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Editor :

Prof. Dr. E. SCHOCKAERT
Department SBG
Limburgs Universitair Centrum
B-3590 Diepenbeek (Belgium)

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

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EDITORIAL

The organisation in November 1990 of a *First Belgian Congress of Zoology* at Antwerp turned out to be a very successful initiative of the Royal Belgian Zoological Society (Koninklijke Belgische Vereniging voor Dierkunde/Société Royale belge de Zoologie), and was followed by subsequent congresses at Diepenbeek in 1991 and at Liège in 1993. During the Liège congress, fruitful discussions with a visiting delegation of the Dutch Zoological Society and contacts with the « Association des Biologistes Luxembourgeois » later on, resulted in the plan to organise a zoological congress at the Benelux level.

On 4 and 5 November 1994, this *First Benelux Congress of Zoology* took place at Leuven, hosted by the Zoological Institute of the Catholic University of Leuven. During a busy two-day programme covering all aspects of zoology, 66 lectures and 98 posters presentations were scheduled,

The enthusiastic attendance of 208 participants from the three Benelux countries resulted in the immediate offer from the Dutch colleagues to organise the *Second Benelux Congress of Zoology* to be held at Leiden in November 1995.

As usual, many of the presentations came from young « newly hatched » researchers presenting their very first results. According to the tradition in place since the 1st Belgian Congress of Zoology one of them was given an awarded for the best oral and one for the best poster presentation. We take here the opportunity to mention all those who have received these awards in last five years :

Best oral presentation :

— 1990, 1 BCZ, Antwerp : Patrick Valckenberg (FUNDP)
— 1991, 2 BCZ, Diepenbeek : K. Van den Branden (UCL)
— 1992, Jongerendag/Journée des Jeunes, Brussels : Hendrik Plompen (UA)
— 1993, 3 BCZ, Liège : Frédérique Luizi (FUNDP)
— 1994, 1 Benelux CZ, Leuven : Farid Dahdouh-Guebas (VUB)

Best porster presentation :

— 1990, 1 BCZ, Antwerp : Hilde Sluyts (UA)
— 1991, 2 BCZ, Diepenbeek : Véronique Delheusy (ULg)
— 1992, Jongerendag/Journée des Jeunes, Brussels : Griet Wouters (KULeuven)
— 1993, 3 BCZ, Liège : Hans De Wolf (UA)
— 1994, 1 Benelux CZ, Leuven : Samantha Delafaille (UA)

For the first time the results of the Congress are collected in a Proceedings volume (issue 125/1 of the Belgian Journal of Zoology). Some 30 contributions were sent in and peer reviewed as normal contributions for the BJZ. Unfortunately nine of them required a major revision and could not be reworked in due time for this issue. Almost all of those came from young and clearly inexpierenced young

people making a first attempt to publish. We most sincerely hope that the rejection of their very first effort does not disappoint them and that it will be more an encouragement to do better next time.

Two contributions came from Luxemburg colleagues (one joint with Belgians) while only three Dutch contributions (of the 25 presentations at the congress) were sent in for these Proceedings. The Belgian Journal of Zoology is clearly insufficiently known by the Dutch zoologists ; we do hope that more Belgians will contribute to the Proceedings of the 2nd Benelux Congress of Zoology (to be published in the Netherlands Journal of Zoology), and that these Benelux Congresses and these Proceedings will lead to a better knowledge and appreciation of our mutual Societies and publications.

Johan BILLEN
Organiser of the 1st BCZ

Ernest SCHOCKAERT
Editor Belgian Journal of Zoology

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COMMUNICATION : THE KEY TO DEFINING « LIFE », « DEATH » AND THE FORCE DRIVING EVOLUTION. « ORGANIC CHEMISTRY-BASED- » *VERSUS* « ARTIFICIAL » LIFE

by

A. DE LOOF (*) and J. VANDEN BROECK
Zoological Institute of the K.U. Leuven,
Naamsestraat, 59, B-3000 Leuven, Belgium

SUMMARY

Plausible definitions of « life » and « death » can be simply derived by combining the principles of compartmental organisation of the living state, with the principles of communication and those of Ilya Prigogine's « dissipative systems ». Prokaryotes have only one compartmental level, but all other living systems have several, up to about fifteen in the tentative classification system we propose. From the principles of communication and information it can be understood why « life » is more than just part-and-parcel of chemistry and physics : information in itself has no units of force or energy. Since communication is the cornerstone of life, a living entity dies when it irreversibly looses its ability to communicate at its highest level of compartmental organization. It is not important that lower levels of compartmental organisation, if present, retain their ability to communicate. Since « death » is the irreversible end of « life », it follows that a compartment starts to live when it acquires the ability to communicate at its highest level of compartmentalisation. Therefore, « life activity » (L) of compartment S at moment t is the total sum of all acts of communication (C) performed by this compartment (with its different levels of organization, from 1 to j) at moment t. This can be mathematically expressed as :

$$L(S,t) = \sum_{1}^{j} C(S,t).$$

Biological life as contrasted to artificial life, cannot be sustained without transmembrane gradients because of their crucial role in communication. Therefore, « Life » could not exist before some primordial aggregate compartmentalized and acquired the ability to sustain a gradient over its limiting membrane and thus established a communication channel. Communication at the level of the plasma membrane requires a moderately « leaky » membrane to make transmembrane ion fluxes possible : thus « life » started with an imperfect (leaky) membrane in combination with a chemical gradient (which is by definition a thermodynamically far-from-equilibrium state) established through the membrane. Sustaining a chemical

(*) Invited speaker.

gradient requires energy, part of which is used to create order out of disorder. These are elements of dissipative systems. Gradient formation, which is a crucial event in life, which is often neglected in many fields of biology, is the primary force to self-selection and evolution. Thus, life on one hand and self selection and evolution on the other are inseparable as the two sides of a coin. Communication is not only the very essence of « life » but at the same time, it is a major driving force of (its own) evolution. This approach leads to a holistic type of biology in which communication plays a central role, and for which the name « dissipative biology » or « non equilibrium biology » is proposed. Our approach also allows to make the distinction between « organic chemistry-based life » and « artificial (man-made) life ».

Key words : definition of life, artificial life, artificial intelligence, dissipative systems, cell biology, endocrinology, cytoskeleton, evolution, Darwinism.

INTRODUCTION

The term « life » as it is daily used has different meanings in *e.g.* « life span », « life quality », « life cycle », « life as the difference making property between inanimate and animate matter », etc. This leads to the erroneous impression that « life » is such an abstract term that it cannot possibly be comprehended by the human mind and could not be defined.

According to SCHEJTER and AGASSI (1994), an adequate definition of « life » should include : « Apart from its not being trite and uninformative (circular, to use a traditional term), it should be neither too wide nor too narrow ; it should not exclude living things and it should not include dead ones. Furthermore, it should not make biology part-and-parcel of chemistry and physics. »

WHAT IS « DEATH » ?

In trying to define life and death, a variety of approaches have been used over the years (*e.g.* SCHRÖDINGER, 1946 ; DE DUVE, 1991) but none of them has as yet yielded a satisfying result. We have used a different approach of asking and answering some simple questions.

1. What is missing in following sentences ?

— « There is joy because of birth ».
— « There is sorrow because of death ».

These sentences are ambiguous because it is not known who or what is born or died : a baby, grandfather, the canary, a cat etc. This means that « death » and « life » have to be connected to what we refer to with a general term as a ***« compartment »*** in order to make sense. This raises the question what types of biological compartments exist in nature. One possible way of bringing some order in the multitude of possible compartments is shown in Fig. 1.

2. At what moment does a living organism, or a living system cease to be alive ? What is the difference between being alive, no longer alive, or not yet being alive ?

a. the multicellular organismal level :

A vertebrate is assumed to be dead upon decapitation, even though some vertebrates such as birds and eels continue to move for a while in an uncoordinated way. Immediately after decapitation the vast majority of the cells, tissues and organs of the body are still alive and can be used in organ transplantation or cell/tissue culture.

b. the eukaryotic cell level :

Upon homogenization a cell is no longer alive although its cell organelles that are prokaryotic in origin such as mitochondria and chloroplasts, may still be functioning. Isolated chloroplasts that are injected into a chicken egg that is properly illuminated, will multiply and colonize the chicken egg.

c. the level of the population :

The same duality is present at the highest level of organization, the population. Imagine that all individual members of a small population of animals are separated from each other until they completely and irreversibly lose contact. The individual members are still alive, they can metabolize, grow, cope with entropy, respond to a variety of stimuli and adapt to short term changing conditions in the environment, evidently within certain limits. Despite all of this, we conclude that the population does no longer exist.

d. the level of the prokaryotic cell or of membrane-limited cell organelles :

At the primitive end of organizational complexity (Fig. 1) dual existence is no longer found. After homogenization nothing remains alive. A mitochondrion, a chloroplast or a prokaryote ceases to live after irreversible disruption of its limiting membrane, *e.g.* by ultrasonic homogenization. Under proper conditions membrane fragments can still perform some metabolic functions, they are complex, but they are not considered to be alive. Disruption of the plasma membrane ends existing gradient(s) across this membrane.

From these four examples we conclude that : **« A compartment dies when it irreversibly looses its ability to communicate at its highest level of compartmental organization ».** It is the **highest** level of compartmentalization that matters. In a population, the highest level of organization is reached when individual members communicate. In the vertebrate the central nervous system makes communication possible at the organismal level : this coordination system is irreversibly destroyed by decapitation. In the eukaryotic cell the highest level of communication is at the level of the plasma membrane ; and in the prokaryote or organelle of prokaryotic origin, communication occurs with the outside world across the limiting membrane. If « Death » is the irreversible loss of the ability of a given compartment to communicate at its highest level of compartmental organization, it follows that a given compartment starts to live from the moment that it acquires the ability to communicate at its highest level of compartmental organization. **Therefore, a compartment is alive when it has the ability to communicate as a whole with its « environment ».**

