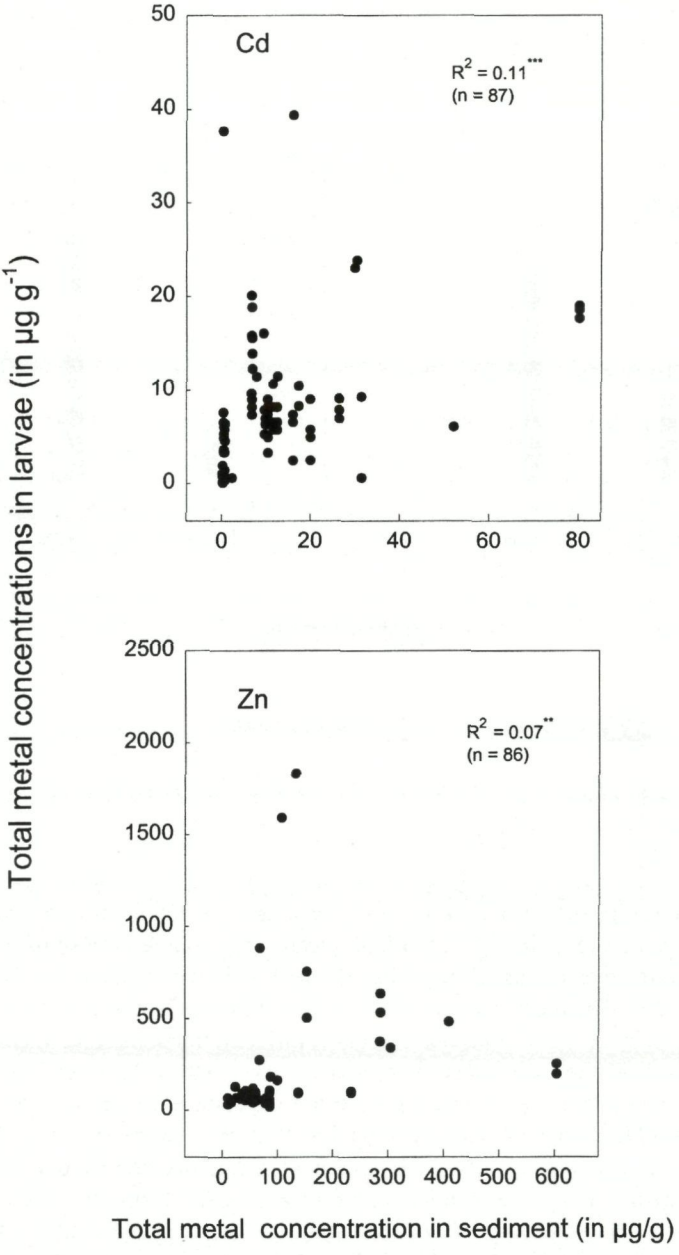

Fig. 2. – Relationships between trace metal levels in sediments and in chironomid larvae in µg g^{-1} dry weight for cadmium and zinc.

Laboratory studies

In Fig. 3 the results of the calculations of the chemical speciation model in defined solutions are summarised. The effects of salinity, temperature and pH on free metal ion activity are presented for cadmium and zinc. In the case of salinity the contribution of the free cadmium ion activity decreased from 72 % at a salinity of 0.24 ppt to 6 % at a salinity of 10 ppt, and the contribution of the free zinc ion activity decreased from 64 % to 31 % over the same salinity range. In the case of pH the contribution of the free cadmium ion activity decreased from 71.6 % at a pH of 5.5 ppt to 0.19 % at a pH of 10, and the contribution of the free zinc ion activity decreased from 69.5 % to 0.03 % over the same range. Temperature had only a small effect on the free metal ion activity over the tested range.

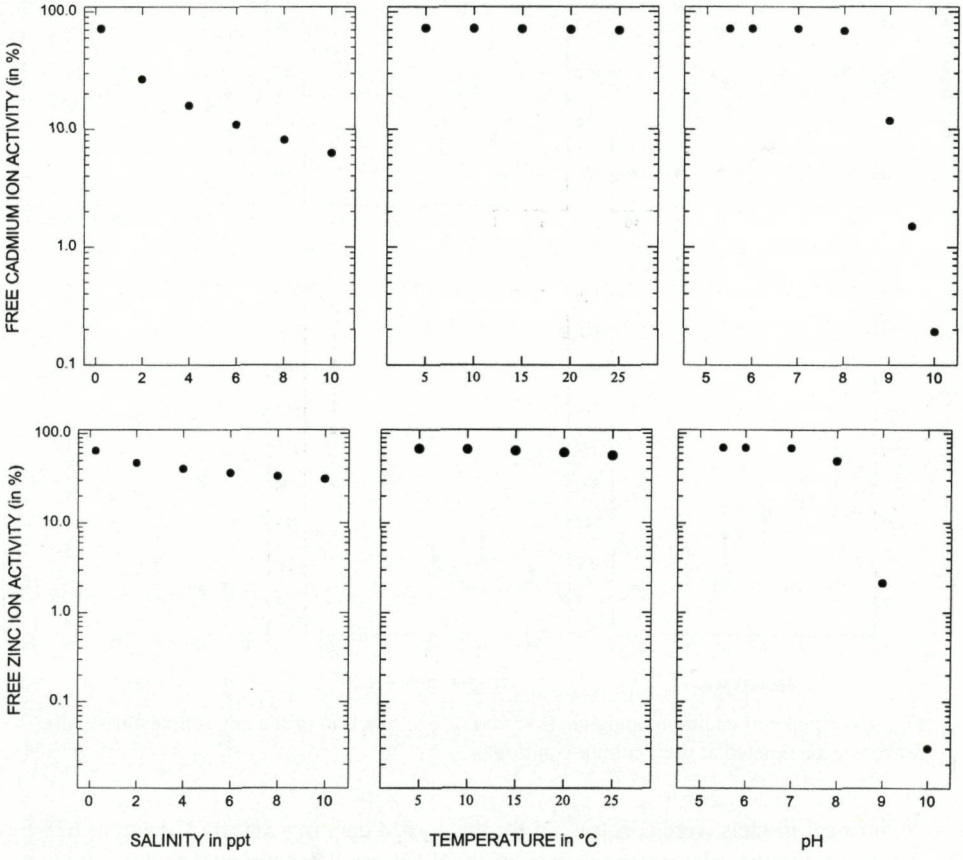

Fig. 3. – Relative contribution of the free metal ions (in %) in function of the environmental factors salinity, temperature, and pH.

In Fig. 4 net uptake as a function of the environmental factors is illustrated for cadmium and zinc. The results of uptake by larvae acclimated at their culture conditions are presented. For all environmental factors an effect was observed on net metal uptake within

each acclimation group (BERVOETS *et al.*, 1995, 1996a, 1996b; BERVOETS & BLUST, unpublished). The net uptake of cadmium and zinc decreased with increasing salinity but increased with increasing temperature. For pH, net uptake increased with increasing pH between 5.5 and 9.0 but decreased between pH 9.0 and 10.0. In most cases, prior acclimation to different environmental conditions had a significant effect on the net uptake but this effect was not always consistent.

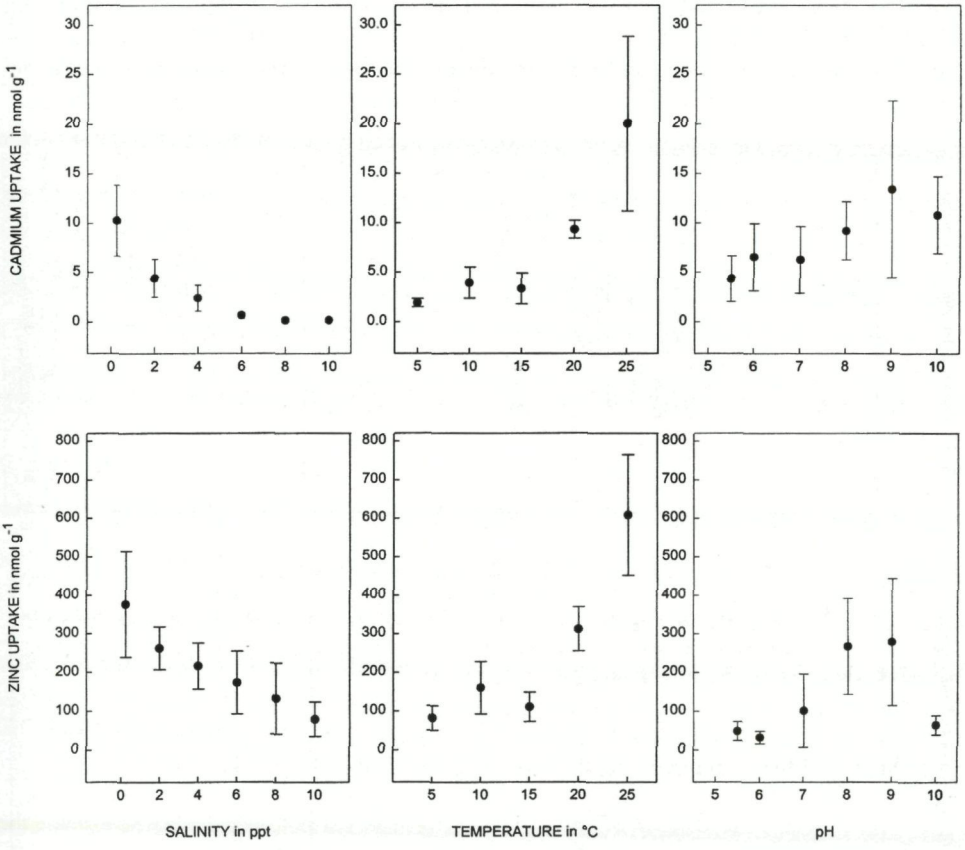

Fig. 4. – Uptake of cadmium and zinc (in nmol. g^{-1}) in function of the environmental factors, for larvae acclimated at their culture conditions.

Non-linear models were constructed for the pooled data (n= 580 for Cd and n=628 for Zn) to determine the relative importance of the different environmental factors and components of those factors on uptake of cadmium and zinc. Factors that were considered in the model were the free metal ion activity, salinity, temperature, and pH of exposure and acclimation, diffusion rate, and calcium ion activity. This was done by starting with a single factor, the metal ion activity, and stepwise adding the other terms, evaluating their contribution to the total variation. Tables 1 and 2 give the results of the non-linear regression analysis. As shown in this table, the free metal ion activity explained 6.2% and 24% of

the variation in metal uptake for respectively cadmium and zinc (Fig. 5). The model that explained most of the variation in net cadmium uptake was:

$$Cd_{midge}=C_f*(Cd^k_{act}*t^l_{exp}*pH^m_{exp}*sal^n_{exp}*Cd_{diff}{}^p*Ca^q*sal^r_{accl}*t^s_{accl})$$

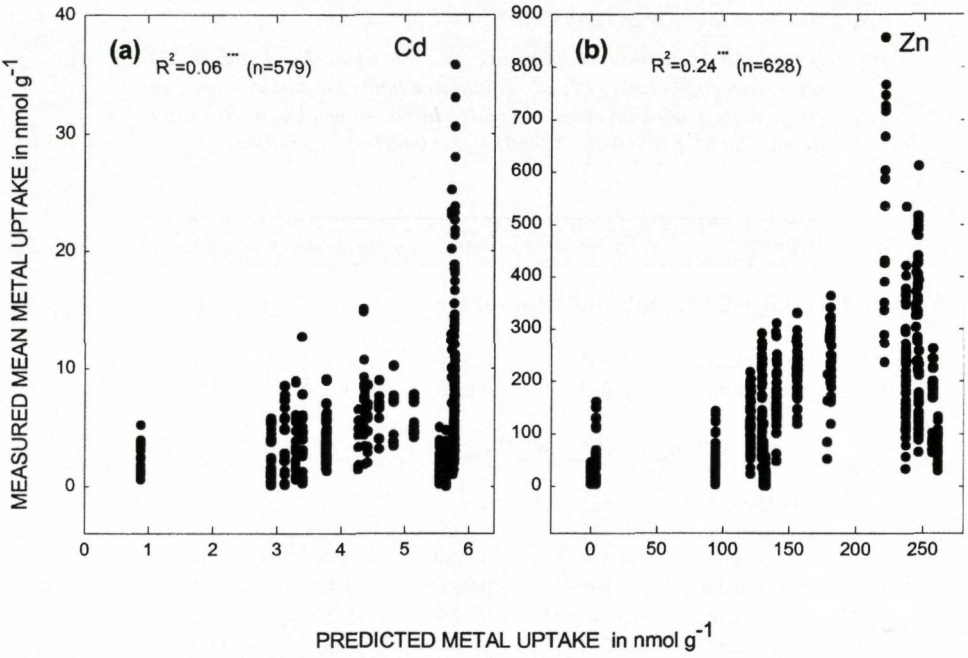

Fig. 5. – Predicted *versus* measured cadmium and zinc uptake by larvae of *Chironomus riparius*, considering only the free metal ion activities.(a) $Cd_{upt} = C_f*(Cd_{act}{}^k)$ ($R^2 = 0.062$**, n = 579); (b) $Zn_{upt} = C_f*(Zn_{act}{}^k)$ ($R^2 = 0.237$**, n = 628).

The model that explained most of the variation in net zinc uptake was:

$$Zn_{midge}=C_f*(Zn^k_{act}*t^l_{exp}*sal^m_{exp}*pH^n_{exp}*Ca^p*sal^p_{accl})$$

where Cd_{act} and Zn_{act} are the metal ion activities, t_{exp}, pH_{exp}, sal_{exp} are the exposure temperature, pH, and salinity, t_{accl}, pH_{accl}, sal_{accl} are the acclimation conditions, and Cd_{diff} is the diffusional rates of the free metal ion. In the case of net cadmium uptake, the factors that accounted for the effect of temperature, salinity, and calcium were negative, indicating that cadmium uptake increased with a decrease in these factors. The models explained 67% and 56% of the variation in uptake by midge larvae for respectively cadmium and zinc (Fig. 6).