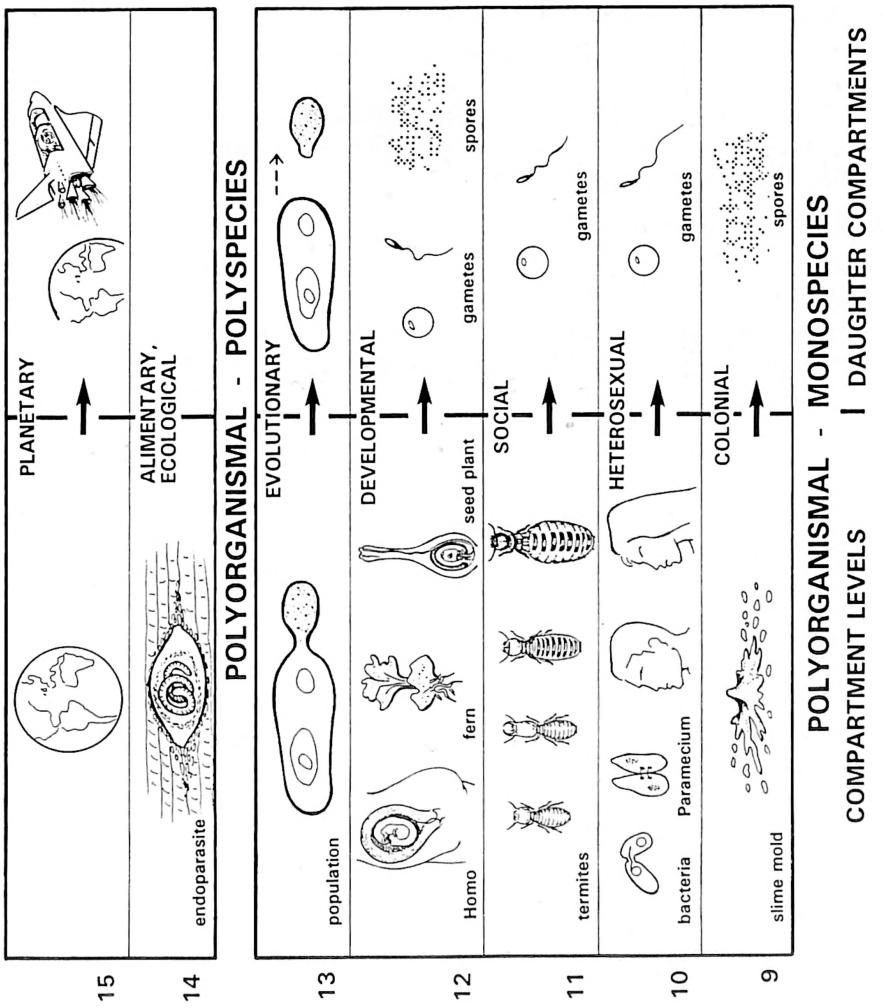
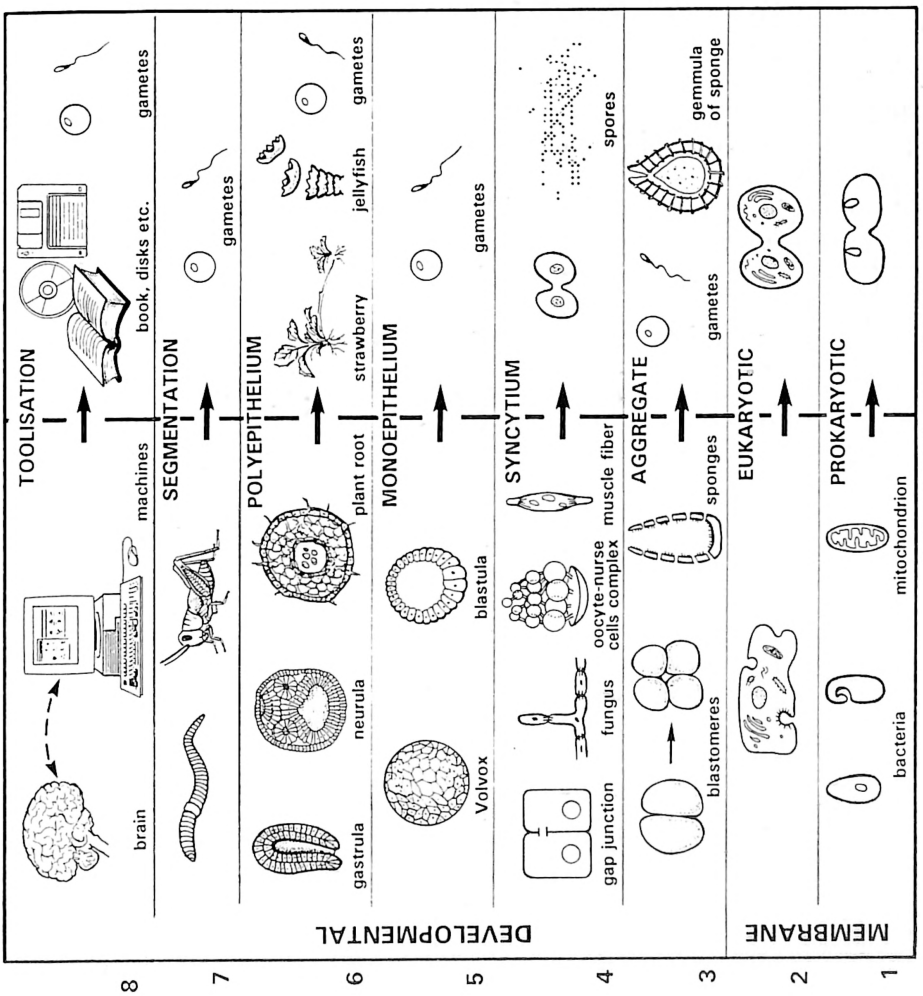


Fig. 1. — Levels of compartmentalization and means for generating daughter compartments.

The simplest level of compartmentalization is the monomembrane type to which belong the bacteria and membrane-limited cell organelles like mitochondria and chloroplasts (1). The most complicated one which is the one with the largest number of different subcompartments, is the planetary one (15). Different levels of compartmentalization are possible within one and the same organism (1-8), within one species- (9-13) and within polyspecies compartments (14-15).

For generating additional levels of compartmentalization, several systems are used. A widely used one is the internalization of novel compartments in existing ones : 1. membrane-limited compartments within an outer membrane compartment (the eukaryotic type, 2) ; 2. epithelium-limited compartments within an outer epithelium (6) ; 3. organism(s) within organism (12, 14) ; 4. subpopulation(s) within population (13). Another one is the aggregation of compartments (3, 4, 5, 9, etc.). The communication between the constituent compartments can be intermittent, *e.g.* through gap junctions between neighboring cells (4a) or more perma-



nent like in case of cytoplasmic bridges, *e.g.* in Fungi, or in meroistic insect ovarian follicles or in myoblasts which fused into a muscle fiber. A third system is segmentation (7). A fourth one is the enlargment of a compartment by tools (*tool* util*isation* or toolisation) (8). A fifth one is the spreading of the genes needed for reproduction over more than one individual (10, 11). For splitting off daughter compartments (reproduction), the variability in systems is rather limited : mitosis, meiosis, systems for asexual reproduction (*e.g.* 3, 6), systems for speciation (12) etc. Evidently, in some systems several mechanisms can be simultaneously operational. Other approaches for categorizing levels of compartmentalization than the one used in this figure are possible. The majority of the levels of compartmentalisation depicted in this figure correspond to revolutionary steps in macroevolution.

Linear biology mainly deals with the description of the generation and functioning of the different levels of compartmentalization as outlined in this figure.

COMMUNICATION IS TRANSFER OF INFORMATION

Since communication is essential for living, a few words about its nature may be helpful for the non-specialists.

The basic anatomy of a communication system is : a *sender* produces a (coded) message which is released into a *communication or transmission channel* through which it is transmitted to a *receiver-decoder-amplifier-responder* (GERAERTS *et al.*, 1994). This is illustrated in Fig. 2.

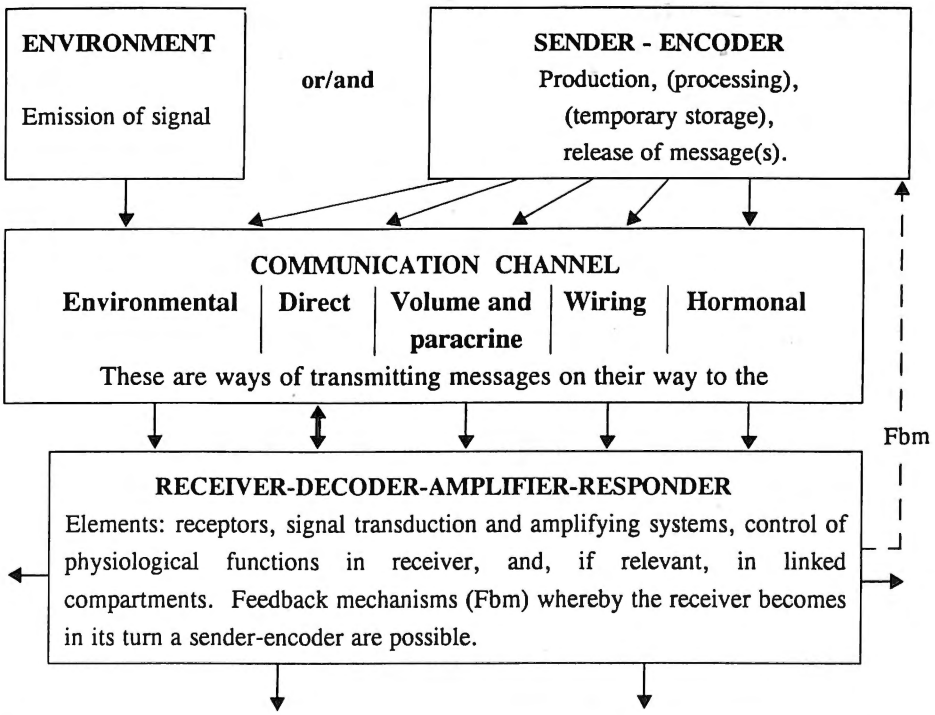


Fig. 2. — Schematic representation of the different elements of a communication system.

Information can be defined as any change within a communication system which affects any component of that system (VAN POECKE, 1994).

The essence of the different aspects of communication can be summarized as follows : « Communication is transfer of information which in itself does not have units of force or energy. This works as follows : either the environment or a sender-encoder delivers some sort of (encoded) message, which is transmitted through a communication channel. After having been perceived by a competent receiver-decoder, the message controls there (and perhaps as well in compartments inter-linked with this receiver through some communication channel) the use of a specific form(s) and quantity of **prestored energy** at a specific time(s). This energy can then

be used for doing some kind of « work » as this is defined in physics, to engage in other acts of communication (*e.g.* feedback mechanisms) and/or to counteract entropy (*i.e.* to prevent the system's break down). » In biological systems the role of communication and signal perception is most probably to adapt or change the use of biochemical and gradient-energy to the present or future needs of the system, especially according to changing « environmental » situations. This does not imply that all communication acts are (equally) useful or meaningful.

INTERMEDIATE ACTS OF COMMUNICATION

A living system communicates with its environment, it reacts upon changes and, in some instances and to some extent, it even learns to control its own direct environment. Biological communication systems are very complex and cannot be discussed in detail in this paper ; a summarizing cartoon is given in Fig. 3. To transform incoming information and translate it so that an organism will react to it, intermediate processes named *« pathways »* and *« networks »* are involved. An example is a hormone that binds to a receptor, causing an ion channel to open, the membrane potential changes, there is a Ca^{2+} explosion in the cytoplasm, the cytoskeleton contracts, protein synthesis is influenced, etc. : such intermediate processes are analogous to different steps in a chain-reaction. We will refer to them later as **« acts of communication ».** Besides the qualitative complexity, there are also quantitative differences : *e.g.* a given « intermediate process » can have a greater impact than other ones. Biological systems manage to **integrate** (« add up ») all these acts of communication. Therefore, the result of this integrative process (the « total sum of all acts of communication ») performed by a given compartment can be regarded as the **« Life activity »** that is produced by this compartment. This Life activity can be considered at a given moment t or over a given interval of time (t_2-t_1).