TABLES 1 and 2

Cadmium uptake (top) and zinc uptake (bottom) by Chironomus riparius*: non-linear regression model for the pooled data.*

*B: partial regression coefficients; SE: Standard Error for partial regression coefficients; L1, L2: confidence limits for partial regression coefficients. Cadmium uptake in midge larvae in nmol/g. ns Not significant; * $0.05 \geq p > 0.01$; ** $0.01 \geq p > 0.001$; *** $p \leq 0.001$*

Variable	*B*	*SE*	*L1*	*L2*
(1) $Cd_{upt} = C_f * (Cd_{act}^{k})$ ($R^2 = 0.062^{**}$, n = 579)				
Coefficient	1.74***	0.394	1.35	2.13
k-exponent	0.281***	0.057	0.224	0.338
(2) $Cd_{midge} = C_f * (Cd_{act}^{k} * t_{exp}^{l} * pH_{exp}^{m} * sal_{exp}^{n} * Cd_{diff}^{p} * Ca_{accl}^{q} * sal_{accl}^{r} * t_{accl}^{s})$ ($R^2 = 0.67^{***}$, n = 579)				
Coefficient	1.62^{-09}	ns		
k-exponent	0.132***	0.044	0.088	0.176
l-exponent	-3.50***	0. 489	-3.99	-2.84
m-exponent	2.84***	0.723	2.12	3.56
n-exponent	-0.245***	0.026	-0.271	-0.219
p-exponent	14.2***	1.18	13.0	15.4
q-exponent	-0.072***	0.011	-0.083	-0.144
r-exponent	0.316***	0.024	0.292	0.340
s-exponent	0.376***	0.055	0.321	0.431
(1) $Zn_{upt} = C_f * (Zna_{act}^{k})$ ($R^2 = 0.237^{**}$, n = 628)				
Coefficient	0.263^{ns}			
k-exponent	0.978***	0.093	0.885	1.071
(2) $Zn_{midge} = C_f * (Zn_{act}^{k} * t_{exp}^{l} * sal_{exp}^{m} * pH_{exp}^{n} * Ca_{p} * sal_{accl}^{p})$ ($R^2 = 0.559^{**}$, n = 628)				
Coefficient	5.67^{-10}	ns		
k-exponent	1.63***	0.261	1.37	1.89
l-exponent	1.46***	0.118	1.34	1.58
m-exponent	0.137***	0.049	0.088	0.186
n-exponent	5.76*	2.44	3.32	8.20
p-exponent	-0.040***	0.007	-0.047	-0.033
q-exponent	0.116***	0.015	0.101	0.131

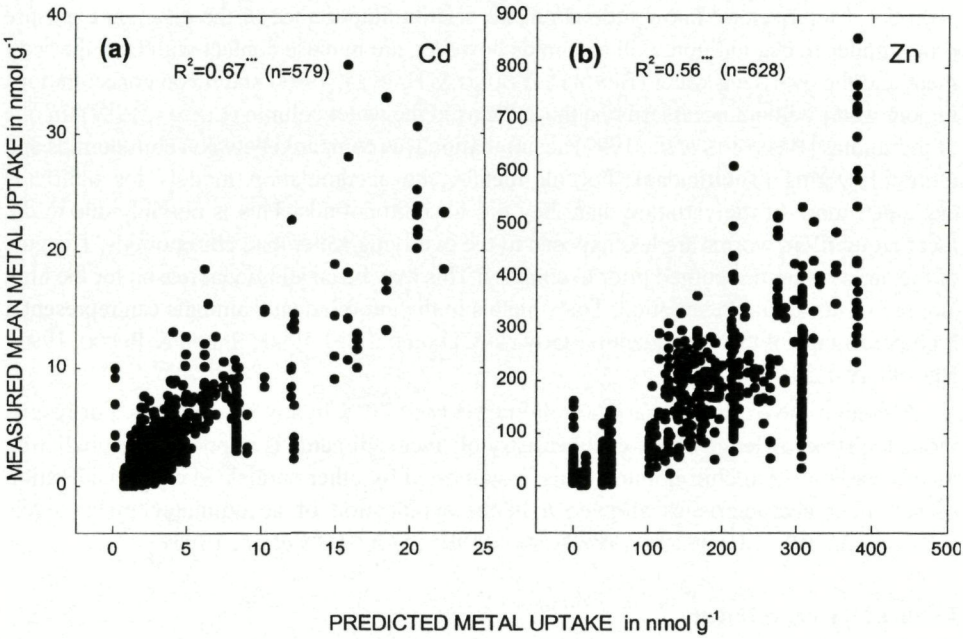

Fig. 6. – Predicted versus measured cadmium and zinc uptake by larvae of *Chironomus riparius*, for the uptake models.(a) $Cd_{midge}=C_f*(Cd^k_{act}*t^l_{exp}*pH^m_{exp}*sal^n_{exp}*Cd_{diff}{}^p*Ca^q*sal^r_{accl}*t^s_{accl})$ (R^2 = 0.67***, n = 579); (b) $Zn_{midge}=C_f*(Zn^k_{act}*t^l_{exp}*sal^m_{exp}*pH^n_{exp}*Cap*sal^p_{accl})$ (R^2 = 0.559**, n = 628).

DISCUSSION

Field studies

Using the pooled data of three studies, we related total cadmium and zinc levels measured in sediment to metal levels in chironomid larvae. For both metals only a poor relationship was found. Total metal concentrations in sediments have been proved to be poor predictors of metal levels in sediment dwelling invertebrates (*e.g.* BENDELL-YOUNG & HARVEY, 1991 ; TESSIER *et al.*, 1993). The absence of a clear relationship is mainly due to changes in bioavailability of metals with changing geochemical characteristics of the sediments. When geochemical sediment fractions were considered as well as sediment characteristics, it was possible to improve the relationship for zinc. For the other metals it was not possible to increase the explained variation in accumulated levels.

The determination of trace metal availability from solid phases is often difficult, even in laboratory experiments. Besides physical and chemical factors, biological processes will affect metal bioavailability, *e.g.* : both animals and plants may alter the metal form prior to bioaccumulation ; feeding strategy influences bioaccumulation. The relatively poor prediction of metal concentrations in the larvae for most metals may have been caused by the large differences in sediment composition. The unexplained variation could be due to numerous environmental factors that were not taken into account, such as sediment pH and redox

potential. Moreover we know little about the relative importance of the different exposure routes under field conditions. Chironomids however, are in close contact with both the sediment and the overlying water (BENDELL-YOUNG & HARVEY, 1991) and metal concentrations in pore water will not necessarily be the same as in the water column (LUOMA, 1989). In one of the studies (BERVOETS *et al.*, 1997) accumulation was compared between chironomids and tubificid worms (Tubificidae). For all metals, the accumulation models for tubificids explained more of the variation than they did for chironomids. This is possibly due to the fact that tubificid worms are less exposed to the overlying water than chironomids. The guts of the larvae were not purged prior to analysis. This may be an additional reason for the high amount of unexplained variation. Trace metals in the gut of aquatic animals can represent a high percentage of the total quantity (GOWER & DARLINGTON, 1990; SAGER & PUSCO, 1991; BROOKE *et al.*, 1996).

Although the explained variation did not exceed 70% in any of the cases, our results indicate that knowledge of the geochemistry of river sediments is important for predicting metal availability to chironomids. This is supported by other studies where consideration of sediment characteristics allowed a better explanation of accumulated metal levels (GUNN *et al.* 1989; BABUKUTTY & CHACKO, 1995; GONZALES *et al.*, 1995).

Laboratory experiments

Generally, the free metal ion is considered as the available metal species. However, relating net metal uptake to the free metal ion activity for the pooled data only explained a small part of the variation; 6% for cadmium uptake and 24% for zinc uptake. In the case of salinity and pH, the environmental factor had a marked effect on the contribution of the free ion activity for both metals over the tested ranges. When only salinity was changed, free ion activity explained 52% of net cadmium uptake (BERVOETS *et al.*, 1995) and 59% of zinc uptake (BERVOETS *et al.*, 1996a). In the case of temperature, the effect on speciation was rather small. Nevertheless temperature of exposure contributed significantly to the explained variation in net uptake of both metals. In the uptake model of the pooled data, the temperature of exposure contributed significantly to the explained variation. The effect of temperature is expected to be the result of the combined effects on the chemical behaviour (speciation and diffusion) and on the physiology of the organism (*e.g.* COSSINS & BOWLER, 1987; BLUST *et al.*, 1994; MOLLER *et al.*, 1994). Several authors have observed an increase in respiration by larvae of *Chironomus sp.* with increasing temperature (*e.g.* JOHNSON & BRINKHURST, 1971; HAMBURGER & DALL, 1990). Also salinity and pH of exposure significantly contributed to the uptake model, supplementary to the effect of these factors on speciation. For salinity this was probably due to a physiological effect (*e.g.* respiration, osmoregulation). For pH, however, net uptake increased with increasing salinity whereas the contribution of the free metal ion decreased. A hypothesis put forward in the literature is that the free metal ions (*i.e.* Cd^{2+} and Zn^{2+}) are in competition with the hydrogen ions at the membrane level and therefore restrict net uptake under acid conditions (CAMPBELL, 1995). In addition, pH also will have a physiological effect.

For the calcium ion activity also, an increase resulted in a decreased net uptake for both cadmium and zinc. As for the hydrogen ion activity, this is probably due to competi-

tion between calcium and metals for the same uptake sites (SPRY & WOOD, 1989; MARKISH & JEFFREE, 1994; HOGSTRAND *et al.*, 1995). In the case of acclimation, only for salinity was an important and consistent effect observed on net cadmium uptake, resulting in an increased uptake with increasing salinity of acclimation.

Although all experiments took place under controlled conditions (*i.e.* chemically defined water, and controlled environment) the integration of the chemical and biological effects of changes in environmental conditions explained, for the pooled data, no more than 67 and 59% of the variation in respectively cadmium and zinc uptake. A possible explanation is that not all effects were considered. The relatively high unexplained variation can also be partially due to the natural variation in metal uptake by midge larvae (SEIDMAN *et al.*, 1986; TIMMERMANS *et al.*, 1992). This is demonstrated when the same non-linear uptake models are constructed with the mean uptake values. With those models, 85% of the net cadmium uptake and 68% of the net zinc uptake could be explained (Fig. 7). A last possible explanation for the unexplained variation is that some environmental factors do not have a consistent effect on metal uptake. This was true for both temperature and pH of acclimation.

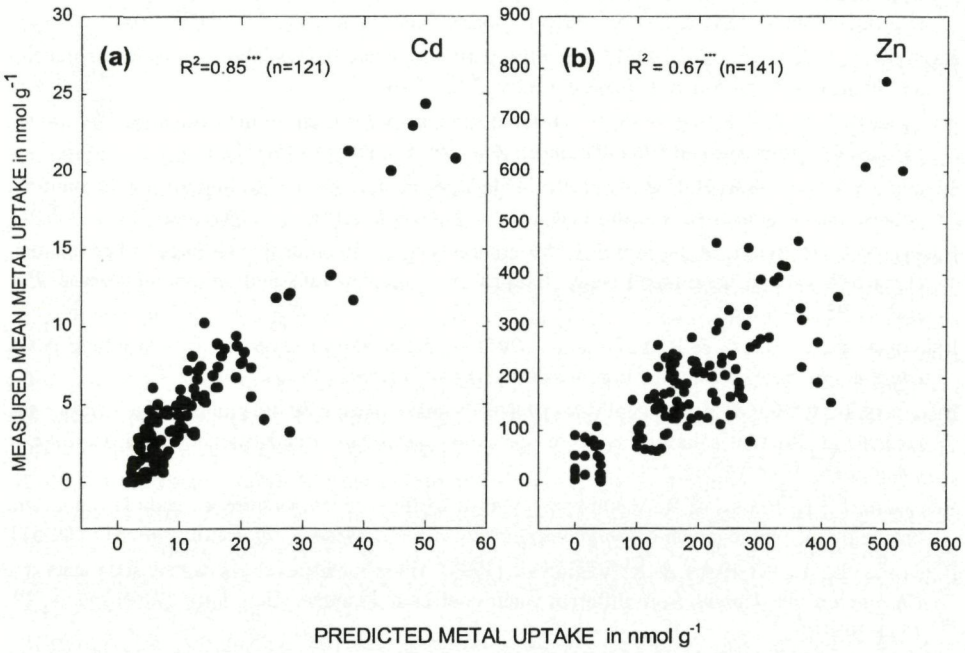

Fig. 7. – Predicted *versus* measured cadmium and zinc uptake by larvae of *Chironomus riparius*, for the uptake models, considering the mean uptake values.
(a) $Cd_{midge}=C_f*(Cd^k_{act}*t^l_{exp}*pH^m_{exp}*sal^n_{exp}*Cd_{diff}{}^p*Ca^q*sal^r_{accl}*t^s_{accl})$ (R^2 = 0.850***, n = 121);
(b) $Zn_{midge}=C_f*(Zn^k_{act}*t^l_{exp}*sal^m_{exp}*pH^n_{exp}*Ca_p*sal^p_{accl})$ (R^2 = 0.671**, n = 144).

From this study it was obvious that both the bioavailability of metals and the physiology of the exposed organisms need to be considered jointly to explain effects of environ-

mental factors on net metal uptake and accumulated levels. When we consider all possible factors contributing to metal bioavailability and when the relative contribution of the different exposure routes is known, it will be possible to explain or even predict metal accumulation by midge larvae under natural exposure conditions.

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INGESTION RATES OF *DAPHNIA MAGNA* STRAUS (CRUSTACEA : BRANCHIOPODA : ANOMOPODA) ON BACTERIOPLANKTON AND PHYTOPLANKTON IN AN AERATED WASTE STABILISATION POND

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Abstract. The impact of zooplankton on waste stabilisation pond (WSP) performance has been poorly studied until now. Zooplankton grazing activity is, however, worth considering as it can control the bacterioplankton and phytoplankton, which are the cornerstone organisms of the WSP treatment technology. The aim of the present study was to determine whether the grazing activity of the dominant zooplankter, *Daphnia magna*, can significantly control phytoplankton and bacterioplankton in a WSP (Differdange, Grand-duchy of Luxembourg). The biomass of phytoplankton (B_{phyto}), bacterioplankton (B_{bact}) and *Daphnia magna* ($B_{Daphnia}$) were estimated fortnightly from January to July 1998. At four key moments during this period, the ingestion rates of phytoplankton (IR_{phyto}) and bacterioplankton (IR_{bact}) by *D. magna* were determined and compared to B_{Phyto} and primary production (P_{prim}), and to Bbact and bacterial production (P_{bact}), respectively. From January to June, IR_{Phyto} varied between 66 and 92 % of $B_{phyto}.day^{-1}$ and between 2 and 90 % of P_{prim}. In July, the grazing impact on phytoplankton increased and reached 171 % of $B_{phyto}.day^{-1}$ and 310 % of P_{prim}, causing a significant drop in B_{phyto}. The grazing impact of *D. magna* on bacterioplankton varied between 0.1 and 18 % of $B_{bact}.day^{-1}$ and between 0.8 and 226 % of P_{bact}. Even when higher than 100% of P_{bact}, the grazing impact did not lead to a significant decrease of B_{bact}, because of a significant input of allochtonous bacterial biomass with the influent.

Key words: *Daphnia magna*, waste stabilisation ponds, ingestion rate, fluorescently-labelled bacteria, fluorescently-labelled algae, gut passage time

INTRODUCTION

Waste stabilisation ponds (WSP) are shallow, man-made basins used for the treatment of organically polluted waste waters. Bacterioplankton and phytoplankton are traditionally pointed out as the cornerstone organisms of this type of water treatment (OSWALD *et al.*, 1957 ; MARAIS & SHAW, 1961). Bacteria degrade organic matter and provide algae with carbon dioxide, while algae provide bacteria with oxygen produced during photosynthesis. Zooplankton often develops in high densities in these hypertrophic water bodies, and planktonic crustaceans often dominate the zooplankton community in WSP that have relatively long water residence times (~ 20 days) (CANOVAS *et al.*, 1996).

There is growing interest in a commercial use for zooplankton living in WSP, for example as food for fish in aquaculture or as a source of chitin (SEVRIN-REYSSAC *et al.*, 1994 ; CAUCHIE *et al.*, 1995). From the viewpoint of commercial applications, the available quantities of zooplankton (*i.e.* its biomass) and its renewal rate (*i.e.* its production) have to be determined. Surprisingly, the production dynamics in WSP have rarely been studied (DABORN *et al.*, 1978 ; MITCHELL & WILLIAMS, 1982 ; JANA & PAL, 1983). As part of a research program dealing with the potential for using WSP planktonic crustaceans as a commercial source of chitin, the production dynamics of the dominant zooplankter *Daphnia magna* have been studied during three years in an aerated WSP located at Differdange (Grand-Duchy of Luxembourg) (CAUCHIE *et al.*, unpubl.data). *Daphnia magna* biomass ($B_{Daphnia}$) and daily production ($P_{Daphnia}$) were found to vary seasonally. It was demonstrated that temperature significantly affected the growth rate and the daily production of *D. magna* (CAUCHIE *et al.*, unpubl.data). However, it proved difficult to demonstrate the effect of food on the production dynamics of the zooplankter.