GENERAL DEFINITIONS OF « LIFE ACTIVITY »

Let us consider the « life activity » (**L**) generated at the level of a given population (**P**). If « **L** » of population P is the « total sum (Σ) of all acts of Communication » at the level of this population and if P is alive, this sum is larger than zero. This can be represented mathematically as :

$$\mathbf{L}(P) = \Sigma \text{ Communication acts } \underset{\text{population level}}{(P)} > 0.$$

Since life is never constant, the acts of communication change all the time. Therefore, one has to indicate the moment (t) at which the acts of communication are considered :

$$\mathbf{L}(P,t) = \Sigma \text{ Communication acts } \underset{\text{population level}}{(P,t)} > 0.$$

If a given compartment (or system) is represented by S and its (highest) level of compartmentalization is j, the general formula becomes :

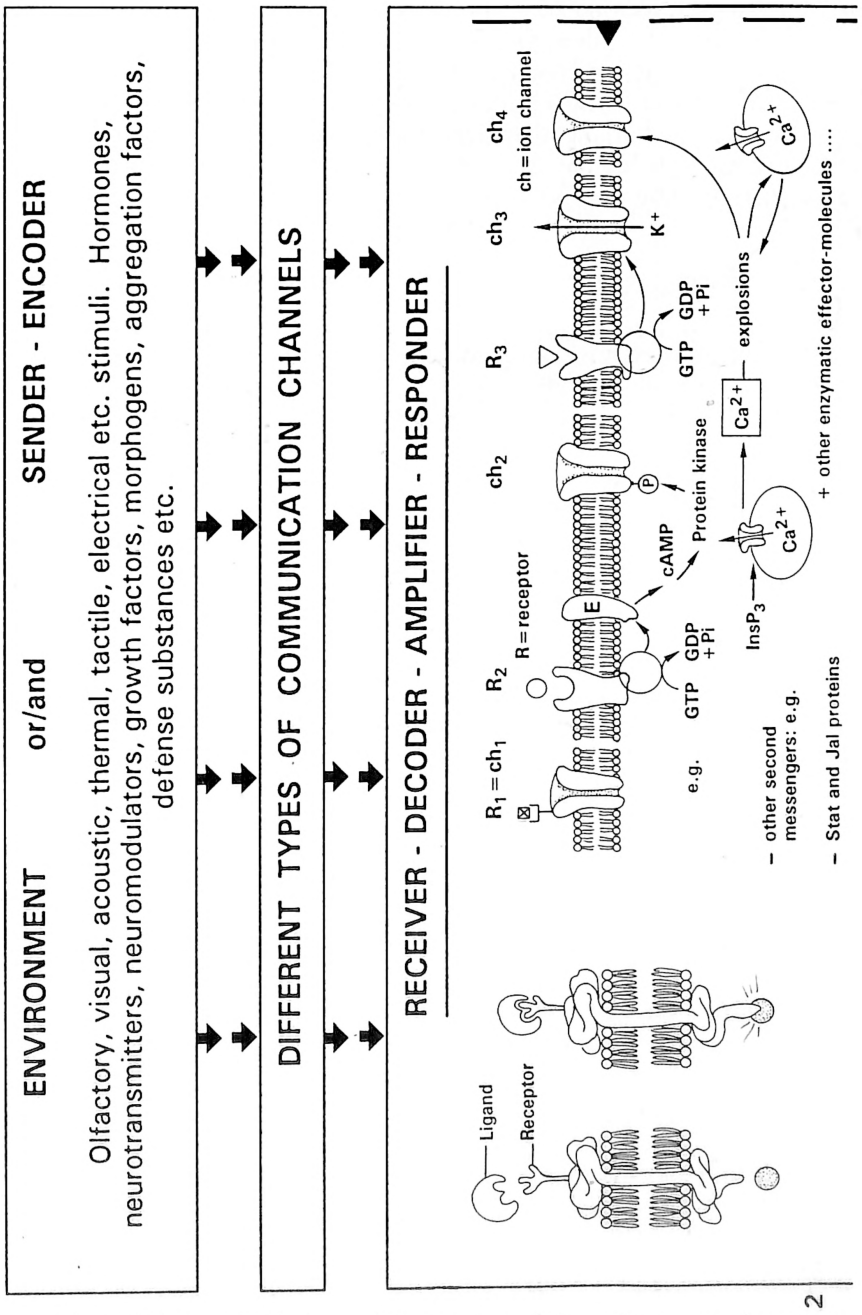
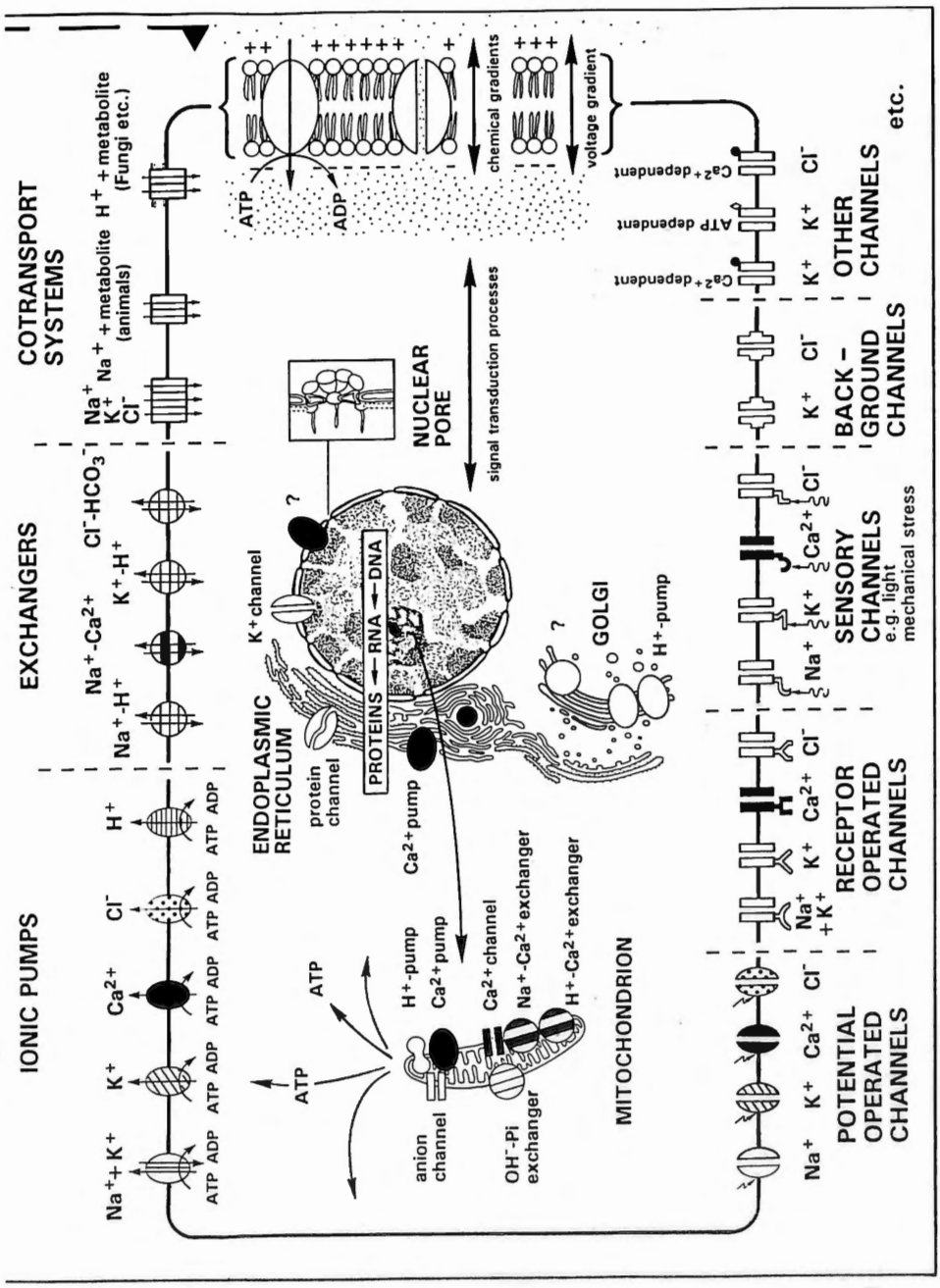


Fig. 3. — Schematic but evidently incomplete overview of the major systems for communication present at the cellular level.

Messages, whatever their nature, have to be « interpreted » either by receptors (R_1, R_2, R_3) present in the plasmamembrane (2a, b, c) or/and in subcellular compartments (1, 2). Inorganic ions/electrical events play a predominant role in communication systems (1, 2 : $ch_{1,2,3,4}$ = ion channels). The arrows in the ionic pumps are not meant to indicate the actual



direction of the transport of the ionic species involved. Other cell organelles than the ones drawn here can also have ion transporting proteins and receptors in their limiting membrane (De Loof *et al.*, 1992).

Dissipative biology mainly deals with the interplay of all the systems for communication as outlined here.

compartmental level j
$L(S,t) = \Sigma$ Communication acts $(S,t) > 0$.

In the preceding formula it has been assumed that, when compartment S is functioning well at level j, all lower levels of organisation that are essential for the well-being of S (at level j) are present. However, when the « Life activities » at all levels of compartmentalization are indeed taken into account, starting from the lowest one (level 1, the cell organelle level) to the highest one (level j), the formula becomes :

$$L(S,t) = \sum_{1}^{j} C(S,t)$$

where **L** = Life activity, **C** = communication act, **S** = system or compartment, **j** = the highest level of compartmental organisation, **1** to **j** = a given level of compartmentalization, **t** = the moment at which the acts of communication are considered. Conditions are that $\Sigma^{j}C\,(S,t) > 0$ **and that, when adding up the acts of communication at successive levels, the same act is added only once.**

We are aware of the fact that « time » is difficult to define and may form a major problem in mathematical expressions of the form, but it is not the task of biologists to solve this philosophical-physical problem. We also have to stress that the life activity of a given system is considered at a given moment t (or time interval) : time only matters (as a variable parameter) as long as the system S remains alive.

THE INTERRELATIONSHIP BETWEEN GRADIENTS, PRESTORED ENERGY, COMMUNICATION AND THE DISSIPATIVE NATURE OF LIVING SYSTEMS

At present, very few biologists are familiar with the name and work of the belgian mathematician Ilya Prigogine who received the Nobel Prize for his innovative concepts in the field of thermodynamics of far-from-equilibrium systems. Prigogines work mainly deals with the question how order can arise out of chaos, and this is evidently of outmost importance for biological systems. Without some knowledge of this theory (PRIGOGINE, 1980), it is impossible to understand the very nature of life. Therefore, we will briefly mention some major points of interest. A most important term, which did as yet not appear in common handbooks of biology, is ***« dissipative system »***. It is a far-from-equilibrium system in which order is created out of chaos by investing energy. All living systems are dissipative in nature : when the investment of energy (food supply) stops, the ordered state changes into chaos. The explanation why living systems are thermodynamically always far-from-equilibrium systems is simple. We have hypothesized earlier that communication is essential for life and that communication is not possible when

there is no ***prestored*** or ***instantly available energy*** in the system. In any cell, part of the energy which is used for communication and doing « work » in general (this includes creating order) is stored in the form of an ***ionic-voltage gradient*** over the plasma membrane. ***Gradients*** are by definition out of equilibrium systems. They are essential in driving communication and evolution at the same time (see text). A second very important term in this field is ***« bifurcation point »***. The term has first been introduced and is commonly used in the nonlinear mathematical literature. When one deals with bifurcations in a rigorous manner, it refers to a situation where beyond some critical parameter value a particular solution becomes unstable and the system spontaneously evolves to another stable regime. In a biological context, the term is loosely used, depicting a point where a choice has to be made (gene a or b to be switched on etc.). But bifurcation points are also very important in communication (see text for the example of the flute and the music) : they are a tremendously rich, but largely overlooked source of variability in communication.

« LIFE » IS COMMUNICATION

The following definitions of « Life » may meet with varying degrees of accuracy the criteria mentioned in the introduction :

— *« Life » of a compartment S at moment t is the « total sum of all acts of communication » performed by this compartment S at moment t.*

— *« Life » of a given dissipative compartment (S) at a given moment of its existence (t) is a dynamic state resulting from the combination of all acts of communication carried out by this compartment S at its different levels of compartmental organisation (from 1 to j) at moment t. This living state should exist at all vital levels of organisation of compartment S.*

— *The term « Life » denotes the state of a thermodynamically open, but compartmentalized dissipative system that has reached a (high) level of internal organisation which allows it to produce coordinated (active) responses to « environmental » changes (including the communicative interactions with other living systems) in order to prolong this state (and to create an even higher level of compartmentalization).*

Our definitions of life imply that :

1. Life and communication are not possible without a **gradient**(s) had been formed first. By definition, a gradient is not a state of stable equilibrium. Exactly because they depend upon gradients, living systems cannot be else than far-from-equilibrium situations, in which order can be created and maintained only by investing energy. Thermodynamically, living systems have the essential properties of **« dissipative systems »**. Irreversible collapse of essential gradients means the end of life. Viruses are not alive because they do not build up an ionic gradient over a plasma membrane.
2. A compartment can only communicate with the outside world or with other compartments if its boundary is not perfectly tight. A cell with a limiting « membrane » made of glass could not be alive. Communication at the level of the

plasma membrane requires a moderately « leaky » membrane to make transmembrane ion fluxes possible. Thus, some 3,7 billion years ago, « life » started as an *imperfection* in a membrane in combination with a chemical gradient over this membrane. The important but usually overlooked role of the cytoskeleton and the distinction between the « prebiotic » and « biotic » era have already been described elsewhere (DE LOOF, 1993).