In the present study, we examined the magnitude of trophic transfers from bacterioplankton and phytoplankton to *D. magna* under four different combinations of bacterioplankton biomass (B_{bact}), bacterial production (P_{bact}), phytoplankton biomass (B_{phyto}), and primary production (P_{prim}), as observed in the WSP of Differdange from January to July 1998. These transfers are discussed in terms of their relative contributions to the daily production of *D. magna*, as well as in terms of the impact of *D. magna* feeding activity on the bacterioplankton and phytoplankton dynamics.

MATERIAL AND METHODS

Study site and sampling strategy

The study site was an aerated WSP located in the Grand-duchy of Luxembourg (49°32'N-5°55'E). It collects the domestic waste water of a small town (15,000 inhabitant-equivalents) after primary treatment (screening and coarsing). Two mechanical aerators continuously oxygenate the pond, which is roughly rectangular. Its length is about 400 metres and its width is 150 metres. Its mean and maximum depths are 2.3 and 4.0 metres, respectively. The sampling site was located along the long axis of the pond, at about 100 metres from the inlet of the pond. At this place, the water column reaches 340 cm.

From January to July 1998, the pond water was sampled twice a month around noon in order to monitor B_{bact}, B_{phyto}, $B_{Daphnia}$, water temperature (TEMP) and dissolved oxygen concentration (DO). The ingestion rates of bacterioplankton (IR_{bact}) and phytoplankton (IR_{phyto}) by *D. magna* were determined on four occasions (January 29, May 03, June 15 and July 09). On these dates, we also determined bacterial production (P_{bact}) and primary production (P_{prim}).

Biomass and production of bacterioplankton

Bacterioplankton was sampled every 40 cm, from the surface to the bottom of the pond, with a 2 l Ruttner bottle and stored in 100-ml autoclaved and acid-washed glass bottles. In the laboratory, bacteria were stained with acridine orange according to HOBBIE *et*

al. (1977) and filtered onto black membranes (pore size = 0.2 µm). They were enumerated and measured to the nearest 0.5 µm using a Leica DMRB epifluorescence microscope equipped with a blue excitation filter block (Leica I3, BP 450-490). Volumetric B_{bact} (fg dry weight (DW) l^{-1}) was determined on the basis of the size class specific density of bacteria, N_i (cells.l^{-1}) and the mean size class specific cell volume of the bacteria, V_i (μm^3) (LOFERER-KRÖSSBACHER *et al.*, 1998):

$$B_{bact} = \sum_{i=1}^{n} 435.N_i.(V_i)^{0.86}$$

Vi was estimated as (LOFERER-KRÖßBACHER *et al.*, 1998):

$$V_i = \left(\left(w_i^2.\frac{\pi}{4}\right).(\ell_i - w_i)\right) + \left(w_i^3.\frac{\pi}{3}\right)$$

where w_i and ℓ_i are the mean width (µm) and mean length (µm) of the bacteria in the size class i. Areal B_{bact} was calculated by integrating volumetric B_{bact} over depth throughout the water column.

Hourly volumetric P_{bact}(µg DW.l^{-1}.h^{-1}) was determined *in situ* on the basis of the incorporation rate of (4,5-^{3}H)-l-leucine according to KIRCHMAN *et al.* (1985). For each sample, triplicate aliquots of 5 ml were incubated with the addition of 83 nM of leucine (10 % (4,5-^{3}H)-l-leucine - 90 % non radioactive leucine). The incorporation rate of leucine was corrected for isotopic dilution using the kinetic approach described by KIRCHMAN *et al.* (1986). Supplementary triplicate aliquots of 5 ml were taken from one sample and incubated in the presence of four concentrations of added leucine in the range of 1-75 nM (10 % (4,5-^{3}H)-l-leucine - 90 % non radioactive leucine). The leucine incorporation rates were plotted against leucine concentration, and the maximum incorporation rate (V_{max}) was determined by fitting a hyperbolic function to the data. The ratio between V_{max} and the incorporation rate for 83 nM of added leucine was used to correct the isotopic dilution, which was supposed to be similar at all depths. Two blanks were made for each triplicate by adding ice-cold TCA (5 % final concentration) immediately after the beginning of the incubation. Incubations were conducted *in situ* during one hour. Incubations were terminated by the addition of ice-cold TCA (5 % final concentration). Samples were then extracted at 85°C during 30 minutes (SERVAIS, 1995) and filtered onto 0.22 µm pore size acetate cellulose membranes. One ml ethylacetate was added to dissolve the filters. After 48 hours of storage, radioactivity associated with the filter was measured using a Beckman LS 6500 scintillation counter and the Beckman Ready Organic scintillation cocktail. Quenching was corrected with the external standard in the Compton edge shift mode (H#). Hourly volumetric P_{bact} (µg DW.l^{-1}.h^{-1}) was calculated from the leucine incorporation rate, assuming a conversion factor of 1,080 gC.mole leucine incorporated^{-1} (SERVAIS & LAVANDIER, 1995) and a carbon-dry weight ratio of 0.54 (SIMON & AZAM, 1989). Areal daily P_{bact} was calculated by integrating volumetric hourly P_{bact} over depth and by multiplying the integrated value by the 24 hours of a day.

Biomass of phytoplankton and primary production

For the determination of B_{phyto}, water was sampled every 40 cm with a 2 l Ruttner bottle and stored in 250-ml autoclaved and acid-washed glass bottles. In the laboratory, water was filtered onto a black polycarbonate membrane (pore size = 0.2 µm) and the filters were observed using a Leica DMRB epifluorescence microscope equipped as noted above. Phytoplankton was distinguished from heterotrophic protists on the basis of its red autofluorescence, and was enumerated and measured to the nearest 1 µm. Considering a carbon to dry weight ratio of 0.524 (OSWALD, 1988), volumetric B_{phyto} (pg DW.l^{-1}) was determined on the basis of the size class specific density of phytoplankton, N_i (cells.l^{-1}) and the mean size class specific cell volume of phytoplankton, V_i (µm^3) as (ROCHA & DUNCAN, 1985):

$$B_{phyto} = \sum_{i=1}^{n} 0.2298.N_i.(V_i)^{1.051}$$

Vi was estimated assuming the cells to be ellipsoids:

$$V_i = \frac{\pi}{6} a_i.b_i.c_i$$

where a_i, b_i and c_i are the mean diameter (µm), the smallest diameter (µm) and the greatest diameter (µm) of the cell in the size class i. Areal B_{phyto} was calculated by integrating volumetric B_{phyto} over depth throughout the water column.

The dominant species in the phytoplankton were determined during the algal bloom observed in June and July. Phytoplankton samples were taken at a depth of 60 cm and preserved in lugol. Determinations were made using a Leitz Laborlux microscope.

Volumetric P_{prim} was determined by the «oxygen light-dark bottle» technique (VOLLENWEIDER, 1969). Phytoplankton samples for P_{prim} were taken every 20 cm with a 2 l Ruttner bottle. Two light and two dark, acid-washed Winkler bottles (volume = 250 ml) were incubated *in situ* for 4 hours around noon. Dissolved oxygen concentration was determined in each bottle before and after the incubation, using a WTW Oxi539 oxymeter equipped with a Trioximatic300 oxygen probe and a magnetic stirrer. The gross photosynthetic rate (mg $O_2.l^{-1}.h^{-1}$) was estimated by summing the oxygen production rate measured in the light bottles and the oxygen respiration rate measured in the dark bottles. The gross photosynthetic rate was converted into hourly volumetric P_{prim} (mg $C.l^{-1}.h^{-1}$), assuming that 1.55 g of molecular oxygen are released in the water when 1 g of algal cell material is synthesised (OSWALD, 1988). Areal daily P_{prim}(mg $DW.m^{-2}.day^{-1}$) was determined by integrating the volumetric hourly P_{prim} over depth and by multiplying the integrated value by the ratio between the total solar irradiance during the day and the solar irradiance during the incubation, both expressed in Einstein units per square metre (MEFFERT & OVERBECK, 1985). Solar irradiance data were obtained from the nearby meteorological station of Belvaux (49°31' N - 5°56' E).

Biomass and production of *D. magna*

Zooplankton samples were taken every 60 cm with a 5 l Van Dorn bottle. They were concentrated using a Nylon net (mesh size = 80 μm) and preserved in 4 % sugar formalin solution (PREPAS, 1978). In the laboratory, *D. magna* specimens were enumerated, measured to the nearest 50 μm using a Leica dissecting microscope equipped with a micrometer, and sorted in 250 μm-wide size classes. Volumetric $B_{Daphnia}$ (mg DW.l^{-1}) was estimated as (RIGLER & DOWNING, 1984):

$$B_{Daphnia} = \sum_{i=1}^{n} N_i.(\overline{w_i})$$

where N_i is the density of *D. magna* in the size class i, expressed in individuals.l^{-1} and $\overline{w_i}$ is the mean weight of the animals in the size class i deduced from the length-weight regressions established by CAUCHIE *et al.* (unpubl.data). The daily net production of *D. magna* ($P_{Daphnia}$) was calculated as (RIGLER & DOWNING, 1984):

$$P_{Daphnia} = \sum_{i=1}^{n} g_i.B_i$$

where g_i is the growth rate of the animals in the size class i, expressed in day^{-1} and B_i is the biomass of animals in the size class i, expressed in mg DW.l^{-1}.

Feeding experiments

Feeding experiments were conducted in the laboratory using fluorescently-labelled bacteria (FLB) and algae (FLA). FLB and FLA were prepared the day before the experiment using 5-(4,6-dichlorotriazin-2-yl) amino-fluorescein (DTAF) according to SHERR *et al.* (1987) and TELESH *et al.* (1995), respectively. Bacteria used for labelling were isolated from pond water by filtration. The alga used for labelling was the chlorophycean *Dictyosphaerium ehrenbergianum* NAEG., cultured in the laboratory. After P_{bact} and P_{prim} had been determined *in situ*, daphnids were collected every 80 cm with a 5 l Van Dorn bottle and brought back to the laboratory in carboys that were kept in incubators at *in situ* temperatures. In the laboratory, B_{bact} and B_{phyto} were determined and pond water was filtered over a polycarbonate membrane (pore size = 0.2 μm) in order to obtain sterile pond water. Feeding medium was constituted by adding FLB and FLA to 50 ml of filtered pond water to obatin B_{bact} and B_{phyto} concentrations similar to those *in situ*. Each feeding experiment was conducted in an incubator at *in situ* temperature and photoperiod, in triplicate glass bottles containing 50 ml of feeding medium.

In order to avoid loss of FLB or FLA through defecation, the incubation time used for the feeding experiments was set lower than gut passage time (GPT). GPT experiments were, therefore, conducted prior to each feeding experiment. GPT was determined by direct observation of the labelled food in the gut. The animals were incubated for an increasing incubation time in the feeding medium. At the end of each incubation, the daph-

nids were narcotised with carbonated water, killed with formalin and rinsed with sterile water. The animals were then individually observed under a Leica DMRB epifluorescence microscope equipped with a blue excitation filter block (Leica I3, BP 450-490). The fullness of the gut of each animal was noted, using the following scores: (1) there were no FLB nor FLA in the gut of the animal; (2) there were FLB or FLA in the first half of the gut; (3) there were FLB or FLA in the second part of the gut but not up to the distal part of the gut; (4) there were FLB or FLA up to the distal part of the gut. GPT was reached when the animal began to defecate fluorescent food.

When gut passage time had been determined, daphnids were incubated in the feeding medium during a time that was approximately 20 % shorter than the gut passage time. The feeding experiments were always conducted around 19:00 on the day of sampling. The incubation was ended by adding carbonated water to narcotise the animals. The animals were killed with formalin and rinsed with sterile water, then grouped according to size and transferred into microvials. One ml of sterile water was added to each microvial which was then shaken vigorously and sonicated until the bodies of the daphnids were destroyed. The suspension was then filtered onto a black membrane, and FLB and FLA were enumerated under a Leica DMRB epifluorescence microscope equipped with a blue excitation filter block (Leica I3, BP 450-490). The hourly IR_{bact} and IR_{phyto} (ng DW.ind^{-1}.h^{-1}) were calculated as:

$$\text{Hourly } IR_{bact/phyto} = \frac{N_{FLB/FLA} \cdot 60 \overline{w}_{FLB/FLA}}{N_{Daphnia} \cdot IT}$$

where $N_{FLB/FLA}$ is the number of FLB or FLA in the microvial (number of FLB or FLA); $\overline{w}_{FLB/FLA}$ is the mean weight of FLB or FLA used in the experiment (ng DW.(FLB or FLA)$^{-1}$); $N_{Daphnia}$ is the number of *D. magna* individuals grouped in the microvial (ind.) and IT is the incubation time (min). Equations describing the relationship between *D. magna* body size and ingestion rates were obtained by fitting a power equation to the data. Daily IR_{bact} and IR_{phyto} by the whole *D. magna* population (mg DW. (g DW *Daphnia*)$^{-1}$.l^{-1}.d^{-1}) were calculated as:

$$\text{Daily } IR_{bact/phyto} = \sum_{i=1}^{n} \left(\frac{\overline{IR}_{bact/phyto\ (i)} \cdot N_i .24}{\overline{w}_i} \right)$$

where $\overline{IR}_{bact/phyto\ (i)}$ is the mean hourly IR_{bact} or IR_{phyto} of the individuals in the size class i (ng DW.ind^{-1}.h^{-1}); Ni is the density of *D. magna* individuals in the size class i (ind.l^{-1}); w_i is the mean weight of the *D. magna* individuals in size class i (µg) determined according to CAUCHIE *et al.* (unpubl.data). The areal daily IR_{bact} and IR_{phyto} were obtained by integrating the volumetric daily IR_{bact} and IR_{phyto} over the water column.

To check whether the ingestion rates measured at 19:00 were representative of the daily mean IR_{phyto}, hourly IR_{phyto} was measured every 3 hours during 24 hours under the conditions prevailing on July 09. Feeding experiments were conducted as described above, using a feeding medium composed of 1.2 µm filtered pond water (*i.e.* pond water con-

taining the natural bacterial community) supplemented with FLA at a biomass similar to the *in situ* B_{phyto} observed on July 09.

RESULTS

The dynamics of areal values of $B_{Daphnia}$, B_{bact} and B_{phyto} are shown in Fig. 1. Low to moderate $B_{Daphnia}$ values (< 2.5 g DW.m^{-2}) were observed from January to May. $B_{Daphnia}$ began to increase steeply in June and reached very high values in early July (60.4 g DW.m^{-2}), before decreasing to about 5 g DW.m^{-2} in late July. The dynamics of areal B_{phyto} showed a first peak in February-March (maximum value = 1.1 g DW.m^{-2}) and a second one in June-July (maximum value = 8.8 g DW.m^{-2}).