3. Life has both **quantitative and qualitative** parameters (types and steepness of gradients).
4. Since in many fast acts of communication **ionic/electrical** processes are involved, life is to a large extent an electrical phenomenon.
5. Communication is transfer of information. Information does not have units of force or energy. This explains why life is not part-and-parcel of chemistry and physics. Life is above the chemistry and physics that are required to form the compartments, the instruments for communication. In our third definition, the term ***coordinated*** is important because it is the coordination which makes the realization of the highest level of compartmentalization functional : this means that **« the whole is more than the sum of its constituting parts »,** a crucial fact which is often overlooked in the philosophy of reductionistic experimentation.
6. In order to allow a system to transfer and integrate incoming messages it has to contain the « information » (or programme) needed to do so. Therefore, living compartments can build up **some kind of memory** (short-term and/or long-term storage of information).
7. A compartment has to communicate to preserve or augment its chances for survival. Therefore, **life and survival are interdependent** and, as will be discussed later, cannot be separated.
8. Life has a different meaning not only for each individual creature, but also at each moment of its existence ; life is never constant. Life started to change and to evolve from its very beginning, and will continue to do so as long as there is life on earth (Evolution).

GRADIENT FORMATION, SELF-SELECTION AND EVOLUTION

The consequence of defining life in terms of communication, implies that **biological evolution includes changes to the communication systems in the course of geological time.** These changes are made by increasing 'complexity', thus by reaching higher levels of compartmentalization by changing the nature of and/or the number of communication acts that can be performed.

Life is the driving force of its own evolution

Life on one hand, and self-selection, natural selection and survival of the fittest (Darwins' terminology) on the other, are as inseparable as the two sides of a coin. The reason for this is simple. Imagine in pregradient era-conditions a compartmentalized aggregate in which the concentration of solutes is the same on both sides

of the limiting membrane. At a given moment this compartment starts to build up a gradient, *e.g.* because a membrane protein starts to cotransport an uncharged amino acid together with the uptake of an inorganic ion, *e.g.* H^+ (theoretical example). If at the same time there are *e.g.* H^+-channels in the membrane through which H^+-ions can leak out at a (slow) rate that depends on environmental conditions, the prerequisites for a primitive communication system with the environment are present. Thus, in our view, this compartment starts to live. If we assume that no strong buffering system is present, the changes in H^+-concentration (pH changes) will be experienced by molecules present in the cytoplasm : enzymes, structural proteins etc... may change conformation and as a result become more active while others may become less active. At the same time the increase in solute concentration in the cytoplasm will lead to osmotic effects. A cell that cannot cope with all these changes will lose its gradient and die. The self-selection of life goes on as long as there is life. This self-selection process which is based on many trials and errors and which can only be achieved in concert with environmental conditions, is probably the most fundamental mechanism that drives evolution. The driving force could be called « gradient drive » or « communication drive ». The entities with the best « communicative skills » (the fittest in neo-Darwinian terminology) have the best chances for survival. « Communication fitness » and « Communication environment » might be useful terms in this context.

Since evolution is driven by the communication drive, it actually means that life is its own driving force (self-selection). This results from following reasoning :

If « Life » is « Communication »
and « Communication » drives « Evolution »,
then it follows that « Life » drives its own « Evolution ».

Thus, life cannot exist without evolving. It drives its own evolution.

« Cultural Evolution »

Is there any form of evolution which is independent from mutations of the genome ? Let us therefore analyze what happened to the social compartment formed by our species, *Homo sapiens*. The behavior of *Homo* (and even to some extent his morphology, *e.g.* the increase in body length) has evolved substantially in the 20th century. This social and economical evolution is due to epigenetic factors (*e.g.* our diet), to the development of specialized tools, to the production of machines that do a great deal of our work, to the drastic changes in communication systems that we can use etc. The speed of acquisition of novel (*e.g.* scientific) information is so fast that genetic evolution is not fast enough to generate larger and better functioning brains. Mechanical and electronic tools (letters, books, journals, radio, television, telephone, fax, computers, compact discs etc.) are used as extensions of the « natural » communication systems (controlled by the brain) to transfer, store, reproduce or select this additional information. In classical terms this type of evolution would be called *« cultural evolution ». But what else is cultural evolution than evolution based on communication and learning at the (highest) level of the* Homo

TABLE I: BASIC PHILOSOPHY OF LINEAR VERSUS DISSIPATIVE BIOLOGY.

	LINEAR BIOLOGY	DISSIPATIVE BIOLOGY
1. study object	living matter	communication
2. basic unit	organism	communicating compartments
3. smallest unit	cell	monomembrane communicating compartment
4. organism	- instrument for metabolism and reproduction - product of its genes and environment - complex because of large number of genes	- instrument for communication - product of its genes and communication environment - extremely complex because of high number of genes and very high number of communication bifurcation points
5. major functions	- growth - development - reproduction	- communication - prevention of transition from order to chaos
6. basic rules biology	DNA --> RNA --> proteins	- idem but Nernst equation equally important - no life without gradients
7. basic physics	- classical linear Newtonian physics - 1st and 2nd law of thermodynamics - time in principle reversible in some processes - mainly deterministic - 4 dimensions of spacetime - living matter consists of aggregated stardust	- modern Newtonian physics - non linear Prigoginean far-from-equilibrium thermodynamics - time in principle always irreversible - indeterministic - idem plus electrical dimension - all matter is creative and self organising

8. basic chemistry	synthesis of organic molecules in "saline" environment	idem plus bioelectrochemistry
9. information carrier	nucleic acids	nucleic acids and memory
10. genetics	eugenetics (Mendel, molecular)	idem plus epigenetics
11. variability due to	- mutations, meiosis, etc. - changes in "macromolecular environment" around the genes	- idem plus bifurcation points - changes in "macromolecular and ionic environment" around the genes
12. evolution	- evolution of macromolecules - changes in genes - mutated protein force - slow - selection follows mutations - survival of fittest	- communicational evolution - changes in communication systems - gradient/commumnication force - fast - self selection results from life itself - survival of fittest in communication - evolution field (Cramer, 1993)
13. definition of life and death	impossible	possible (this paper)
14. scientific approach thinking	- reductionistic experimentation and holistic thinking - sum of parts approximates the whole	- reductionistic experimentation and - the whole is more than the sum of its parts
15. feedback systems	for coordinated functioning	for interactive communication
16. feelings, emotions, etc.	largely irrelevant	where present, essential part of communication

compartment ? This process is not restricted to the *Homo sapiens* compartment, but it is a basic and thus general feature of all living systems.

Changes in some communication acts may be due either to mutations or to another almost completely overlooked but nevertheless very important source of variability, namely the bifurcations which are inherent to far-from-equilibrium systems (dissipative systems such as living beings). Mutations allow evolution to proceed at a slow rate, whereas bifurcations at a very fast rate.

With respect to communication at the level of the plasma membrane, bifurcations are especially important in those processes where fluxes of *inorganic ions play a role and which are not directly controlled by genes.* For the relationship between bifurcations and selection, we refer to KAUFFMANN (1993).

Bifurcations, the overlooked companion of mutations

The following analogy between life and music may help to clarify the point we want to make, namely that for driving evolution, mutations or genomic changes in general are only part of the story. *To produce music* **(communication)**, one needs an instrument **(compartment)** *and one has to play* **(to live)**. This means that in order to understand what drives evolution *one has to make the distinction between the evolution of the compartment, and the communication produced by this compartment :* these are quite different things, but they depend on each other. Imagine a musician who plays a flute. At first, the flute has only one finger hole. The resulting music will be rather monotonous. If a second finger hole is introduced, which means a ***mutation*** for the flute, the resulting complexity (***degrees of freedom***) of the music does not double, but multiplies. The reason for this is that the musician can now continuously decide whether to use the upper hole or the lower or both, and for how long : thus he makes use of the possibilities of *an imaginary « bifurcation point »* (an important term in the Prigoginean thermodynamics of systems far-from-equilibrium, see later) located between the two holes. The introduction of a third fingerhole results in a much higher complexity of the music. The more fingerholes the greater the possibilities, provided the musician manages to coordinate the movements of his fingers. But once there is a hole for each finger, the music can nevertheless further evolve in the course of time without additional fingerholes. The instrument could perhaps still be improved by changing the size or location of the holes. The higher the possible number of bifurcation points, the less important are changes in the instrument itself, and the greater the possibilities for evolution of the music. The situation is very similar in biological systems : gene mutations will have drastic effects in simple systems, but in more complex systems there can be situations where additional changes are only to a minor extent responsible for changes in communication.

An almost literal biological equivalent for the fingerholes in the flute are the holes in the plasma membrane through which inorganic ions can flow (ion channels, ion pumps etc.). In addition to these, other components of the communication system can also « generate » imaginary bifurcation points (Fig. 3). Because of the high number of ion transporting membrane proteins in each cell and other com-

ponents of communication systems, the number of possible bifurcation points is very high. This results in nearly infinite variability and low predictability in organisms consisting of many cells, especially in organisms with well developed brains (*e.g.* our brain consists of about 100 billion cells). No organism « knows » what all his acts of communication will be the next day, not even the next hour, minute or fraction of a second : it all depends on the interplay of circumstances that contribute to decide which bifurcation points will be involved, and which one of its two sides will be chosen. The communication-future is thus unpredictable. Bifurcation points allow to explain phenomena we are familiar with but which cannot be easily explained by genetics. Imagine that genetically identical twins are born in Brussels. One baby stays in Brussels and is raised in a bilingual dutch-french family, the other is moved to Tokyo and raised there by a japanese family. Despite their genetic identity, the twins will eventually speak totally different languages and behave each according to the cultural standards of their local environment. Mutations do not play any role in generating the differences because the instruments for communication, the bodies of the twins, do not change by being raised either in Brussels or in Japan. Nevertheless the communication produced by the twins is different under the influence of the environment and learning : these do not act through mutations but through bifurcations just like different musicians can produce totally different music with the same flute. Imagine now that genetically identical twin lambs are treated the same way. The mèèèèèèè-language of the japanese lamb, once grown up to a sheep, will probably not be that different from its counterpart in Brussels. The reason for this difference humans-sheep is to a large extent due to the number of possible bifurcation points in the brain of humans, allowing a much higher flexibility corresponding with a higher number of degrees of freedom. This allows the modification in the cited example to occur. Evidently, the muscles involved in sound production play an important role.

The neo-Darwinistic theory of Evolution versus our holistic « double continuum » approach

Evolutionary biologists usually define evolution as the *change in the genetic make-up of a population* with time. The centerpiece of the neo-Darwinian synthesis is that natural selection, acting upon individual variations within a population to substitute one allele (= a variant of a gene at the same locus) for another, is the major force driving adaptive evolutionary change (KAUFFMAN, 1993). By doing so, neo-Darwinists only take into account relatively small evolutionary steps due to genomic changes that are fixed in a population.

We defined Evolution as the *change in compartments and in their acts of communication* in the course of geological time. Due to the « communication drive », which is — in our opinion at least — the most prominent selection principle, this process leads to changes in the nature and/or in the number of possible bifurcation points. This approach is not at all in contradiction with neo-Darwinism, but it integrates the principles of this neo-Darwinism with those of thermodynamically far-from-equilibrium systems (such as living compartments) and of communication.

It takes into consideration the entire interplay of elements affecting the communication acts that can be generated by a given compartmentalized system and the way by which these can be integrated (« added up »). These elements include both the gene products as well as the other organic (*e.g.* lipids, carbohydrates, steroids, amino acids, nucleotides, second messengers etc.) and inorganic molecules (*e.g.* ions arranged by the system into gradients across membranes) which are part of the system's communicative machinery.

In the neo-Darwinian view, however, biological systems are generally still considered as « discontinuous » systems which get born, live and die and which eventually transfer their genetic information to a next generation. In our holistic approach, life and survival are highly interdependent. Therefore, life can be considered as a ***double continuum***. First, there is the continuity of the physical compartments, which are the instruments which are needed for communication. Second there is also the continuity of communication and some types of information themselves. Both do not necessarily overlap because some types of information and communication present in a population can last longer (or shorter) than the individual organisms of which a population consists : *e.g.* the information present in a book can last longer than the person who wrote it. Neo-Darwinism largely limits itself to the study of the evolution of the « physical compartments » as such : it is a « ***single continuum*** » theory.

The communication which is produced is never constant and changes in concert with environmental as well as internal conditions. Gene duplications and mutations are just the mechanism by which long-term changes in the properties of such continuously evolving communication systems can be created and preserved. These duplications and mutations generate a higher variability by which a population may have better chances for its survival : the « fittest » individuals will survive.

The process of reproduction circumvents the problems that arise by system breakdown (death) : life of an individual far-from-equilibrium system is not endless, since it will have to cope with entropy sooner or later. The population, however, may survive during longer periods of time via reproductive processes. An offspring is usually created via pinching off daughter compartments that then may « regenerate » to form novel adult compartmentalized systems. In the case of sexual reproduction, the zygote obtains mixed genetic information derived from maternal and paternal compartments. This allows more variation by generating different genetic combinations within a population. Besides that, what else is sexual reproduction than an extreme form of regeneration through a process which we call embryonic development ? A mature oocyte is an example of a daughter compartment that is pinched off from the maternal system. It contains the information and the programme that is necessary to « regenerate » into a novel fully developed system (in concert with some essential environmental and/or social conditions). This daughter compartment formation process as well as embryonic development are the result of a long evolutionary process. They make use of the genetic information (DNA is a form of phylogenetic molecular memory which contains the sequential information to build up complicated macromolecules) and of the communicational machinery which is essential for life (cfr. our definition of « Life ») and thus for sur-

vival. The entire oocyte system is organized in such a way that the « life activity » that it produces, will normally lead to the formation of a regenerated system via a certain developmental programme. It is not surprising at all that this (ontogenetic) developmental programme appears to be a fast recapitulation of the system's phylogenetic history.

In our approach, selection is evidently very important : it can act at many different levels of organization. However, probably like chemists and physicists, we have difficulties in accepting the rigourous statements of KAUFFMAN (1993) that selection is both the driving force of evolution and essentially the only source of order in the biological world.

« ARTIFICIAL LIFE » ?

The technological innovations which have been developed in fields such as molecular biology, electrophysiology with artificial membrane vesicles but especially in the field of cybernetics make the question whether the *Homo sapiens* can make « artificial life » relevant. Molecular biologists have the tools to genetically transform existing life, but not to create new life. Collaborating teams of molecular biologists and membrane physiologists may be able in the near future to make a very primitive form of a communication system in artificial membrane vesicles. But, most of all, some man-made mechanical and/or electronic devices (or robots) have become so sophisticated that they start to look like being alive : *e.g.* imagine a man-made computer in which the electrical energy comes from a solar cell. A system like this can have all the necessary elements of a communication system as outlined above (stockpiled energy, a built-in programme to act as a receiver-decoder-amplifier and the possibility to use energy for carrying out specific types of work). When a signal via a key board is send, the system will work. In principle, such a system is not basically different from the one in which a hormone binds to its receptor (= the equivalent of typing on the key board the right code) which initiates signal transduction to mobilize previously stockpiled energy for doing work. Intuitively everybody will reject the idea that the computer is alive, although we sometimes use a similar vocabulary : *e.g.* we say the computer is « dead » like we say that the radio is « dead », meaning that its communication system has failed.

Do we have good arguments to firmly say that a computer is not alive as we think we have ?

1. *Computers cannot reproduce themselves.* But neither can a castrated bull, and nobody will claim that this bull is not alive. Some parasites and symbionts cannot reproduce without a host. On the other hand, it is possible to make identical copies of all information on computerdiskettes, when the right « signals » are given. Reproduction only comes into play when life is considered *over a period longer than one generation :* for time spans not exceeding the lifetime of a compartment, reproduction is not an essential parameter of the living state (« short-term » life versus « long-term » life : DE LOOF, 1994).

2. *A computer has no DNA.* Red blood cells of mammals eject their nucleus when their cytoplasm gets filled with hemoglobin. Such « cells » (erythroplastids) have no DNA but nevertheless may continue to live for a few weeks. A fertilized egg of an amphibian can be enucleated, thereby loosing its DNA (but retaining the DNA present in its mitochondria). Such an egg can still undergo a number of cleavages like a normal one.
3. *Computers use different principles of communication.* Not true : similar principles as outlined in Fig. 2. are used in computers and in our brain. Computers are designed to take over some of the communication in our brains.
4. *A computer cannot think, it is not autonomous and it has no emotions.* Can an amoeba, a red blood cell, a plant, a fungus think ? How autonomous are they, and do they have feelings ? A computer equipped with a solar cell coupled to a battery for storing the solar energy can be autonomous for a longer time than an amoeba that has to find enough food. Specialists in the field of artificial intelligence think that it will not take that long before « emotional phenomena » will be generated in computers. Any kind of feeling is mediated through the nervous system, and invariably involves ionic-electrical phenomena.
5. *A computer cannot cope with entropy, it will break down after some time.* In that it does not differ from living systems which also age and get sick.
6. *A computer does not carry out metabolic reactions.* It is true, but not a good argument, because the main purpose of metabolic reactions is to mobilize energy and use it for doing work. In our example the computer uses light energy while organisms usually use chemical energy. However, this chemical energy provided in the form of food is the same as solar light energy that plants transformed into chemical energy during photosynthesis.
7. *A computer cannot come and stay into existence without the help of humans.* True of course, but we could not come into existence or stay alive as youngsters either without the help of our parents. A more valid argument is that we and our parents belong to the same species, the computer needs help from outside the « computerworld ».
8. Computers do not use carbon chemistry like true living beings do. True again and a good argument. The basic chemistry of computers is inorganic chemistry as opposed to the organic chemistry of true life. Computer chemistry is based on silicon and metals, with some organic polymers which are mainly used for insulation purposes. Since computers are very recent inventions, there is a possibility that in the future carbon chemistry may perhaps become more important in computer technology.
9. *Computers use a different carrier of electricity.* True and again a good argument. In biological systems electricity, which is the movement of charges, is carried by inorganic ions, in computers it is carried by electrons. Electrons can move at the speed of light, namely at about 300 000 km per second while ion-carried electricity is many orders of magnitude slower. This is the major reason why computers are so efficient : they can work much faster than our brain.

10. *Computers cannot adapt themselves to a changing environment.* If one analyzes the success story of computer development, one cannot escape the conclusion that computers seem to adapt « themselves », of course with the help of humans, much faster than organisms do. Research in the domain of artificial intelligence shows that in certain circumstances man-made robots adapt themselves and even install a sort of hierarchy among each other without having been programmed to do so. It is not that difficult to make computer programmes that allow changes and errors.

What is then the point ?

The point is that one should not a priori discard the possibility that novel man-made forms of « life » can (have) emerge(d). The entire human compartment has become populated with mankind on one hand, and its tools on the other. Computers are electronic-mechanical extensions of the brain, man-made tools which only exist for a few decades. Nevertheless, they have become so sophisticated that they start to look like being alive. One could say that man created a novel type of life, « artificial or man-made life ». The entire *Homo* compartment has therefore reached a higher order of organisation (toolisation : level 8 in Fig. 1) containing « organic chemistry-based » life forms, « artificial » life forms (as « symbionts ») and non-living elements (tools). The « Life activity » produced by this supercompartment thus is the result of the integration of acts of communication which are performed by biological as well as by artificial components. Communication in our body uses organic chemistry and ion-carried electricity, while our electronic-mechanical « companions » are metal- and silicon-based and their electricity is carried by electrons. To make the distinction between « organic chemistry-based » life forms and « artificial » life forms, the formula that represents « Life activity » of a given compartment can be made more specific as follows :

$$\mathbf{L}(\mathbf{S}_{(\mathbf{TC},\mathbf{TE})},\mathbf{t}) = \sum_{\mathbf{1}}^{\mathbf{j}} \mathbf{C}(\mathbf{S}_{(\mathbf{TC},\mathbf{TE})},\mathbf{t})$$

where **L** = « Life activity », **S** = a given system or compartment which uses the **T**ype of **C**hemistry, TC, and the **T**ype of **E**nergy, TE, to produce its communication acts **C**. The condition is that $\Sigma^{j} C > O$ and that acts of communication are « added up » only once.

In his evolution, *Homo sapiens* has reached the point that his coordination center, the human brain, has become so highly developed that the mutational evolution rate (that could result in bigger and better functioning brains) cannot keep pace any longer with the fast increase in information-processing capacity and variability that can be achieved by expanding his communicational avenues via cultural evolution and via the creation of novel and very efficient tools for communication. Whether one likes the idea or not, the fact is, that transfer of information (*e.g.* from one generation to the next) and work can be carried out very efficiently by « artificial »

life forms and/or electronic-mechanical tools. This is (and will probably remain) one of the big problems of our times, leading to unemployment with all its deleterious effects.

CONCLUSIONS

The combination of the principles of communication and of dissipative systems may result in a more *holistic* philosophy of biology for which the name « **dissipative biology** » or « **non equilibrium biology** » as opposed to the « **classical** » or « **lineair** » biology (Table I) is proposed. This is analogous to the terminology which is used in physics (CRAMER, 1993). Linear biology, which is the *reductionistic* biology as it is taught today, mainly deals with the formation, functioning and evolution of the physical support (cells, organisms etc.) of life in an ever changing physical environment. It describes the different levels of compartmentalization and their functioning. Its basic unit is the « cell ». Dissipative biology mainly deals with communication of compartments in an ever changing communication environment. Its basic unit is the « communicating compartment », and its smallest unit is the « monomembrane communicating compartment ». It studies the interplay of the communication systems which are described by linear biology. Linear and dissipative biology as observed for their historical context, are not at all mutually exclusive, but *complementary*. An easy way to understand and compare « linear » and « dissipative » biology is to have a second look again at figures 1 (for linear biology) and 3 (for dissipative biology).