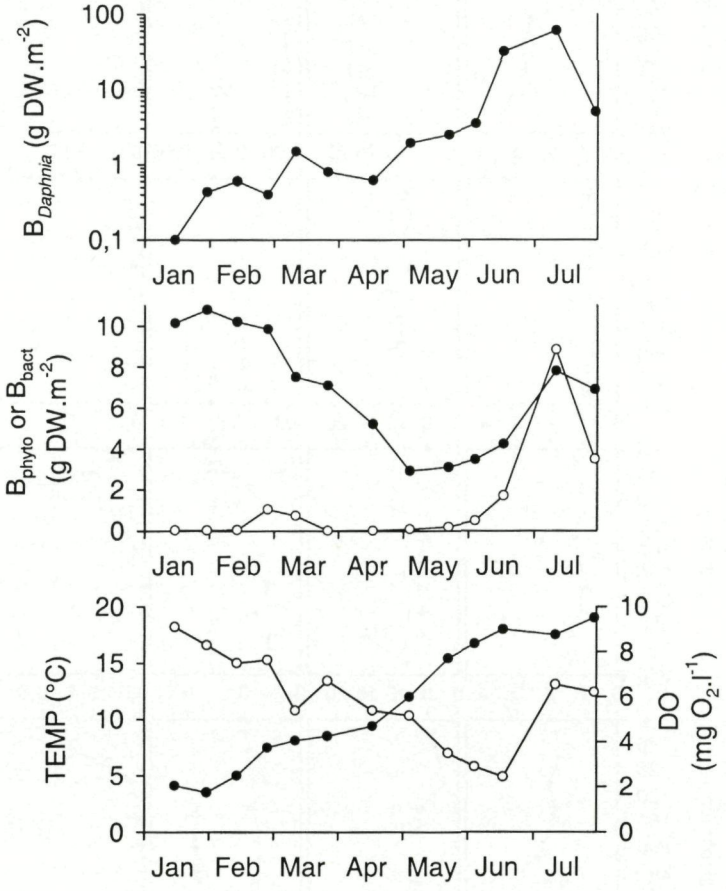

Fig. 1. – Variation of *Daphnia magna* biomass ($B_{Daphnia}$), bacterioplankton biomass (B_{bact}), phytoplankton biomass (B_{phyto}), water temperature (Temp) and dissolved oxygen concentration (DO) in the waste stabilisation pond of Differdange from January to July 1998. Middle panel: black circles = B_{bact}; open circles = B_{phyto}. Lower panel: black circles = Temp; open circles = DO.

The second, main peak of phytoplankton was dominated by the unicellular green alga *Planktosphaeria gelatinosa* SMITH. B_{bact} decreased from about 10 g DW.m^{-2} in January to 3 g DW.m^{-2} at the beginning of May. It then increased to 7.8 g DW.m^{-2} at the beginning of July. Water temperature gradually increased from 3.5°C in January to 19°C in July.

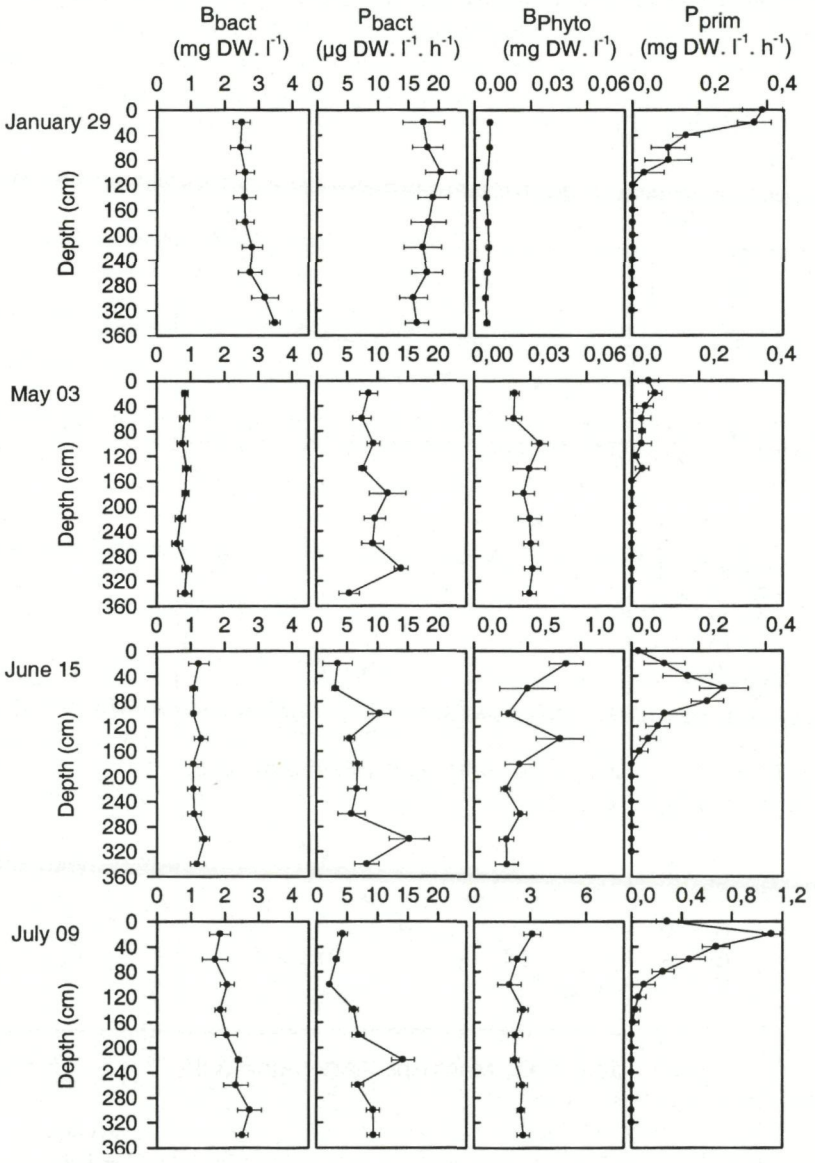

Fig. 2. – Vertical profiles of bacterioplankton biomass (B_{bact}) and production (P_{bact}), phytoplankton biomass (B_{phyto}) and primary production (P_{prim}) in the waste stabilisation pond of Differdange on the dates of the feeding experiments.

Dissolved oxygen concentration decreased from 9.1 mg $O_2.l^{-1}$ in January to 2.5 mg $O_2.l^{-1}$ in June. It increased to 6.5 mg $O_2.l^{-1}$ in July. Pearson Moment Product correlations between the variables presented in Fig. 1 are shown in Table 1. Significant positive correlations were found between $B_{Daphnia}$ and B_{phyto} and between B_{bact} and dissolved oxygen concentration. Negative correlations were found between B_{bact} and water temperature, and between water temperature and dissolved oxygen concentration.

TABLE 1

*Correlation matrix (Pearson Product Moment Correlation) for variables measured bimonthly in the WSP of Differdange from January to July 1998 ns = not significant (P>0.05), * P<0.05, ** P<0.01, *** P<0.001*

	$B_{Daphnia}$	B_{bact}	B_{phyto}	Temp.
B_{bact}	– 0.08 NS			
B_{phyto}	0.88 ***	0.06 NS		
Temp.	0.56 *	– 0.70 **	0.55 NS	
DO	– 0.20 NS	0.89 ***	0.01 NS	– 0.74 **

B_{bact} and B_{phyto} values showed weak vertical variation during the day, except in June when outlier values of B_{phyto} were observed at the surface and at a depth of 120 cm (Fig. 2). By contrast, P_{bact} and P_{prim} generally varied significantly with depth. P_{prim} displayed typical profiles, with maximum values observed between 20 and 60 cm deep. Daytime $B_{Daphnia}$ vertical distribution (Fig. 3) appeared quite patchy in May and June, whereas it was homogenous in January and July. Water temperature and dissolved oxygen concentration did not vary substantially over the water column (Fig. 3). In the absence of significant variation in vertical distribution of food and physico-chemistry, *in situ per capita* IR_{bact} and IR_{phyto} were assumed to be constant throughout the water column, and only one set of feeding experiments was performed per date, using mean volumetric values of $B_{Daphnia}$, B_{bact} and B_{phyto}. The feeding experiments were performed under low (January 29), intermediate (May 03), high (June 15) and very high (July 09) $B_{Daphnia}$ values (Table 2). Moreover, the ratio of B_{bact} on B_{phyto} varied widely among these dates, decreasing from 390 in January to 29 in May, 2.5 in June and 0.9 in July.

TABLE 2

Areal values of bacterioplankton biomass (B_{bact}) and production (P_{bact}), phytoplankton biomass (B_{phyto}), primary production (P_{prim}) and Daphnia magna biomass ($B_{Daphnia}$) and production ($P_{Daphnia}$) in the aerated waste stabilisation pond of Differdange on the dates of the feeding experiments. TEMP = water temperature, DW = dry weight.

Date	*TEMP* *°C*	*Bbact*	B_{phyto} *g DW.m^{-2}*	$B_{Daphnia}$	P_{bact}	P_{prim} *gDW.m^{-2}.day^{-1}*	$P_{Daphnia}$
January 29	3.5	10.152	0.026	0.431	1.536	1.390	0.047
May 03	12.4	2.910	0.102	1.947	0.792	0.314	0.344
June 15	18.0	4.234	1.701	31.961	0.624	1.644	18.518
July 09	17.5	7.801	8.837	60.386	0.600	4.889	54.601

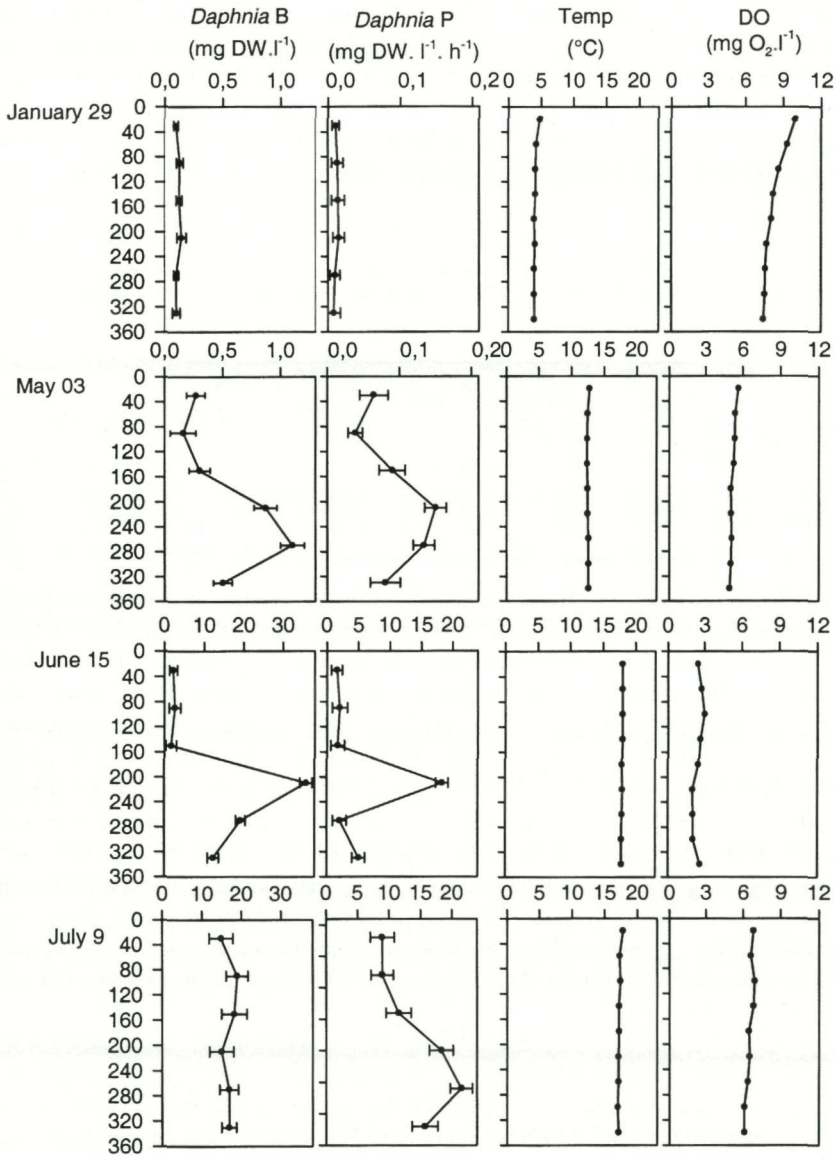

Fig. 3. – Vertical profiles of *Daphnia magna* biomass ($B_{Daphnia}$) and production ($P_{Daphnia}$), water temperature (Temp) and dissolved oxygen concentration (DO) in the waste stabilisation pond of Differdange on the date of the feeding experiments.

The results of the GPT experiments are shown in Fig. 4. In the January experiments, the proportion of non-feeding individuals, *i.e.* individuals which had not ingested fluorescent food, decreased from 100 to 35 % within the first 15 minutes of incubation. It then stayed around 35 % for incubation times up to 60 minutes. Individuals which had begun

to defecate fluorescent food were only observed in experiments lasting 60 minutes. In the three other GPT experiments, animals defecating fluorescent food were observed within 5 to 9 minutes from the beginning of the incubation. In May, June and July experiments, the proportion of the non-feeding individuals remained as high as 10-15 % for incubations longer than 15 minutes. On the basis of the results presented in Fig. 4, the incubation duration was set at 30 minutes for January experiments and 4 minutes for the three other sets of experiments.

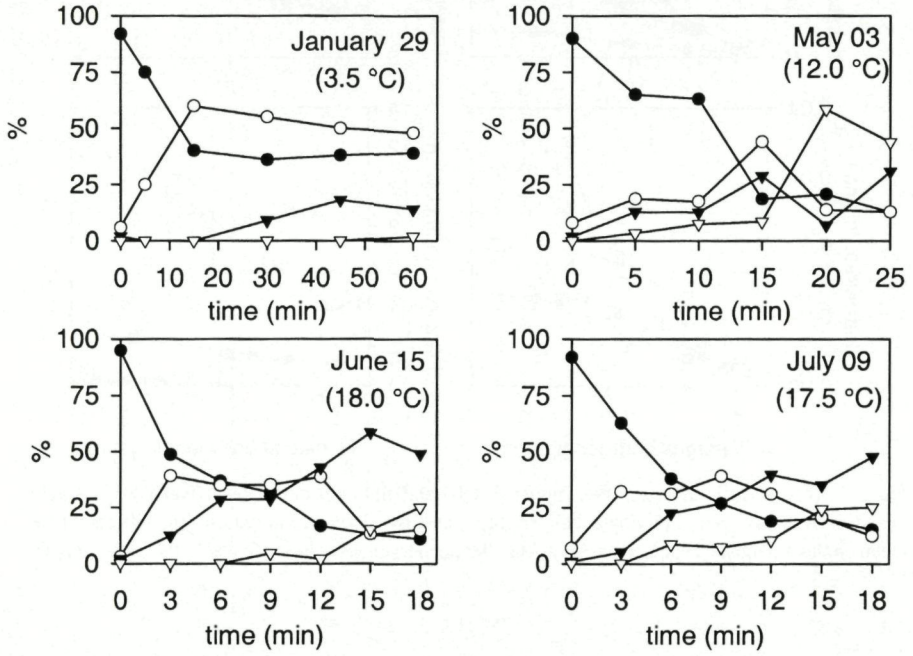

Fig. 4. – Evolution of the proportion of the individuals having no fluorescently labelled algae (FLA) in the gut (black circles), FLA in the first half part of the gut (open circles), FLA in the second part of the gut but not up to the distal part (black triangles) or FLA up to the distal part of the gut (open triangles) as a function of increasing incubation time in *Daphnia magna* kept under constant conditions in the laboratory and provided with *in situ* concentrations of FLA. Water temperature indicated in brackets.