In dissipative biology links can be made that were not evident before : by its very nature it is *holistic* biology and therefore it allows a much more coherent concept of Life and Nature than is possible with the classical, reductionistic or « linear biology ». No doubt, because of their *indeterminate* nature, dissipative systems are more difficult to analyze than linear ones. This is the major reason why relatively few biologists are engaged in this type of research. However, several basic principles of dissipative biology have already been reported, some of them decades ago : the principles of basic membrane physiology (the Nernst- and related equations *e.g.* : see HAROLD, 1986), the chemiosmotic theory of MITCHELL (1979), ionic-electrical control of development (JAFFE and NUCCITELLI, 1977) and gene expression (VANDEN BROECK *et al.*, 1992), the electrical dimension of cells and self-electrophoresis (WOODRUFF and TELFER, 1980 ; DE LOOF, 1986), epigenetics (LØVTRUP, 1974), the evolution field (CRAMER, 1993), self-organisation as applied to biological systems (PRIGOGINE, 1980 ; PRIGOGINE and NICOLIS, 1971) and the hypercycle theory of EIGEN and SCHUSTER (1977, 1978) are some examples. Some of them were awarded a Nobel Prize. What has been missing so far is the conceptual context in which these « peripheral aspects » of biological functioning, as some of them have been considered for shorter or longer spans of time, fit in. One of the goals of this paper is to fill this gap. Another goal is to show that in biology, as in physics, both reductionism and holism have their merits. As a result of the successes of reductionistic experiments, biology has rapidly evolved towards a technological science. If it could

become more centralized around its key issue, which is communication, a more harmonious view of the place of man in Nature and in the cosmos would result. Teaching biology as « dissipative biology » or « non equilibrium biology » might substantially contribute to achieve this goal.

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REFERENCES

CRAMER, F. (1993) — *Chaos and Order.* The complex structure of living systems. VCH Verlaggesellschaft mbH, Weinheim, New York, Basel, Cambridge, Tokyo : 249 pp.

DE LOOF, A. (1986) — The electrical dimension of cells : the cell as a miniature electrophoresis chamber. *Int. Rev. Cytol.*, **104** : 251-352.

DE LOOF, A. (1993) — Schrödinger 50 years ago : « What is Life ? » « The ability to communicate », a plausible reply ? Int. J. Biochem., **25,** 1715-1721.

DE LOOF, A., P. CALLAERTS and J. VANDEN BROECK (1992) — The pivotal role of the plasma membrane-cytoskeletal complex and of epithelium formation in differentiation in animals. *Comp. Biochem. Physiol.*, **101A** : 639-651.

DE DUVE, C. (1991) — *Blueprint for a cell. The Nature and Origin of Life.* Neil Patterson Publishers, Burlington, USA : 275 pp.

EIGEN, M. and P. SCHUSTER (1977) — The hypercycle. A principle of self-organization. Part A : emergence of the hypercycle. Naturwissenschaften, **64** : 541-565.

EIGEN, M. and P. SCHUSTER (1978) — The hypercycle. A principle of self-organization. Part B : the abstract hypercycle. Naturwissenschaften, **65** : 7-41.

EIGEN, M. and P. SCHUSTER (1978) — The hypercycle. A principle of self-organization. Part C : the realistic hypercycle. Naturwissenschaften, **65** : 341-369.

GERAERTS, W.P.M., A.B. SMIT and K.W. LI (1994) — Constraints and innovations in the molecular evolution and neuronal signaling : implications for behavior. In : *Flexibility and Constraints in Behavioral Systems (*R. J. Greenspan and C.P. Kyriacou, eds.), John Wiley and Sons Ltd, New York : 209-235.

HAROLD, F. M. (1986) — *The vital force : a study of bioenergetics.* W. H. Freeman and Company, New York : 577 pp.

JAFFE, L.F. and R. NUCCITELLI (1977) — Electrical controls of development. *A. Rev. Biophys. Bioeng.*, **6** : 445-476.

KAUFFMAN, S.A. (1993) — *The Origin of Order. Self-Organisation and Selection in Evolution.* Oxford University Press, New York : 709 pp.

LØVTRUP, S. (1974) — *Epigenetics.* J. Wiley and Sons Ltd., London, New York, Sydney, Toronto : 547 pp.

MITCHELL, P. (1979) — Compartmentation and communication in living systems. Ligand conduction : A general catalytic principle in chemical, osmotic and chemiosmotic reaction systems. *Eur. J. Biochem.,* **95** : 1-20.

PRIGOGINE, I. (1980) — *From Being to Becoming. Time and Complexity in the Physical Sciences.* W.H. Freeman and Company, San Francisco : 272 pp.

PRIGOGINE, I. and NICOLIS G. (1971) — Biological order, structure and instabilities. *Quat. Rev. Biophys.,* **4** : 107-148.

SCHETJER, A. and J. AGASSI (1994) — On the definition of life. *J. gen. Philos. Sci.,* **25** : 97-106.

SCHRÖDINGER, E. (1946) — *What is Life ?* Cambridge : Cambridge University Press : 194 pp.

VAN POECKE, L. (1994) — *Non verbale communicatie.* Garant, Leuven, Apeldoorn (in press).

VANDEN BROECK, J., A. DE LOOF and P. CALLAERTS (1992) — Electrical-ionic control of gene expression. *Int. J. Biochem.,* **24** : 1907-1916.

WOODRUFF, R.I. and W.H. TELFER (1980) — Electrophoresis of proteins in intercellular bridges. *Nature (London),* **286**: 84-86.

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FUNCTIONAL COMMUNICATION BETWEEN THE ENDOCRINE AND IMMUNE SYSTEM AT THE PITUITARY : PLEIOTROPISM OR TRANSIENT DIFFERENTIATION STAGES WITHIN THE FOLLICULO-STELLATE AND DENDRITIC CELL GROUP

by

WILFRIED ALLAERTS
DEPARTMENT OF IMMUNOLOGY, ERASMUS UNIVERSITY ROTTERDAM,
P.O. BOX 1738, NL-3000 DR ROTTERDAM (THE NETHERLANDS)

ABSTRACT

Functional communication between the endocrine and immune systems has been described in terms of the common expression of signalling molecules, receptors and coupling proteins. However, information transfer in immune-endocrine interactions may also result from transgression of diffusion barriers in the body by immune effector cells. The presence of myeloid cells in the anterior pituitary (AP) of mouse and rat under non-pathological conditions, illustrates the importance of immune accessory cells in immune-endocrine interactions in the AP. Using double immunogold-labelling of ultrathin cryo-sections of rat AP, we demonstrate the presence of MHC class II^+ $S100^+$ cells with ultrastructural characteristics of pituitary folliculo-stellate (FS) and dendritic cells (DC). Our data suggest the heterogeneity of the group of $S100^+$ FS cells, in that this group may contain a subpopulation of lymphoid DC, which are also present in other endocrine organs. Moreover, our data indicate that dispersed cell populations of the rat AP contain a fraction of immunocompetent cells, that are capable of stimulating T lymphocyte proliferation *in vitro*. The latter cell fraction is also enriched in $S100^+$ and MHC class II^+ cells. Furthermore, in the present study a review is given of the problem of the nomenclature of pituitary folliculo-stellate cells and dendritic cells, whereby special attention is given to the quite different ways in which both cell types are defined.

Key words : anterior pituitary, folliculo-stellate cell, dendritic cell, mixed leukocyte reaction assay, ultrastructural heterogeneity.

INTRODUCTION

Cross-talk between the endocrine and immune systems has been described in terms of a variety of phenomena, occurring at different organismal levels, including the anterior pituitary (AP). Due to the pivotal role of the AP as an endocrine

regulation centre, immune-endocrine interactions at this level may have a profound effect on endocrine regulatory systems. Cross-talk phenomena between the two systems have been characterized in terms of the common expression of signalling molecules (classical hormones, cytokines, growth factors, ...), of receptor molecules, of binding proteins and of other effector molecules ; in addition, the specificity of the information transfer in these immune-endocrine interactions was believed to rely on local signal decoding mechanisms based on target cell specific post-receptor transduction (AUDINOT *et al.*, 1992). On the other hand, it is well known that immune reactions of the body against non-self are exerted by cellular as well as humoral responses, and that diffusion barriers in the body may, in certain conditions, be transgressed by immune effector cells.

The presence of myeloid cells in the AP of mouse and rat under non-pathological conditions (ALLAERTS *et al.*, 1991), suggests a role for immune accessory cells in immuno-endocrine interactions in the AP. Our present data indicate that these normally resident myeloid cells of the AP indeed are immunocompetent cells, and that they are capable of stimulating T lymphocyte proliferation *in vitro*.

Using double immunogold-labelling of ultrathin cryo-sections of rat AP, we demonstrated the presence in the AP of MHC class II-positive and S100-positive cells with ultrastructural characteristics of pituitary folliculo-stellate (FS) cells and dendritic cells (DC) (ALLAERTS *et al.*, 1995 ; in press ; present data). In the present paper we speculate on the heterogeneity of the group of FS cells, an S100-positive resident cell group of the AP with endocrine regulatory function (ALLAERTS, et al., 1990), and suggest that this group contains a subpopulation of DC. DC are a cell group with prominent immune accessory function (INABA and STEINMAN, 1987), they originate from the bone marrow (STEINMAN *et al.*, 1974) and they are present in several endocrine organs under non-pathological conditions (KABEL *et al.*, 1988 ; VOORBIJ *et al.*, 1989 ; ALLAERTS and DREXHAGE, 1994).

The attribution of endocrine regulatory and immune accessory functions to the group of folliculo-stellate and/or dendritic cells, raises the question whether these cells represent a pleiotropic cell group or whether they are a heterogenous group of cells with divergent functional states. We call this the PLEIOTROPISM-HETEROGENEITY problem. Related to this first question is the question of the embryological origin of these cells, and whether the different phenotypes encountered represent ultimate or transient differentiation stages within a cell lineage. As is well known in case of the monocyte-macrophage-dendritic cell lineage, the possible answer to the latter question is provided by the stability of immunohistochemical marker expression and functional activity related to maturation within a given cell lineage. In the subsequent paragraph we will briefly summarize the history of the problem of the embryological origin and immunohistochemical markers of the folliculo-stellate and dendritic cells. The clinical relevance of our findings of myeloid and/of immune accessory cells in the AP is discussed with respect to the role of dendritic cells in the onset of endocrine autoimmune diseases (ALLAERTS and DREXHAGE, 1994).

HISTORY OF NOMENCLATURE

Folliculo-stellate (FS) cells of the AP represent a non-hormone-secreting pituitary cell type with stellate morphology and surrounding follicular cavities (VILA-PORCILE, 1972). Previous studies on intercellular communication between rat pituitary cell types have revealed that FS cells attenuate the action of both stimulatory and inhibitory secretagogues on hormone-secreting cells *in vitro* (BAES *et al.*, 1987 ; ALLAERTS and DENEF, 1989), and therefore FS cells are involved in paracrine communication in the AP. FS cells have also been designated as the local interleukin-6 (IL-6) producing cells of the AP (VANKELECOM *et al.*, 1989, 1993). Some controversy exists whether FS cells originate from the neuroectoderm because of the expression of the S-100 protein (NAKAJIMA *et al.* 1980 ; COCCHIA and MIANI, 1980) or whether precursors of these cells are already present in the stomodeal ectoderm, called Rathke's pouch (GON, 1987). The S-100 protein was first described as a protein characteristic of the nervous system (MOORE, 1965), but thereafter immunohistochemical studies revealed S-100-immunoreactivity in various cell types not derived from the neuroectoderm (TAKAHASHI *et al.*, 1984), nor from the neural crest, as for instance in mesodermal chondrocytes (STEFANSSON *et al.*, 1982). It was suggested that S-100 expression may result from neuroectodermal induction (STEFANSSON *et al.*, 1982), which hypothesis was strengthened by the observation that immunoreactive S-100 in pituitary FS cells is first detected in the marginal cell layers of the pars tuberalis and pars intermedia, which regions are in close contact with the pars nervosa (SHIRASAWA *et al.*, 1983 ; COATES and DONIACH, 1988).

However, a number of studies revealed that S100 protein is also expressed in bone marrow-derived DC such as in the skin Langerhans cell (LC) (COCCHIA *et al.*, 1981), in DC in the airway epithelium (ZEID and MULLER, 1993) and in DC at various locations (UCCINI *et al.*, 1986). DC with the exception of follicular dendritic cells (TEW *et al.*, 1990) are defined as a distinct class of cells originating from a precursor(s) in the bone marrow (STEINMAN *et al.*, 1974). DC display a dendritic morphology and abundantly express HLA-DR (MHC-class II) antigens (NUSSENZWEIG *et al.*, 1981).

They are found in lymphoid as well as non-lymphoid organs and in peripheral blood and lymph (DREXHAGE *et al.*, 1979 ; HOEFSMIT *et al.*, 1982 ; KNIGHT 1984). Although a common progenitor of all cell types representing the DC class has yet to be found (KAMPERDIJK *et al.*, 1994), SANTIAGO-SCHWARZ *et al.*, (1992) have shown that granulocyte-macrophage colony-stimulating factor (GMCSF) in combination with tumor necrosis factor (TNF) induce the differentiation of DC together with monocytes and macrophages (mø) from progenitors in the $CD34^+$ stem cell compartment in neonatal cord blood. It was suggested that DC morphology and marker expression may fluctuate in culture or are transient features acquired by certain cells of the monocyte-mø lineage (SANTIAGO-SCHWARZ *et al.*, 1992). DC may become distinguishable from the monocyte compartment by their weak expression instead of abundant expression of the CD14 antigen (THOMAS *et al.*, 1993). DC also differ from mø's and monocytes by their prominent instead of dim antigen presenting capacity (STEINMANN and NUSSENZWEIG, 1980) and by

their limited instead of abundant (mø) acid phosphatase activity (KAMPERDIJK *et al.*, 1985). Typically, in DC an acid phosphatase spot is found in juxtanuclear position, close to the groove of the indented nucleus (ARKEMA *et al.*, 1991b). Recently also some characteristic, — although not unique —, markers of the DC class have been identified, such as the L25 antigen in the human DC (ISHII *et al.*, 1985) and the OX62 antigen in the rat DC (BRENAN and PUCKLAVEC, 1992).

To conclude, from the present literature survey it appears that, although FS cells and DC share morphological features and may both express the S-100 protein, the two cell types are defined in two quite different manners. FS cells are regarded as an ultimately resident cell type of the AP with enduring regulatory function in the endocrine gland and presumably originating from the (neuro) ectoderm ; DC are regarded as an ubiquitous cell type with possibly transient features and belonging to the bone marrow-derived monocyte-mø lineage. The presence of the latter cell type in the endocrine glands is rather regarded as an expression of the immune surveillance function of these cells, or of the immune accessory potential of these cells which will develop into an ongoing immune response in particular pathological conditions.

MATERIALS AND METHODS

Light Optical Immunohistochemistry

LO immunohistochemistry was performed on cryo-sections of mouse, rat and human anterior pituitaries and paraffin sections of human pituitaries. Human pituitaries were obtained at autopsy from subjects that died from non-endocrine causes. Mouse and rat pituitaries were from Balb/c and C57bl strains and Wistar, BioBreeding (BB) and Lewis strains respectively. Technical details of the immunostaining methods used are published elsewhere (ALLAERTS *et al.*, 1995).

Electron Microscopical Immunohistochemistry

EM immunohistochemistry was performed on cryo-sections of Wistar rat pituitaries fixed in periodate-lysine-paraformaldehyde (PLP) solution (MCLEAN and NAKANE, 1974). Halved pituitaries were fixed for 1 hr on ice, and thereafter were transferred into 2.3 M sucrose in 0.1 M phosphate buffered saline (PBS) on ice for cryo-protection (GRIFFITHS, 1993). After 60-90 min incubation in the cryo-protectant, the tissue blocks were mounted on specimen holders of the Ultracut FC4 ultramicrotome (Reichert Jung, Leica) and plunged by hand into a dewar vessel with liquid nitrogen (LN2).

Immunolabelling of the pituitary tissues was according to the Tokuyasu thawed cryo-section technique (post-sectioning immunolabelling) (GRIFFITHS, 1993) and immunogold staining method (DE MEY *et al.*, 1981). Ultrathin cryo-sections of 80-90 nm were cut with the FC4 ultramicrotome with diamond knife at $-100°C$. Sections were transferred to copper grids that previously had been covered with a

carbon-coated Formvar film (Formvar 1595E ; Merck). To transfer the sections to the grids, a wire loop containing a drop of 2.3 M sucrose was quickly brought into contact with the sections (TOKUYASU method) (TOKUYASU, 1973), thereby preventing the sucrose from freezing, which would hamper the stretching of the sections. Subsequently, the sucrose drop was removed from the sections by laying the grids with the sections faced down on top of a 2 % (w :v) gelatin layer (Merck) in 0.1 M PBS.

Sections were labelled according to the immunogold staining method described in detail elsewhere (ALLAERTS *et al.*, 1995). Primary antisera used were the anti-rat MHC-class II monoclonal antibody (moAb) OX6 (gift of dr. N. Barclay, Oxford, U.K.) in dilutions between 1 :2 and 1 :10 and the polyclonal anti-bovine-S100 (Dakopatt's, Glostrup, Denmark) diluted 1 :100 to 1 :25. Dilutions of antisera and immunogold complexes were made in 0.1 M PBS containing 0.05 % (w :v) of the acetylated and partly linearized bovine serum albumin (BSA) combined with 0.05 % (v :v) Tween-20 (Aurion BSA-C ; Aurion, Wageningen). Goat anti-mouse IgG-25 nm gold complex (Aurion) diluted 1 :20 was used as the first step conjugate. Double immunostaining was performed by first incubating the grids with the OX6 moAb followed by first step conjugate and rinse in PBS with 0.2 % glycine (Fluka), and a second staining sequence with the anti-S100 serum followed by a protein A-15 nm gold complex (Aurion) (diluted 1 :20).

Contrasting and air-drying of the sections was performed according to TOKUYASU's method (1978) for heavy metal staining in an organic polymer scaffold, in order to prevent the collapsing of the sections upon air-drying. After three rinses in distilled water the grids were placed on a drop of filtered 4 % (w :v) uranyl acetate (pH 8.0) in 0.3 M oxalic acid for 3 min, and subsequently placed on a drop of 1.2 % (w :v) low viscosity methyl cellulose (0.25 poise ; Sigma) and filtered 1 % uranyl acetate (pH 4.0) in distilled water for 3 min. The methyl cellulose powder was dissolved at 90°C in distilled water, matured in the refrigerator for 2 days under stirring and then centrifugated at 100,000 g at 4°C in a Sorvall Ultracentrifuge OTD-75 B (Dupont, Wilmington, Delaware, USA). Grids were removed from the methyl cellulose-uranyl acetate mixture using 3 mm wire loops, and excess fluid was removed by carefully blotting the grids on filter paper, yielding a final interference colour of the dried film between gold and blue. Grids were examined in a Philips EM 301 electron microscope.

Functional studies with pituitary Dendritic Cells and Folliculo-Stellate Cells

Populations enriched in FS and dendritic cells were obtained from Wistar rat anterior pituitaries as previously described (ALLAERTS *et al.*, 1993 ; BAES *et al.*, 1987 ; ALLAERTS *et al.*, 1995, in press). Anterior pituitary lobes were enzymatically dissociated into single cells and separated into enriched cell populations by velocity sedimentation at unit gravity in a linear BSA gradient (ALLAERTS *et al.*, 1994), and by a magnetic cell separation system (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) using a moAb against rat MHC-class II determinants (OX6 from Seralab, Sussex, England).

The accessory function of pituitary derived DC or FS cells was evaluated using the autologous mixed leukocyte reaction (MLR) assay, with Wistar rat spleen T lymphocytes as responder cells, and Wistar rat anterior pituitary cells as stimulator cells. DC from Wistar rat spleen, prepared according to the method of KNIGHT *et al.*, (1986) modified after HAVENITH *et al.*, (1992), were used as a positive stimulator cell control population. Concanavalin A (Con A ; Sigma) was used as an internal standard for stimulating T cell proliferation. Pituitary cells were either used as freshly dispersed cell populations, or as enriched cell populations obtained by the BSA gradient sedimentation or MACS separation method. Aliquots of 1.5×10^5 responder T cells in 100 μl volumes were mixed in flat-bottomed microtitre plates with 100 μl volumes of stimulator cells, in a stimulator-responder cell ratio varying between 1 :2 and 1 :32. In some experiments even lower stimulator-responder ratio's were tested. Stimulator cells were X-irradiated for 18 min (2000 rad) before mixing with the T lymphocytes. After 5 days in culture, 0.5 μCi ^{3}H-thymidine was added to each well, and, after 16 hr incubation at 37°C, cells were harvested on filter paper and ^{3}H-thymidine incorporation measured in a liquid scintillation counter (1205 Betaplate, LKB Wallac). Control DC-enriched cell populations were prepared from minced and enzymatically digested spleens. Splenic cell suspensions were cultured overnight at 37°C in a 5 % CO_2 incubator in adherent conditions (KNIGHT *et al.*, 1986), whereafter non-adherent cells were further enriched for DC using a discontinuous Nycodenz gradient (14,5 % w/v Nycodenz, Nycomed As, Oslo, Norway) (HAVENITH *et al.*, 1992). This gradient was centrifuged at 600 g for 10 min, the interphase yielding the DC enriched population. T cell-enriched populations were prepared from Wistar rat spleen according to the method of HAVENITH *et al.*, (1992).

RESULTS

Presence of myeloid markers in mouse, rat and human anterior pituitary

In Table 1 an overview is given of myeloid markers found in the mouse, rat and human AP using LO immunohistochemistry. These data are collected and adapted from previous publications of our group (ALLAERTS *et al.*, 1991, 1993, 1995). Several of these markers reveal overlap between immunopositive cell populations as previously described (ALLAERTS *et al.*, 1995).

Ultrastructure of S100 and MHC-class II expressing cells in rat anterior pituitary

Ultrathin cryo-sections of rat AP labelled with the OX6 moAb (MHC class II specific) and stained following the immunogold technique, revealed the presence of readily detectable numbers of gold particles on cells with the following characteristics (Fig. 1-4) : $OX6^+$ cells all displayed a narrow cytoplasmic rim around the nucleus and a varying number of cytoplasmic processes. The numbers of gold particles per cell section was rather low, namely of an order of magnitude of 10^1 to 10^2. Background labelling was absent, for granule-bearing endocrine cells were not

TABLE 1

Pattern of LO immunohistochemical staining with moAb's against myeloid antigens present in mouse, rat and human anterior pituitaries

MoAb	*Antigen*	*Reference*	*Presence in pituitary cells*
MOUSE			
BMDM1	aminopeptidase	LEENEN *et al.* 1992	+
M5/114	class II, MHC		+
M1/42	class I, MHC		+
30G12	T200 antigen (common leukocyte antigen)	LEDBETTER and HERZENBERG, 1979	+
N418	CD11c antigen (p 150, 90 antigen)	METLAY *et al.*, 1990	−
RAT			
OX6	class II, MHC	HART and FABRE, 1981	+
OX62	integrin-like antigen (α subunit)	BRENAN and PUCLAVEC, 1992	+
ED1,2	?	DIJKSTRA *et al.*, 1985	+
HUMAN			
CD1a	T6 antigen (common thymocyte antigen)	FITHIAN *et al.* 1981	+
CD14	Phosphatidyl-inositol glycan linked antigen	FREUDENTHAL and STEINMAN, 1990	+
CD45	T200 antigen	FREUDENTHAL and STEINMAN, 1990	+
OKIa	class II, MHC	REINHERZ *et al.*, 1979	+
TB1-4D5	L25 antigen	ISHII *et al.*, 1985	+
RFD1	MHC class II-associated antigen	POULTER *et al.*, 1986	+

labeled with the gold particles. The gold label was frequently found associated with the cell membrane (Fig. 1a), but was also found within the intra-cellular vesicles (ARKEMA *et al.*, 1991a) (Fig. 1b). Exact quantification of the density of gold particles was not possible due to freeze ruptures of the cryo-sections, and due to the fact that high resolution images were selected to reveal the OX6 labelled cells. Therefore, a qualitative description is given.