The relationships between *D. magna* body length and IR_{bact} and IR_{phyto} (Fig. 5) could be adequately described by a power function (Table 3). The exponent b ranged from 1 to 2, except for January IR_{bact} where it was 3.12 and for May IR_{phyto} when it was 0.87. Areal daily IR_{bact} and IR_{phyto} increased exponentially from January to July (Table 4). The proportion of B_{bact} and B_{phyto} ingested per day by *D. magna* varied from 0.1 to 17.6 % and from 65.7 to 171.1 %, respectively. *D. magna* ingested daily a small percentage of P_{bact} in January (0.8 %) and May (0.9 %), and a large percentage of P_{bact} in June (119.7 %) and July (226.0 %). The percentage of P_{prim} ingested daily by *D. magna* increased from 1.7 % in January to 319.2 % in July.

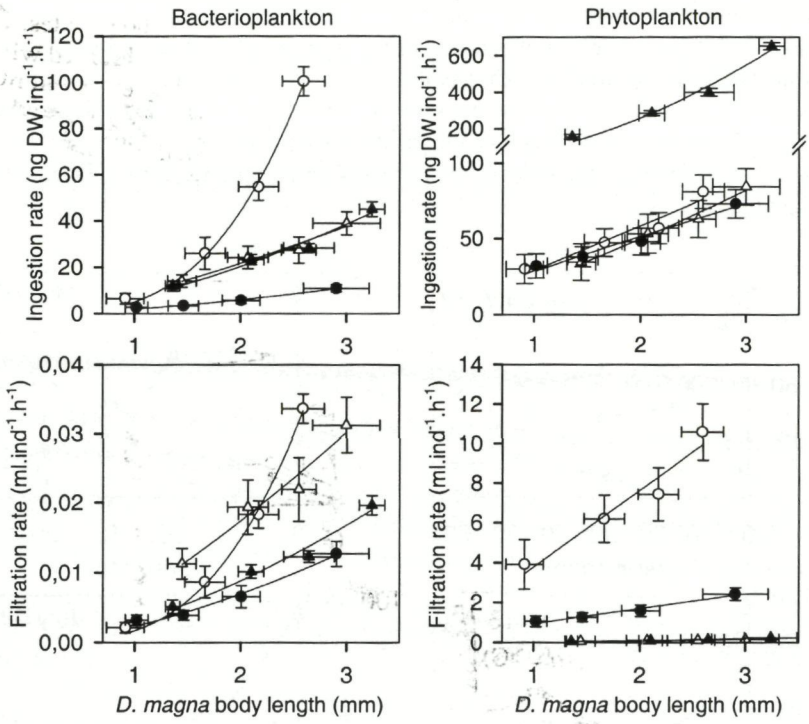

Fig. 5. – Size specific ingestion rate (upper panels) and filtration rates (lower panels) of bacterioplankton (left panels) and phytoplankton (right panels) by *Daphnia magna* on four different dates. Open circles = January 29; black circles = May 03; open triangles = June 15; black triangles = July 09.

TABLE 3

Cell concentrations of bacterioplankton and phytoplankton used in the feeding experiments and power functions describing the dependence of the ingestion rate (IR) and the filtering rate (FR) on the Daphnia magna *body length (L). Equation: IR or FR = a . L b ; For units, see Fig. 5*

	Concentration (cells.ml^{-1})	*IR*	*FR*	r^2
Bacterioplankton				
January 29	3.3 10^7	0.0017 $L^{3.12}$	1.70 $L^{3.12}$	0.997
May 03	1.0 10^7	0.0024 $L^{1.57}$	2,35 $L^{1.57}$	0.984
June 15	1.4 10^7	0.0068 $L^{1.36}$	6.78 $L^{1.36}$	0.963
July 09	2.6 10^7	0.0030 $L^{1.58}$	2.95 $L^{1.58}$	0.972
Phytoplankton				
January 29	650	28.96 $L^{1.01}$	3.78 $L^{1.01}$	0.944
May 03	2,500	28.21 $L^{0.87}$	0.94 $L^{0.87}$	0.972
June 15	41,700	21.43 $L^{1.22}$	0.04 $L^{1.22}$	0.979
July 09	220,000	73.42 $L^{1.83}$	0.03 $L^{1.83}$	0.983

TABLE 4

Ingestion rates and filtering rates of the Daphnia magna *population on bacterioplankton and phytoplankton in the aerated waste stabilisation pond of Differdange*

	January 29	*May 03*	*June 15*	*July 09*
Bacterioplankton				
FR_{bact} ($l.m^{-2}.day^{-1}$)	4.0	8.2	599.9	591.00
IR_{bact} (g DW m^{-2} day^{-1})	0.012	0.007	0.747	1.356
IR_{bact} (% of B_{bact} day^{-1})	0.1	0.2	17.6	17.4
IR_{bact} (% of daily P_{bact})	0.8	0.9	19.7	226.0
Phytoplankton				
FR_{phyto} ($l.m^{-2}.day^{-1}$)	3137.3	2233.3	2970.2	5816.5
IR_{phyto} (g DW m^{-2} day^{-1})	0.024	0.067	1.486	15.117
IR_{phyto} (% of B_{phyto} day^{-1})	92.3	65.7	87.4	171.1
IR_{phyto} (% of daily P_{prim})	1.7	21.3	90.4	309.2

The diel pattern of variation in IR_{phyto} is shown in Fig. 6. The diel pattern was unimodal for 1.0 mm long *D. magna* and bimodal for 2.5 mm long ones. For 1 mm long animals, the mode was located at 13:00. For 2.5 mm long animals, the modes were observed between 7:00 and 10:00, and at 19:00. The relative amplitude of hourly IR_{phyto}, δ, (*i.e.* the ratio between the highest and the lowest IR_{phyto} values observed over the nycthemeron) reached 2.09 and 2.46 for 1 mm long daphnids (juveniles) and 2.5 mm long daphnids (adults), respectively (Table 5). The ratios between the IR_{phyto} calculated from all hourly measures made during the nycthemeral monitoring and the IR_{phyto} measured at 19:00 (*i.e.* when the ingestion experiments were performed on January 29, May 03, June 15 and July 09) were quite close to 1.

TABLE 5

Diel relative amplitudes of hourly IR_{phyto} over the 24 hours cycle (δ) and comparison of the daily IR_{phyto} calculated from all the hourly IR_{phyto} values measured every 3 hours and the daily IR_{phyto} extrapolated from the hourly IR_{phyto} measured at 19 00. d is the daily maximum hourly IR_{phyto} divided by the daily minimum hourly IR_{phyto}

D. magna body length (mm)	δ	Daily IR_{phyto} *(µg DW. ind-1.d-1)*		Calculated on *Extrapolated ratio*
		Calculated	*Extrapolated*	
1.0	2.09	5.88	6.08	0.97
2.5	2.46	6.32	5.85	1.08

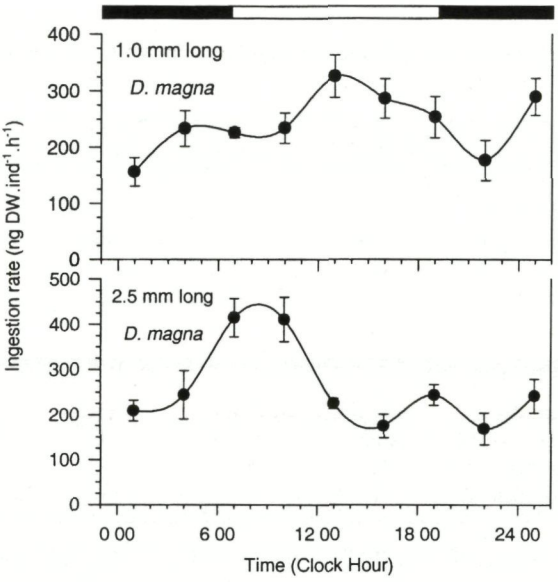

Fig. 6. – Diel variations of the hourly IR_{phyto} (mean ± standard deviation) of 1.0 and 2.5 mm long *D. magna* under the feeding conditions observed on July 09.

DISCUSSION

On the basis of the dynamics presented in Fig. 1 and the correlations shown in Table 1, the variation in $B_{Daphnia}$ in the pond of Differdange appeared tightly linked to the availability of algal food and, to a lesser extent, to water temperature. The importance of phytoplankton in the daily ration of *Daphnia magna* will be discussed below on the basis of the results of the feeding experiments. The correlation between $B_{Daphnia}$ and water temperature reflected the strong dependence of the growth rate of *D. magna* on water temperature (BOTTRELL *et al.*, 1976). An increase of water temperature from 10 to 20°C shortens the generation time from more than 20 days to less than 5 days (CAUCHIE *et al.*, unpubl.data).

B_{bact} apparently varied independently of $B_{Daphnia}$, but was correlated with dissolved oxygen concentration and water temperature. The decrease in dissolved oxygen concentration from January to June resulted from a gradual decrease in the input of well-oxygenated rain water in the sewage, as rainfall decreases from winter to spring. As a consequence, anaerobic bacteria most probably became dominant over aerobic ones. The slower metabolic rate of anaerobes compared to aerobes (DROSTE, 1997), coupled with an increase in the density of bacterivorous protozoans generally observed in spring in WSP (CANOVAS *et al.*, 1996), most probably caused the continuous decrease of B_{bact} observed from January to May. The release of organic material by phytoplankton is a major source of carbon for bacterioplankton. This may explain why the increase of B_{phyto} observed in June and July was followed by a significant increase of B_{bact}.

The homogeneity of the vertical profile of B_{bact} and B_{phyto} throughout the water column probably resulted from efficient mixing of the water column by the mechanical aerators. The vertical distribution of $B_{Daphnia}$ appeared patchy during May and June. Because the aerators induced an efficient mixing of the water, hydrodynamic factors did not cause this patchiness. On the other hand, the swarming of *D. magna* was not an adaptation to the presence of predators since neither fish nor other common daphnid consumers were present in the pond. In the presence of homogeneously distributed food, the vertical distribution of daphnids might also be expected to be homogeneous (PIJANOWSKA & DAWIDOWICZ, 1987). It is therefore not known why *D. magna* tended to aggregate near the bottom in May and in June.

GPT experiments revealed that feeding activity was neither synchronised nor continuous in the *D. magna* population. Such high variability in GPT has been observed in other *Daphnia* species (ZANKAI, 1983 ; MURTAUGH, 1985 ; GERRITSEN *et al.*, 1987). This variability could have a purely experimental origin. When transferred from one medium to another with different characteristics (food concentration, water temperature, etc.), animals can be stressed and may need from 15 to 30 minutes to recover constant physiological rates (BURNS, 1968 ; PORTER *et al.*, 1982). We transferred animals carefully during the feeding experiments and the animals did not present any signs of stress (rejection of food from the filtering appendages with the postabdomen, prostration or increase in swimming speed) during or after transfer. However, the animals were transferred from a medium containing unfiltered pond water to a feeding medium containing filtered pond water to which bacteria and algae were added, but which did not contain detritus and protozoans, both abundant in the pond (H.M. CAUCHIE, pers. obs.). Daphnids are indeed known to feed not only on bacteria and algae but also on detritus and protozoa (*e.g.* PETERSON *et al.*, 1978 ; PORTER *et al.*, 1983 ; JÜRGENS, 1994). Total food availability was therefore certainly lower in the feeding medium than in the pond and could have caused a variation in the feeding behaviour of the animals. Nevertheless, the persistence of a significant proportion of non-feeding animals during long experiments (> 15-30 minutes) suggests that, besides methodological biases, an actual inter-individual variation exists in the feeding activity of *D. magna*.

In *Daphnia*, the distance between the setules of the filter combs increases with body length (KORINEK *et al.*, 1986 ; LAMPERT & BRENDELBERG, 1996). As a consequence, small individuals can retain small particles such as bacteria more efficiently than large individuals, whereas large animals can handle large particles more easily than small animals. High values of the exponent *b* are therefore observed for *Daphnia* when large green algae and cyanobacteria are offered as food (HOLM *et al.*, 1983 ; BRENDELBERGER, 1985 ; STUCHLIK, 1991). By contrast, low *b* values are observed when bacteria are offered as food (PETERSON *et al.*, 1978 ; PORTER *et al.*, 1983). Except on January 29 for IR_{bact}, intermediate to low *b* values were observed as a consequence of the small size of the FLB (length = 1 µm ; width = 0.5 µm) and FLA (diameter = 5 µm). The high value of *b* observed for IR_{bact} in January probably reflects an adaptation of the filtering apparatus to low food during winter. Daphnids are indeed able to increase their filtration area and to reduce the open distance between the setules of the endites of their third and fourth thoracic limbs (KORINEK *et al.*, 1986). As a consequence, an increase of the value of *b* is observed under conditions of low food availability (LAMPERT & BRENDELBERGER, 1996).

Even when B_{bact} was significantly higher than B_{phyto}, *D. magna* predominantly fed on algae. This is in good agreement with the bulk of evidence presented earlier (PETERSON *et al.*, 1978; GELLER & MÜLLER, 1981) and reflects the low ability of this species to capture bacteria compared to algae (BRENDELBERGER, 1985). This difference in capture efficiency resulted in a lower impact of *D. magna* on the bacterial community than on the algal community. From January to June, B_{phyto} was maintained at a low level because the *D. magna* population consumed a high proportion of B_{phyto}. Despite this high grazing pressure, the green alga *Planktonsphaeria gelatinosa* was able to develop a high biomass. In early July, $B_{Daphnia}$ reached an uncommonly high value resulting in an overgrazing of the phytoplankton and a collapse of B_{phyto}. The high grazing pressure of *D. magna* is certainly a major cause of the weak phytoplankton development generally observed in waste stabilisation ponds (NAMÉCHE, 1998). Significant grazing pressure on bacterioplankton was only observed when $B_{Daphnia}$ reached high values. In June, the *D. magna* population ingested one fifth of B_{bact} daily and more than 100 % of the P_{bact} daily. However, Bbact did not decrease because of the input of allochtonous B_{bact} with sewage. The grazing pressure on P_{bact} was maximum in early July, resulting in a decrease in B_{bact}. Because, however, such high $B_{Daphnia}$ are only transient in waste stabilisation ponds, the grazing pressure of the *D. magna* population on the bacterioplankton dynamics must be considered, on the whole, as marginal.

The gross production efficiency (GPE) of *D. magna* on the combined biomass of bacterioplankton and phytoplankton ($P_{Daphnia}$ / (IR_{bact} + IR_{phyto})) reached 130, 465, 830 and 331 % in January, May, June and July, respectively. Such obviously too high values of GPE indicate, as far as $P_{Daphnia}$ was correctly estimated, that the total quantity of food ingested by *D. magna* was underestimated. This may indicate that the detritic particulate matter constitutes a significant part of the *D. magna* ration in waste stabilisation ponds. The next stage of our trophodynamic study of waste stabilisation ponds will therefore involve the labelling of detritus and the estimation of its ingestion rate by *D. magna*.

The extrapolation of daily ingestion rates from a few short-term experiments is questionable. The monitoring of diel variation of IR_{phyto} revealed, however, that a single measure of IR_{phyto} made at 19 :00 correctly reflected the mean IR_{phyto} over 24 hours. Diel patterns of IR_{phyto} in adults were similar to those observed in other *Daphnia* species (STARKWEATHER, 1975, 1983; HANEY & HALL, 1975), with the highest ingestion rates being generally observed during the night or at light-to-darkness or darkness-to-light transition. By contrast, the daytime maximum of IR_{phyto} observed in juveniles was quite uncommon. Visual predation by fish is often pointed out as a major cause of the occurrence of maximum feeding rates at night (STARKWEATHER, 1983). In the WSP of Differdange, this type of predation was non-existent and there was thus no disadvantage of feeding during the day. Under these conditions, the temporal separation of the maximum feeding activity of juveniles from that of adults can be hypothesised to be a niche shift leading to a reduction of intra-specific competition for food.

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THE KINEMATICS OF VOLUNTARY STEADY SWIMMING OF HATCHLING AND ADULT AXOLOTLS (*AMBYSTOMA MEXICANUM* SHAW, 1789)

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Abstract. Axolotls swim throughout post-hatching ontogeny. This coincides with an approximately twentyfold range in total body length (L), which may imply unfavourable differences in encountered flow regime (viscous *versus* inertial) during ontogeny. Using high-speed video (500 fields s^{-1}), we analysed the kinematics, mechanical efficiency, swimming speeds and flow regime of swimming hatchlings (approximately 0.01 m L, « stage 1 »), 2 week old animals (approximately 0.02 m L, « stage 2 ») and 20 week old animals (approximately 0.08 m L, « stage 3 »), and compared the data with similar data from adults (0.135-0.238 m L, « stage 4 »).

All stages swim by passing waves of lateral curvature down the body. The kinematics, described by the characteristics of this wave (speed, frequency, length, amplitude) are largely comparable in all four stages : within each stage, swimming speed is increased by increasing the wave frequency only. Mechanical swimming efficiency, estimated by means of Lighthill's elongated-body theory, is about 5 % lower in hatchlings than in adults.

The most striking result is that the observed, voluntary absolute swimming speeds from stage 1 to stage 4 are much more similar than would be expected given the twenty-fold L range. Possible explanations are ecological and/or hydrodynamical. Firstly, predator escape success increases as the swimming speed increases. Secondly, by adopting high speeds, axolotls increase Reynolds numbers, and thus avoid having to swim in the unfavourable viscous flow regime.

Key words : *Ambystoma mexicanum*, axolotl, swimming, ontogeny, flow regime.

INTRODUCTION

Axolotls, being neotenic salamanders, maintain a larval habitus throughout ontogeny (BRUNST, 1955). Total body length (L), however, ranges from about 0.01 m (post-hatching) to 0.25 m or more for adults. This paper deals with the effect of body size on locomotion and, more specifically, with the mechanical consequences of swimming at the full range of post-hatching body lengths.

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The thrust and drag (which are quantitatively equal in the case of steady swimming) during swimming arise from two different forces acting on the animal's body and in the water close to the body (WEBB, 1975): inertial forces and viscous forces. Their ratio (inertial/viscous forces) is the Reynolds number (Re). Re indicates the governing flow regime and is of crucial importance to an understanding of the dynamic environment in which the animal moves. It is calculated as follows:

$$Re = \frac{L.U}{\mu/\rho} \quad (1)$$

with L being a relevant length (mostly, and in this paper, total body length), U the swimming speed, π the viscosity and ρ the density of the medium. The ratio μ/ρ is the kinematic viscosity and equals $1.002\ 10^{-6}\ m^2s^{-1}$ for fresh water at 20°C (calculated from μ and ρ data in WEAST, 1974). Changes in the kinematic viscosity result from changes in water salinity (*e.g.* typically 3.5% lower in sea water than in fresh water) and more importantly, from changes in temperature (FUIMAN & BATTY, 1997). However, for most biological studies, Re is estimated from L and U only, keeping kinematic viscosity constant. At low Re (< 1), viscous forces dominate and inertial forces can be neglected (this is the case for small and slow animals, e.g. swimming bacteria, sperm cells). At high Re (e.g. swimming fish, Re typically around 10^4-10^5), inertial forces dominate, and viscous forces are only quantitatively important in the animal's boundary layer (WEBB, 1975). Briefly, low Re indicates a « viscous » flow regime, high Re an « inertial » flow regime, with the threshold around a Reynolds number of 200 (WEIHS, 1980; WEBB & WEIHS, 1986). Theoretical (WEIHS, 1980) and experimental (VYMEN, 1974) studies indicate that swimming in the two flow regimes is accompanied not only by considerable differences in swimming style, but also in body morphology (*e.g.*, see MÜLLER & VIDELER, 1996), because constructional demands also change with the flow regime (cfr. OSSE, 1990, and references therein). Therefore, if larvae could swim in an inertial flow regime, they would be subject to similar physical constraints as the adults (WEBB & WEIHS, 1986), and design changes (which likely require a lot of energy) would be unnecessary.

Throughout their ontogeny, axolotls are likely to encounter a wide range of Reynolds numbers, because their L varies approximately 20-fold. D'AOÛT & AERTS (1997) found that adult axolotls voluntarily swim at approximately 1 L/s. If this remains true for larvae of 0.01-0.015 m, Re would range from 80 to 200, and axolotls would have to switch from viscous to inertial swimming very soon after hatching, when energy allocation is crucial and predation pressure high.

Many fish larvae hatch in the viscous flow regime and reach the inertial flow regime around first feeding (MÜLLER & VIDELER, 1996). MÜLLER & VIDELER (1996) found that larvae from all analysed species do not, however, adapt to their flow regime, but grow disproportionally in length to escape from the viscous regime into the « safe harbour » (SHINE, 1978) of inertial swimming as fast as possible.

Apart from increasing body length, there is another way to reach the inertial flow regime: increasing swimming speed. D'AOÛT & AERTS (1997) found that, within the limited observed size range of adults (0.135-0.238 m), small individuals swim relatively faster than big individuals. If this trend holds true for larvae, they may indeed escape from the viscous flow regime by swimming faster. This would, apart from the hydrodynamic

advantages, also have an ecological advantage, which is absent in the case of increased length growth: the higher the animal's speed, the higher its escape success will be (GARLAND, 1994). High relative locomotor speed (in body lengths per second) would further enhance escape probability of larvae in a stage where it is especially prone to predation (VAN DAMME & VAN DOOREN, 1999).

This paper focuses on swimming kinematics and speeds, and investigates the following points: (1) does the swimming style of larval and adult axolotls differ, (2) do larvae swim relatively faster than adults and (3) do they succeed in escaping the viscous flow regime?

MATERIALS AND METHODS

Experimental animals

Prior to experiments, Mexican axolotls, *Ambystoma mexicanum* Shaw, 1789 (Amphibia: Caudata: Ambystomatidae), were kept in an aerated freshwater tank at room temperature. After spawning of one individual, eggs were transferred to another tank and regularly checked. Dead or fungus-infected eggs were immediately removed. About 80% of the eggs hatched.

First-day hatchlings, defined here as « stage 1 » individuals, were randomly selected and used for video recordings. After 2 and 20 weeks, a random selection of individuals was made (defined here as « stage 2 » and « stage 3 » individuals, respectively) and these were again used for video recordings (see also Table 1). « Stage 4 » animals are adults from 0.135-0.238 m total body length, previously analysed by D'AOÛT & AERTS (1997). In this way, early post-hatching stages (stages 1 and 2, see aims in the Introduction) can be compared with adults (stage 4) and with specimens of smaller body size, but with an adult habitus (stage 3).

Recording of the swimming sequences

During recording sessions, animals were placed in an appropriate set-up consisting of a petri dish (diameter 0.19 m, depth 0.03 m; stages 1 and 2) or of two open aquaria, interconnected by a closed glass tunnel (height = 0.1 m; width = 0.15 m; length = 1 m; stages 3 and 4). Both set-ups included reference grids. Animals were video-taped in dorsal view at 500 fields s^{-1} using a NAC-1000 high-speed video system equipped with a Fujinon 12.5-75 mm zoom lens. In the cases of the stage 1 or 2 animals, B&H (Schneider-Kneuznach) close-up diopters were fitted to the lens to increase magnification. Animals were sometimes stimulated to swim by gently touching them with a fine probe, but they were free to select their swimming speed (« preferred » or « voluntary » speed) as they were not chased, or made to swim against an externally induced flow.

Water temperature was 20 ± 2 °C during both the acclimatisation and the experiments. Kinematic viscosity differences in this range are about ± 5% and are neglected; for Re calculations the kinematic viscosity value of 1.002 10-6 m^2s^{-1} was used (see Introduction).

Analysis of the high-speed video sequences

For analysis, only rectilinear swimming trials consisting of at least three complete cycles were used. Three representative swimming bouts from each stage were thus selected for a complete analysis and compared with 28 « stage 4 » sequences (D'AOÛT & AERTS, 1997).

All animals swam at the bottom in all analysed sequences, representing the normal behaviour in aquaria (pers. obs.) and most likely also in nature, where axolotls live at the bottom of lakes (SMITH & SMITH, 1971). For each frame, the dorsal midline was digitised using a NAC-1000 XY coordinator connected to a PC. For details about further data processing, we refer to D'AOÛT & AERTS (1997). The dorsal midlines of the animals are described by 20 segments of 0.05 L, delimited by 21 points (the « body points », BP), point 1 being the snout tip and point 21 being the tail tip.

We studied the following parameters: (1) swimming speed U (m/s), the forward speed of the animal calculated as the displacement of the snout tip divided by the corresponding time period; (2) wave speed V (m/s), the speed of the wave of propulsion, relative to the animal's body (thus, not to the external frame of reference); (3) wave frequency f (Hz), the reciprocal value of the wave period, *i.e.* the duration of one complete cycle; (4) stride length, the distance travelled in one complete cycle (*i.e.* with one tailbeat; VIDELER, 1993), (5) wave length λ, the length of the propulsive wave on the body, and (6) the amplitudes A (m) of the 21 body points (BP). The maximal amplitude A (= maximal lateral deflection from the mid-position) was determined for every body point; the graph representing these amplitudes as a function of position along the body is further referred to as the amplitude profile. To allow comparison of U, A and λ different-sized animals, these parameters were scaled to the total body length yielding specific speed U_{sp}, specific amplitude A_{sp} and specific wave length λ_{sp}.

Because of the range of animal sizes (L) and swimming speeds, fluid dynamical regimes may differ drastically. To account for differences in flow regime, Reynolds numbers (Re) were calculated (see Introduction). In addition, dimensionless frequency was calulated as follows:

$$f_{dl} = \frac{f.L^2}{\mu/\rho} \qquad (2)$$

In this way, dimensionless frequency (f_{dl}) times dimensionless stride length (the distance travelled with exactly one complete cycle, normalised for L) yields Re.

Based on the kinematical data, the (hydro)mechanical efficiency of swimming was calculated, i.e. the ratio of propulsive power to the mechanical power produced by the swimming animal. LIGHTHILL's (1960) elongated body theory is widely used and has, for tadpoles of *Rana catesbeiana*, proven to give accurate estimations in quantitative terms (*e.g.*, LIU *et al.*, 1996). The elongated-body theory has a number of inherent limitations (for details, see D'AOÛT & AERTS, 1997) but, since the body shape of larval and adult axolotls does not change drastically, this theory can be used to compare mechanical swimming efficiencies. Also propeller efficiency (or slip factor), defined as U/V, was calculated. Since V is always greater than U during steady swimming, the propeller efficiency can range from zero to values close to the theoretical maximum of one.

In order to have a wider range of swimming speeds and frequencies for stage 1 larvae, 30 rectilinear, steady sequences of 3-9 cycles were randomly selected (in addition to the three analysed in detail, see above), and mean U and mean f were calculated. These additional data extended the data set on Reynolds numbers of hatchling axolotls.

TABLE 1

Overview of the kinematic variables and calculated efficiencies for three sequences of stages 1-3. Data can be compared with literature data on stage 4 (D'AOÛT & AERTS, 1997). For details, we refer to the Material and Methods section. L, total body length (m); U, swimming speed (m/s); U_{sp}, specific swimming speed (L/s); V, wave speed (m/s); f, swimming frequency (Hz); wave length λ (m); A_{sp}, specific tail tip amplitude (L); U/V(dimensionless), propeller efficiency (slip factor; dimensionless); EBT, efficiency using LIGHTHILL's (1960) elongated-body theory (dimensionless); strl, stride length (m); $strl_{sp}$, specific stride length (bodylengths); λ_{sp}, specific wave length (bodylengths).

seq	*stage*	*L*	*U*	U_{sp}	*V*	*f*	λ	A_{sp}	*U/V*	*EBT*	*strl*	$strl_{sp}$	λ_{sp}
d54	1	0.010	0.106	10.2	0.190	18.7	0.010	0.368	0.563	0.782	0.006	0.549	0.976
d58	1	0.010	0.104	10.0	0.211	19.3	0.011	0.289	0.500	0.750	0.005	0.520	1.038
d53	1	0.011	0.115	10.8	0.242	23.1	0.010	0.199	0.494	0.747	0.005	0.470	0.937
	mean	0.010	0.108	10.3	0.214	20.37	0.010	0.285	0.519	0.760	0.006	0.513	0.984
	s.e.	0.0006	0.0059	0.416	0.026	2.39	0.0006	0.085	0.038	0.019	0.00058	0.040	0.0509
d55	2	0.018	0.078	4.29	0.156	9.13	0.017	0.276	0.504	0.752	0.009	0.470	0.956
d56	2	0.018	0.097	5.39	0.217	13.0	0.016	0.156	0.451	0.726	0.007	0.414	0.874
d57	2	0.022	0.097	4.33	0.198	10.9	0.019	0.183	0.499	0.750	0.009	0.399	0.855
	mean	0.0193	0.091	4.67	0.190	11.0	0.017	0.205	0.485	0.743	0.008	0.428	0.895
	s.e.	0.0023	0.011	0.62	0.031	1.94	0.0015	0.063	0.029	0.014	0.001	0.037	0.054
d50	3	0.066	0.271	4.10	0.553	11.7	0.049	0.186	0.492	0.746	0.023	0.349	0.748
d52	3	0.085	0.151	1.78	0.306	5.75	0.047	0.082	0.496	0.748	0.026	0.309	0.548
d51	3	0.088	0.132	1.50	0.248	4.50	0.068	0.105	0.534	0.767	0.029	0.333	0.767
	mean	0.080	0.185	2.46	0.369	7.32	0.055	0.124	0.507	0.754	0.026	0.330	0.688
	s.e.	0.012	0.075	1.43	0.162	3.85	0.012	0.055	0.023	0.012	0.003	0.020	0.121

RESULTS

According to BREDER's (1926) definition, axolotls of all stages have an anguilliform swimming mode, characterised by a wave of curvature travelling down the body faster than the animal's swimming speed (Fig. 1).

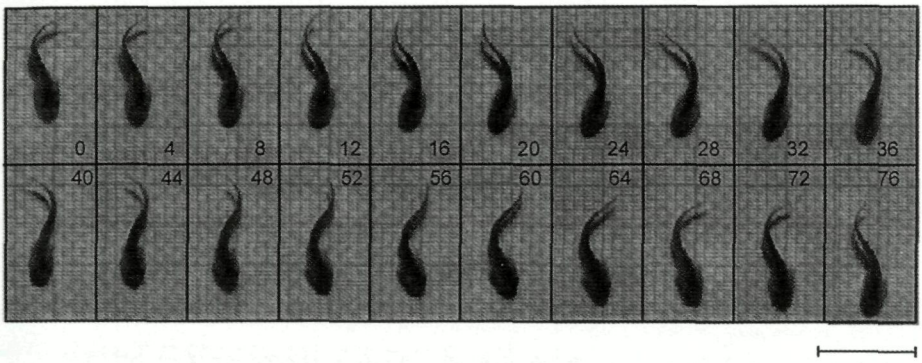

Fig. 1. – Dorsal-view video images illustrating approximately one swimming cycle of a stage 1 individual of *Ambystoma mexicanum*. The time interval between successive frames is 4 ms, the scale bar is 0.01 m. The swimming style of this hatchling animal is qualitatively similar to adults (see fig. 2 in D'AOÛT & AERTS, 1997).

The amplitude of the wave of propulsion is not constant, but changes as a function of the location along the body: the head region oscillates least, although still considerably (*e.g.* approximately 10% L in stage 1), and the amplitude increases to reach a maximum at the tail tip (Fig. 2). Maximal specific tail tip amplitudes decrease during ontogeny and are approximately 0.285 L for stage 1, 0.205 L for stage 2, 0.124 L for stage 3 (Table 1) and 0.097 L for

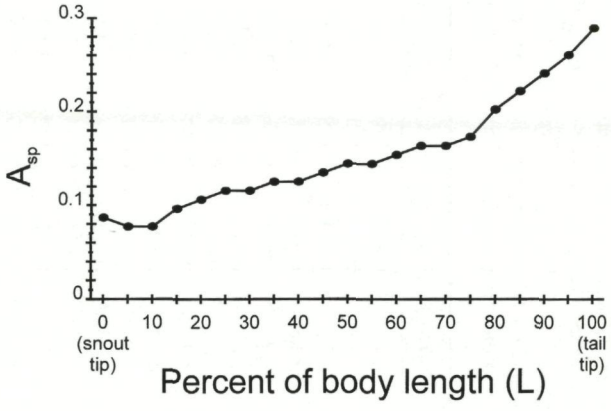

Fig. 2. – Specific amplitude as a function of position along the body, for a representative sequence of a stage 1 individual. Amplitude is smallest (but not zero) in the head region and increases towards the tail, where amplitude is maximal.

stage 4 (D'AOÛT & AERTS, 1997). Specific stride length is clearly higher in stages 1 and 2 (0.513 and 0.428, respectively), than in stages 3 and 4 (0.330 and 0.345, respectively).

In stages 1 to 3, the frequency of the propulsive wave (f) increases linearly with U_{sp} (Fig. 3), as it does in the size range represented by stage 4 (D'AOÛT & AERTS, 1997). It is, however, important to focus on dynamically similar conditions when kinematic variables between different-sized animals at different speeds are compared. Therefore, we plotted the dimensionless frequency as a function of Reynolds number, for all available data of the four stages (after log-log transformation, see Fig. 4). Clearly, f_{dl} relates directly to Re, and the previously observed linear relationship between frequency and speed remains valid when the fluid dynamical regime is taken into account. Note that early stages have a low Re, but high U_{sp} (compare Figs 2 and 3).

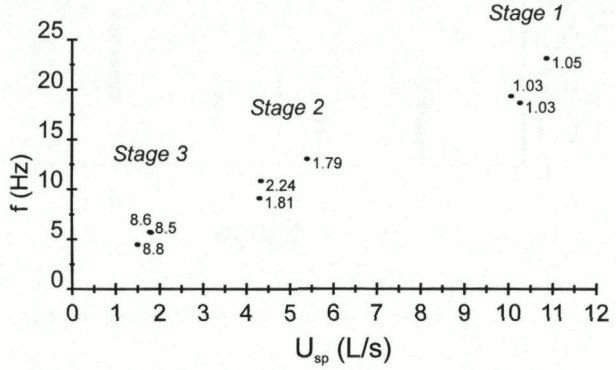

Fig. 3. – Swimming frequency as a function of specific swimming speed for 3 sequences each of stage 1-3 axolotls. L (cm) is indicated at the data point. Note that smaller individuals have higher specific swimming speeds. As in adult axolotls (stage 4), specific swimming speed is directly related to frequency.

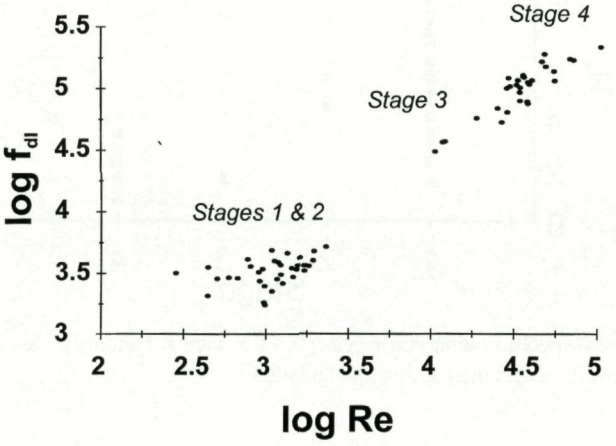

Fig. 4. – Dimensionless frequency as a function of Reynolds number (log-log transformed) for stage 1-4 individuals (including the 30 sequences of stage 1 that were not analysed in more detail). Animals with a 20-fold range in body length have a similar relationship between dimensionless values (Re resp. f_{dl}).

Absolute voluntary swimming speed increases from stage 1 to stage 4 (Fig. 5 and Table 1), but it should be noted that the ontogenetic variation in swimming speeds is much smaller than the differences in body length between the four stages. Fig. 6 and Table 1 show that U_{sp} (which accounts for L differences) reaches much higher values for small larvae (stages 1, 2) than for large larvae and adults (stages 3 and 4, respectively).

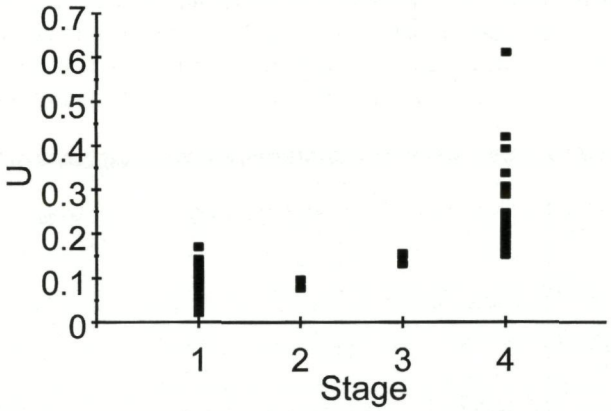

Fig. 5. – Observed swimming speeds (U) for stage 1-4 axolotls. Larval axolotls clearly swim more slowly than adults, but the difference is less than the 20-fold difference in L.

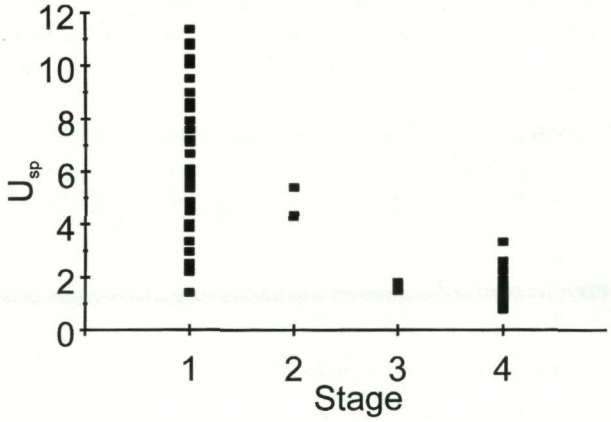

Fig. 6. – Observed specific swimming speeds (U_{sp}) for stage 1-4 axolotls. Larvae can swim at much higher specific swimming speeds than adults.

Fig. 7 depicts the observed log-transferred Reynolds numbers for the different stages. Reynolds numbers range from 2.48 10^2 to 1.98 10^3 for stage 1 (N = 36), and from 1.59 10^4 to 8.63 10^4 for adults (N = 28). Stages 2 and 3 have intermediate Reynolds numbers (N = 3, for both stages).

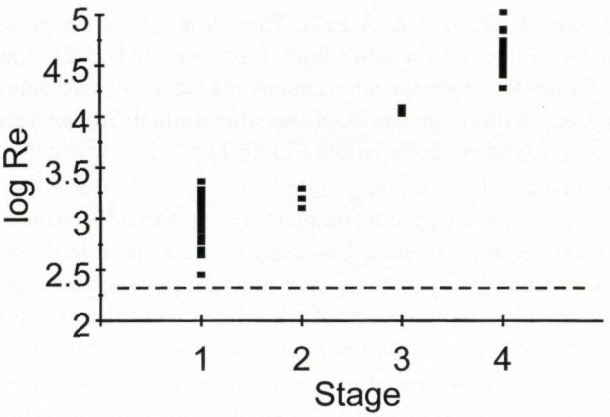

Fig. 7. – Observed Reynolds numbers (Re) for stage 1-4 axolotls. Note that, even for the slowest observed sequences of stage 1 animals, Re is higher than 200. As a consequence, axolotls swim in the inertial flow regime throughout ontogeny.

Mechanical swimming efficiencies, estimated by LIGHTHILL's (1960) elongated-body theory, differ little between stages 1 to 3, and are, with mean values of 0.743 to 0.760, slightly (respectively, 4.5 % to 6.7 %) lower than the mean efficiency of stage 4 animals.

DISCUSSION

In this paper, we compared the swimming style, efficiency, speeds and flow regime of larval versus adult axolotls. Qualitative comparison (Figure 1) and analysis of several kinematic variables show that the swimming style of axolotls remains remarkably similar throughout ontogeny and can be compared with literature data of other anguilliform swimmers (*e.g.* adult axolotls, D'AOÛT & AERTS, 1997; *Siren intermedia*, GILLIS, 1997; *Anguilla rostrata*, GILLIS, 1998). Axolotls of all post-hatching ontogenetic stages have a largely comparable swimming style, characterised by the presence of a body wave, travelling from head to tail with increasing amplitude. The specific swimming speed correlates linearly with wave frequency, as in most fishes (VIDELER, 1993). A striking difference, however, is the amplitude of this wave, which is larger for larvae than for adults (see Table 1). This causes an important head yaw due to angular recoil forces (BATTY, 1981), also found in anuran tadpoles (WASSERSUG, 1989).

Using LIGHTHILL's (1960) elongated-body theory, we have shown that larval stages (1-3) swim slightly less efficient (by approximately 5 %) than do adults, and about 10 % less efficient than do anuran tadpoles (WASSERSUG & HOFF, 1985). A puzzling finding, however, is that relative stride length is clearly longer for larvae than for adults (see Table 1). While adults need about three cycles to travel their own body length (specific stride length 0.345, D'AOÛT & AERTS, 1997), stage 1 larvae need only about two (specific stride length 0.513, Table 1). Since the slip factor (or propeller efficiency), and elongated-body efficiency of all stages are similar, this finding has to be the result of differences in specific wave length λ_{sp}. Indeed, the specific wavelength decreases from about 0.98 at stage 1 (this

paper) to 0.60 at stage 4 (D'AOÛT & AERTS). Thus, less waves are present on the larval body (approximately 1) than on the adult body (approximately 1.7) at any instant of the swimming cycle. Evidently, since the slip remains the same, a wave that travels down the full length of the body within one cycle, propels the animal further than a similar wave that travels over only 0.6 of the body length (Table 1).

Apart from similarities in swimming style, small axolotls were observed to swim at much higher specific swimming speeds (up to 11.4 L/s) than adults (up to 3.3 L/s). Since, at all stages, a linear relationship was observed between preferred specific swimming speed and frequency, frequencies of larvae are much higher, accordingly. This is in contrast to fish larvae, the swimming speeds of which increase linearly with size (BLAXTER, 1986), so that specific swimming speed remains approximately constant.

The relatively high swimming speed of larvae has important consequences in relation to the flow regime the animal encounters, since Reynolds number increases linearly with swimming speed (Equation 1). Like fish larvae, axolotl larvae can, at first glance, be assumed to swim in the viscous flow regime ($Re < 200$), which is most likely not beneficial (see Introduction). WEBB & WEIHS (1986) argued that most biological functions in fish larvae occur at higher Re (> 200), because then, constraints towards hydrodynamical activities (feeding, breathing, swimming) would remain similar throughout ontogeny. Fish larvae acquire relatively high Re by increased length growth (MÜLLER & VIDELER, 1996; see also WEBB & WEIHS, 1986; FUIMAN, 1983; OSSE, 1990), or by kick-and-glide swimming (WEIHS, 1980). Axolotl larvae acquire $Re > 200$ from the moment of hatching because of their high swimming speed. Thus, both fish and axolotl larvae succeed in escaping from the viscous flow regime, but by different means. Axolotls swim in the inertial flow regime from the instant of hatching, so they do not have to switch from viscous to inertial swimming during ontogeny. Hence, physical constraints towards the swimming mode would remain similar, and similar forms would correlate with similar (swimming) behaviour (WEBB & WEIHS, 1986).

Escaping the viscous flow regime by speed increase has an advantage, of an ecological nature, that length increase does not offer: increased escape success. Larvae are highly predated, and mortalities are high (*e.g.* see PEPIN, 1991, for fish larvae). Obviously, a higher absolute speed will increase the chance of escape from a predator (GARLAND, 1994). Since axolotl larvae are able to swim at least as fast as the preferred speed of adults (Fig. 5, and D'AOÛT & AERTS, 1997), they would likely have good escape chances. Recent evidence (VAN DAMMEN & VAN DOOREN, 1999) argues that specific speed is an even better predictor of escape success than absolute speed. In this case, escape succes of axolotl larvae would increase even more, since specific speeds are very high (Fig. 6).

To conclude, the relatively high swimming speed acquired by axolotl larvae is both hydrodynamically and ecologically beneficial, i.e. to escape the viscous flow regime, and to increase the chances of successful escape from predators.

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SMALL OUTDOOR INSECTARIES AS A TOOL FOR LIFETIME STUDIES ON DAMSELFLIES

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Abstract. Damselflies are suitable subjects for examination of a variety of biological questions, but most research has been carried out in the field. Several questions are hard to test because of the uncontrolled conditions inherent in field studies. This can be circumvented by studying populations in semi-natural outdoor insectaries. We assessed the suitability of such insectaries by comparing the survival and adult behaviour of *Ischnura elegans* (Vander Linden) in insectaries and in the field. Our results showed that damselflies behaved differently under experimental conditions. Nevertheless, outdoor insectaries can be regarded as a valuable tool to elucidate questions concerning life history traits since they offer the possibility to eleminate predation, emi- and immigration and hidden life.

Key words : Odonata, damselflies, insectaries, experimental setup.

INTRODUCTION

In the last two decades, it has become clear that damselflies are suitable model organisms to study a diversity of behavioural and ecological topics (*e.g.* FINCKE *et al.*, 1997). One of their advantages over vertebrates is their variety in reproductive strategies and the possibility they provide for the collection of lifetime data over a short timespan. Housing larval damselflies has been succesfully conducted by several researchers (see JOHNSON, 1991). In contrast most research on imagines has been performed in the field.

Several problems and limitations apply to conventional field studies. First, in the field up to half of a study population may disappear after marking and releasing (*e.g.* FINCKE, 1988 ; CORDERO, 1997). As a result, conclusions may not be based on a random sample of the population. A second problem is the occurrence of unknown successful dispersal to other ponds (MICHIELS & DHONDT, 1991a). Third, examining several populations simultaneously is difficult because of habitat complexity ; and problems (*e.g.* due to weather conditions) may arise when life history is studied in different periods or years (*e.g.* BANKS & THOMPSON, 1985). Fourth, observed frequencies of copulation and oviposition in the field are often an underestimation of the reality (VAN NOORDWIJK, 1978 ; CORDERO *et al.*, 1997). Finally, it is extremely difficult to control and vary the variables sex ratio, age distribution and density (but see SIVA-JOTHY, 1995) which are important for testing hypotheses (*e.g.*

MICHIELS & DHONDT, 1991a; FINCKE, 1994). Some of these problems have been avoided by observing populations in large outdoor cages (MICHIELS & DHONDT, 1991a; DUNHAM , 1994). However, testing more than two populations at the same time remains difficult. Here, we present an inexpensive method for housing damselflies in small outdoor insectaries, enabling the simultaneous study of several populations with chosen population ecological parameters.

A major possible criticism of this type of experiment is the possibility that unnatural behaviour may occur. Therefore we examined the differences between small insectaries and the field. We observed *Ischnura elegans* (Vander Linden) in insectaries and, simultaneously in the field, and compared our findings with literature data. The insectary experiments described herein are part of a broader study on sexual strategies in that species.

MATERIAL AND METHODS

Four dining shelters (Partytent, marketed by Bricomarkt B.V.B.A.; size: 3x3x2.5 m), were covered with bee-netting (mesh size: 2x8 mm, marketed by B.V.B.A. Ranschaert) (Fig. 1). In contrast with former studies (FINCKE, 1987; FORBES & LEUNG, 1995) the netting allowed passage of small insects and avoids the need to supply food. To allow enough light, we replaced triangular parts of the plastic roof with the bee-netting. Insectaries were secured against heavy wind by stretching tent-ropes and by digging in the undersides of the bee-netting. The total price of an insectary was approximately 2000 BEF (65 USD) at the time of the study.

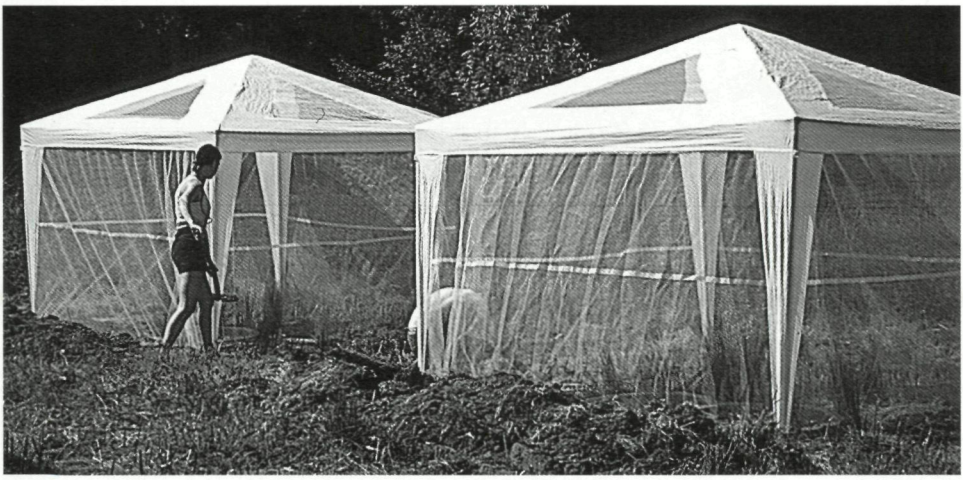

Fig. 1. – Insectary.

Each insectary contained two small pools (plastic shells, Boubsy N.V.) with a perimeter of approximately four meter each, to serve as oviposition sites and supply of water on hot days. Around the pools we planted *Juncus effusus* as roosting sides (GEIJSKES & VAN TOL , 1983) The remaining vegetation consisted mostly of *Arrhenaterum elatius, Artemia*

vulgaris, Cirsium arvense, Dipsaccus fullonum, Plantago major and *Urtica dioica* with heights between 20 and 80 cm.

The insectaries were placed in a field called « De Biotuin », property of the University of Antwerp. Experiments were performed during the summer of 1998. To avoid predation, the insectaries were cleared of spider webs and frogs. The animals used for the experiments were collected approximately 10 km away from our study site (« De Walenhoek », Niel). Captured animals were transported in cages (size : 30x30x30 cm). All animals were marked with an individual number in black ink on the left forewing (Staedtler Pancolor, 0.3 mm pen). Observations were made with binoculars from outside the insectaries (Opticron, 10 x 42).

In a first experiment we examined whether the food availability in the insectaries was sufficient to exlude mortality due to starvation and cannibalism (ROLFF & KRÖGER, 1997). We released 63 freshly emerged tenerals (34 males and 29 females) to one insectary and counted the number of survivors after maturation (five days). Survival of adults was analysed on a new group of animals in the second experiment. The combination of both experiments should produce an idea of the adequacy of the food available during the study.

In the second experiment we examined survival and adult behaviour including daily activity pattern and timing, duration and frequency of copulation and oviposition of adult males and females in four insectaries. We performed field studies on *I. elegans* during the summers of 1996 and 1997 (unpublished results), and compared both findings. We daily recorded interactions (tandem formation, copulation, oviposition) (from 8h00 until 18h00). Individuals in one insectary were observed during 15 min, whereafter we focused on the next insectary. Observations on insectaries were made in a daily random sequence. On three sunny days we observed damselfly activity between 7h00 and 8h00 at dawn. Hours are given in Mid European Summer Time.

Because of small sample sizes, we compared the daily activiy pattern between insectaries and the field by grouping frequencies of copulations and ovipositions per two hours. The analysis was performed using a Fisher Exact test. We analysed the effect of insectary and sex on the survival of adult *I. elegans* with a Cox proportional Hazard model (KLEIN & MOESCHBUERGER, 1997) using proc PHREG in SAS 6.12. Ties were handled with the EXACT procedure. Means are reported with one standard deviation.

RESULTS

Survival

In the first experiment we found that mortality of tenerals was low, and similar for males and females (respectively three and four damselflies died ; Fisher Exact test, df = 1, p = 0.69). We have no comparative field data. Survival of adult animals did not differ between insectaries and sexes (log likelihood test, (χ_2= 3.24, df = 4, p = 0.52 ; Fig. 2). Average adult lifespan was 17.67 ± 7.45 days. In the field study we observed an average lifespan of only 3.2 ± 0.5 days (unpublished results), without a difference in survival between males and females. We did not observe cannibalism in insectaries or field.

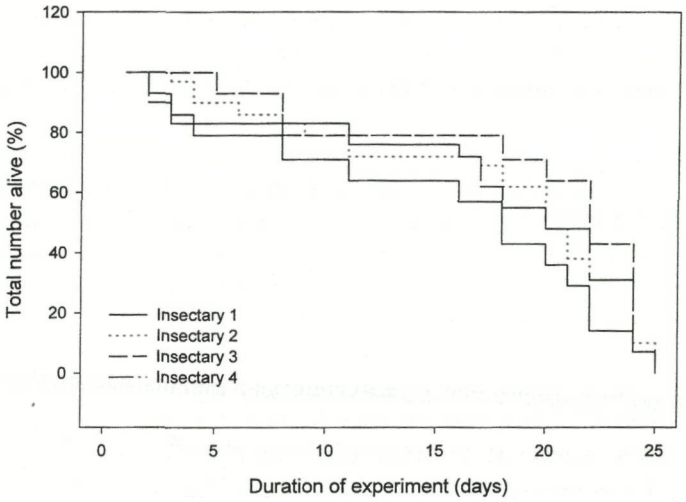

Fig. 2. – Survivorship curves of adult *Ischnura elegans* in outdoor insectaries.

Adult behaviour

At night animals roosted on the vegetation of the insectary. During the day, sunshine resulted in males making long flights, inspecting vegetation looking for females. Unwilling females prevented males from mating by spreading their wings and curling their abdomen. At the end of a copulation, pairs perched in tandem for periods ranging from 1 min up to an hour. Long post-copula tandems were mostly observed close to oviposition sites. After post-copula tandem, animals separated and sometimes continued to perch alone at the same spot. Females oviposited without male mate guarding. They were aggressive towards intruding males or females, attacking, and sometimes even clashing with them, thereby temporarily reserving an oviposition site for their exclusive use. Field observations were similar, except that at night, damselflies were found roosting only on emergent aquatic or littoral vegetation.

Daily activity pattern

On sunny mornings, the first individuals began to flutter and to fly to the tops of the vegetation at 7h15 while the insectaries were shadowed and temperatures just above 15° C. Males grasped perching females and formed tandem linkages when sunshine entered the insectary. These tandems usually lasted a few minutes on a sunny morning but up to two hours when it was cloudy and/or rainy. The first copulations were observed at 8h05 and became numerous by 10h00. Most copulations were initiated before 11h00, except on cloudy days. When weather was bad, most activities were retarded by one to two hours except oviposition which, in contrast, occurred up to two hours earlier. Only in extremely bad conditions (harsh rain and strong wind) was no activity observed. Most oviposition took place from 16h00 onwards. Exceptionally, oviposition was observed earlier in the afternoon and only twice before 9h00. Under field conditions we observed a similar daily

distribution of ovipositions (Fisher Exact test, df = 4, p = 1.0) (fig. 3). We found, however, a shift in the daily distribution for copulations (Fisher Exact test, df = 4, p = 0.034) whereby copulations in insectaries were initiated and terminated earlier (Fig. 3).

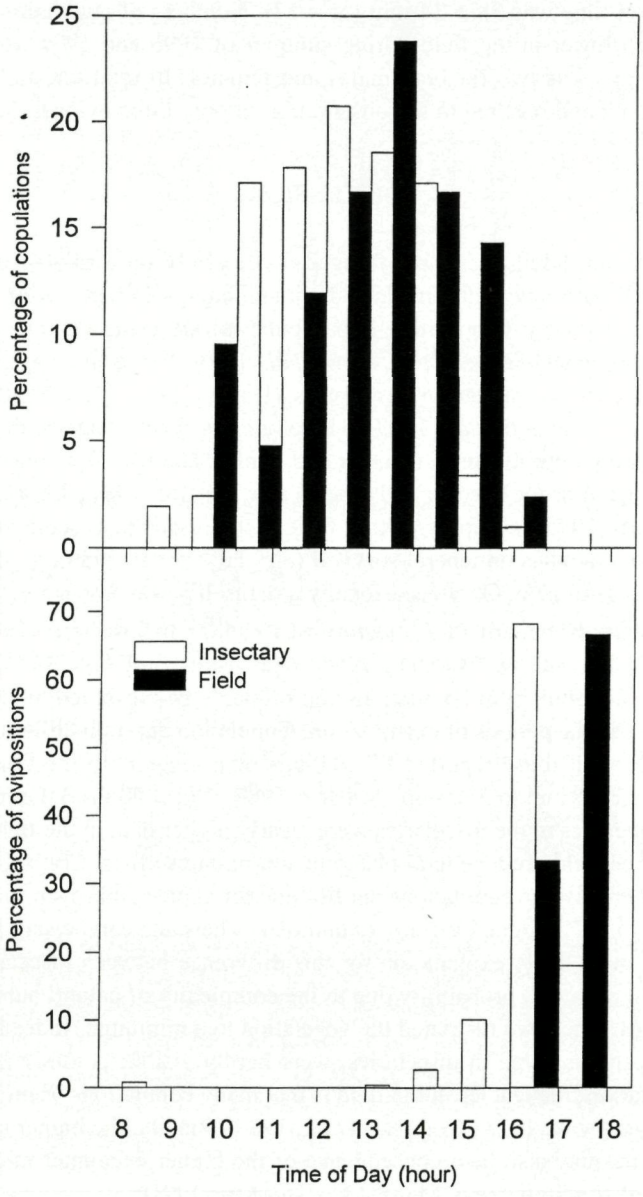

Fig. 3. – Daily distribution of copulations (upper panel) and ovipositions (lower panel) in adult *I. elegans* in insectaries and field.

Duration and frequencies of copulation and oviposition

Observed lifetime copulation frequency in the insectaries ranged from zero to twenty in males, and from zero to thirteen in females. Mean copulation time was 292 ± 19 min (N = 11). The maximum number of ovipositions observed per female was fifteen, while mean oviposition time was 38 ± 24 min (N = 17). Numbers of copulations and ovipositions were much lower in the field during summer of 1996 and 1997. Here, maximum number of matings was two for both males and females. In total, the field studies took more than a month and resulted in the observation of only three ovipositing females.

DISCUSSION

Both teneral and adult *I. elegans* foraged successfully on available prey. Mortality rates for tenerals were low and animals had enough energy to mate, while females were seen ovipositing regularly. Ovipositing in particular is very energy demanding due to the continuing development of eggs (*e.g.* ANHOLT *et al.*, 1991). Moreover, the survival of adult damselflies is generally higher in insectaries (LORD, 1961; VAN NOORDWIJK, 1978; HINNEKINT, 1987; CORDERO *et al.*, 1997). Therefore, our results suggest that stress due to food limitation was improbable in tenerals and adults. The use of bee-netting offers the advantage that we did not have to add insects (see FINCKE, 1987; LANGENBACH, 1993; FORBES & LEUNG, 1995) or supply rotting fruit to the insectaries (see FINCKE, 1987). In contrast with most studies on teneral survival (*e.g.* THOMPSON, 1989), predation pressure could be kept close to zero. Only occasionally a damselfly was trapped in a spiderweb.

The daily activity pattern of *I. elegans* was similar in insectaries and in the field (MILLER, 1987; HILFERT & RÜPPELL, 1997; see results and Fig. 3). The differences observed in the distribution of copulations can probably be attributed to varying weather conditions between the periods of examination. Copulation duration observed in this study was comparable with those reported for matings of *I. elegans* in the field (KRIEGER & KRIEGER-LOIBL, 1958: up to 340 min; MILLER, 1987: 324 ± 90 min). On the other hand, copulation frequencies in the insectaries were clearly higher than in the field. Low mating frequencies in the field are a general phenomenon in damselflies. CORDERO *et al.* (1997) found a maximum of three copulations per lifetime for a male and seven for a female, and PARR & PALMER (1971) found even lower numbers. The same can be said for oviposition frequency. The most likely explanation for this difference between insectary and field is the difference in detection probability due to the complexity of natural habitats compared to our insectaries, where we restricted the vegetation to a minimum. Indeed many copulations and ovipositions, even in insectaries, were hardly visible. Another aspect of lower observed copulation frequencies in the field is that many copulations occur away from the water (VAN NOORDWIJK, 1978; CORDERO *et al.*, 1997). Finally, the higher observed copulation frequencies may also be a consequence of the higher encounter rates between the sexes in encaged conditions (*e.g.* MICHIELS & DHONDT, 1991b).

The higher observed mating and oviposition frequency offers a great advantage. It increases the possibility to find differences between individuals or groups of individuals.

Moreover, the observation of all copulations and ovipositions is required if one wants to study a fitness component like lifetime reproductive success.

An additional advantage of working with insectaries is the possibility to eliminate or introduce mortality due to predation. In the field, predation often strongly reduces the variance in phenotypic characters, which makes it difficult to detect any sexual selection upon them (WADE & KALISZ, 1989). While one solution to circumvent this is to artificially increase the variance in the population for a character upon which both survival and sexual selection might act (*e.g.* Anholt 1991), another is to retain the variance in the population and differ the predation pressure. The latter can be achieved in insectaries where the researcher can examine a range of predator frequencies in different insectaries. Moreover, in the described small insectaries, several populations can be monitored simultaneously while controlling factors such as sex ratio and population density to test specific hypotheses regarding their effects on fitness. Therefore, we conclude that outdoor insectaries are a powerful tool to study questions concerning life time characteristics of damselflies.

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