On the basis of the morphology of the cells, three $OX6^+$ cell phenotypes could be distinguished in the AP. Intermediate phenotypes were also found, therefore the following descriptions are to be interpreted as characteristic rather than distinct phenotypes occurring within the morphological spectrum of this cell group. The first cell phenotype displayed an elongated cell body and nucleus, with one or more long cytoplasmic processes, exceeding the length of the cell nucleus (Fig. 2). A second cell phenotype was found to have a more rounded morphology and many short cytoplasmic processes, often also a heavily indented nucleus and one or more

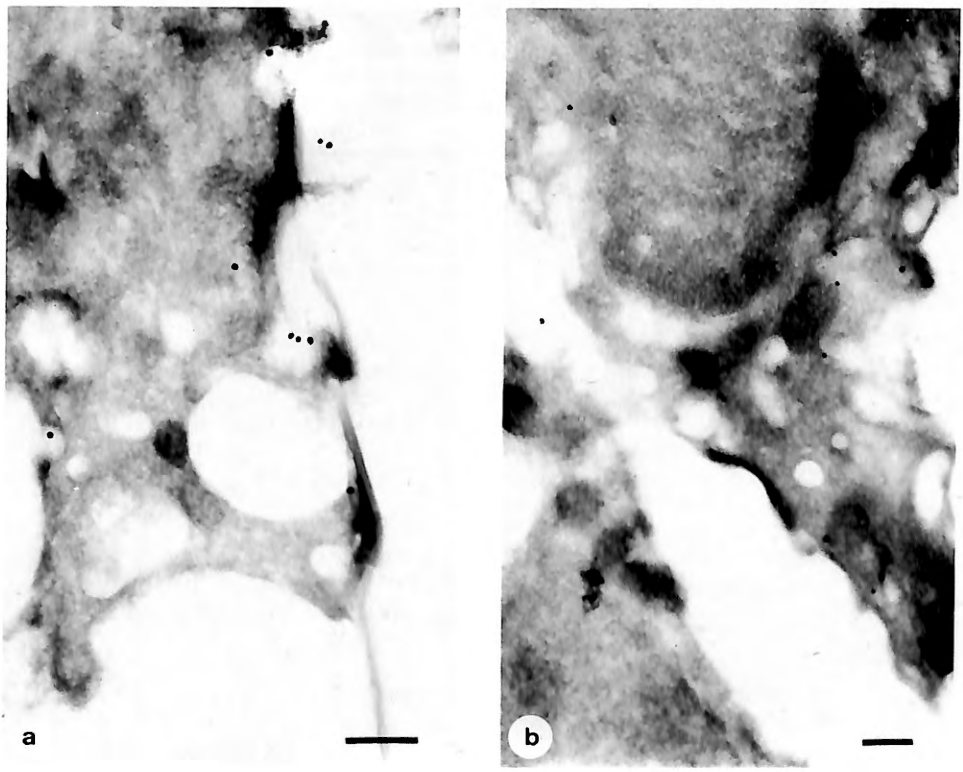


Fig. 1. — Immuno-EM of ultrathin cryo-sections of rat anterior pituitary labelled with the OX6 moAb (MHC-class II specific). The gold label was observed at the cell membrane (3a) (× 58000) or within the intracellular vesicles (3a,b) (× 42000 ; Bars = 0.2 μm).

large vesicles located in juxtanuclear position (Fig. 3) (ARKEMA *et al.*, 1991b). A third cell phenotype with elongated or triangular shape on section was frequently found surrounded by a number of hormone-secreting parenchyme cells (Fig. 4). This cell displayed a more or less indented nucleus, microvillous projections (Fig. 5 a,b), many large intracellular vesicles, including lipid droplets and phagolysosomes, elongated or vermiform mitochondria (VILA-PORCILE, 1972) (Fig. 6a) and a centriole (Fig. 6b). In the AP these characteristics are classically ascribed to FS cells (VILA-PORCILE, 1972 ; SCHECHTER *et al.*, 1988). However, according to STEINMAN and COHN (1973) some of these characteristics may also be found in DC (lipid droplets, microvilli and centrioles), whereas phagolysosomes have been characterized in DC *in situ* (KAMPERDIJK *et al.*, 1985). Birbeck granulae (BIRBECK *et al.*, 1961), the hallmark of the Langerhans cell, were not found in the $OX6^+$ pituitary cells, although they are present in many DC representatives (KAMPERDIJK *et al.*, 1994). The degree of labelling with the OX6 moAb was variable in these three cell phenotypes, but clearly exceeded background, since gold particles were rarely found in a location different from that within these cells.

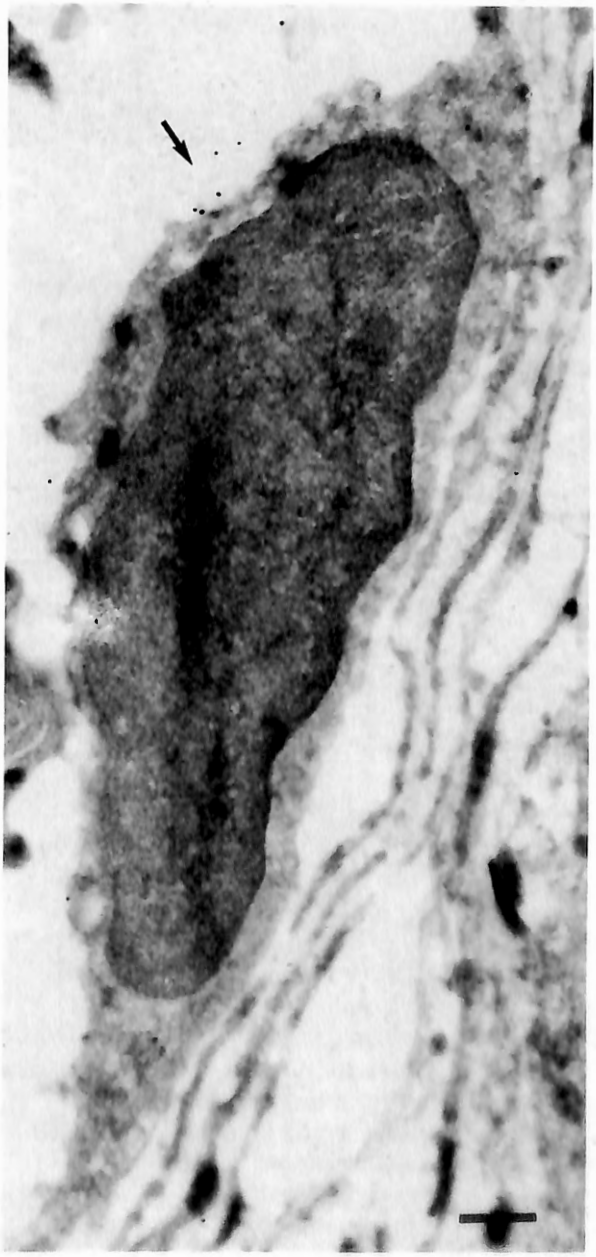


Fig. 2. — Immuno-EM of cryo-section of rat AP labelled with the OX6 moAb : example of *type I* MHC-class II-expressing cell (arrow). The *type I* cell is characterized by an elongated cell body and nucleus, with a narrow rim of cytoplasm and long cytoplasmic processes. This cell is located at the periphery of the epithelial parenchyme cord and is flanked by connective tissue (× 19500 ; Bar = 0.5 μm).

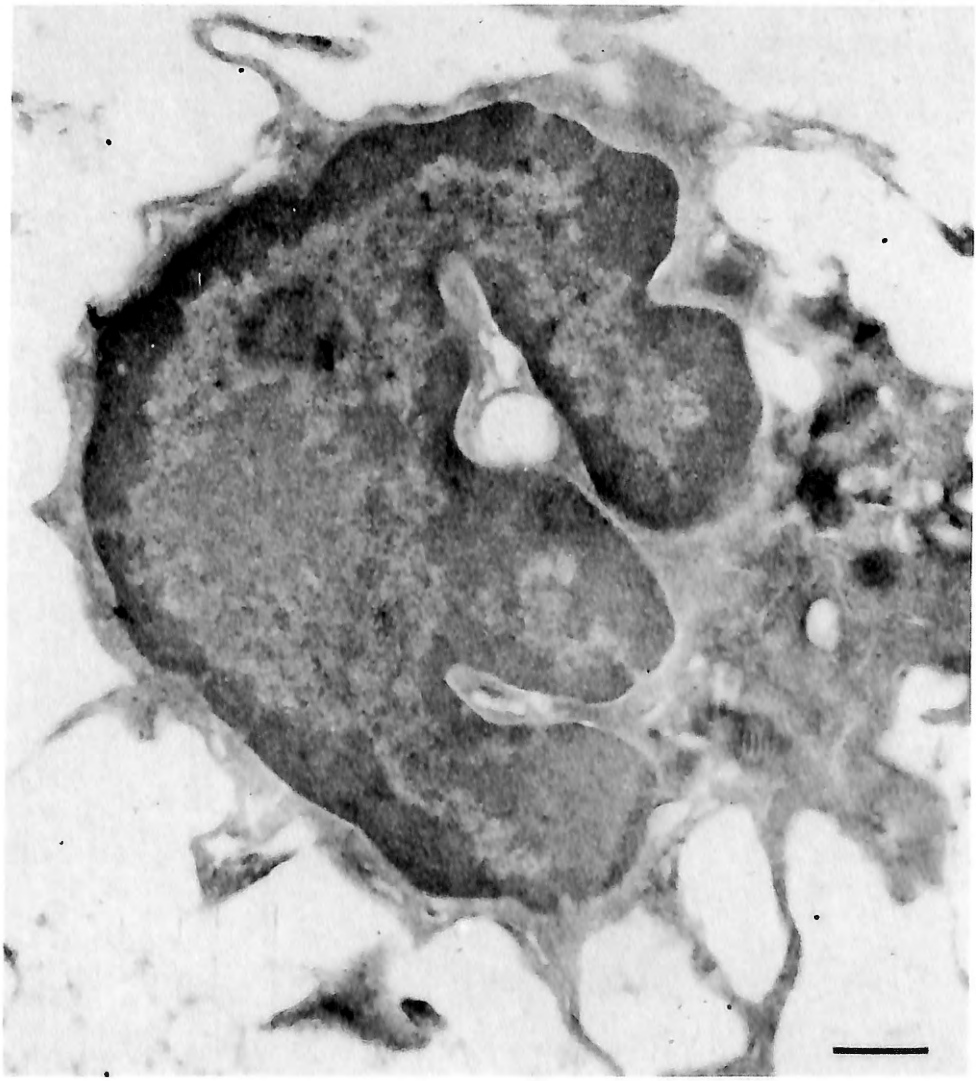


Fig. 3. — Immuno-EM of cryo-section of rat AP labelled with the OX6 moAb : example of *type II* MHC-class II-expressing cell, reminiscent of the « classical » DC. The *type II* cell is characterized by relatively short cytoplasmic processes, a heavily indented but not fragmented nucleus and a few large vesicles in juxtanuclear position, possibly corresponding to phagolysosomes ($\times$ 32000 ; Bar = 0.5 μm).

Immunogold labelling with the polyclonal S100 antiserum of ultrathin cryo-sections (Fig. 5,7) revealed a cell type reminiscent of the third OX6$^+$ phenotype described above (Fig. 4). Immunogold label was more frequently found within the nucleus (Fig. 7, inset) of these cells than within the cytoplasm, although both

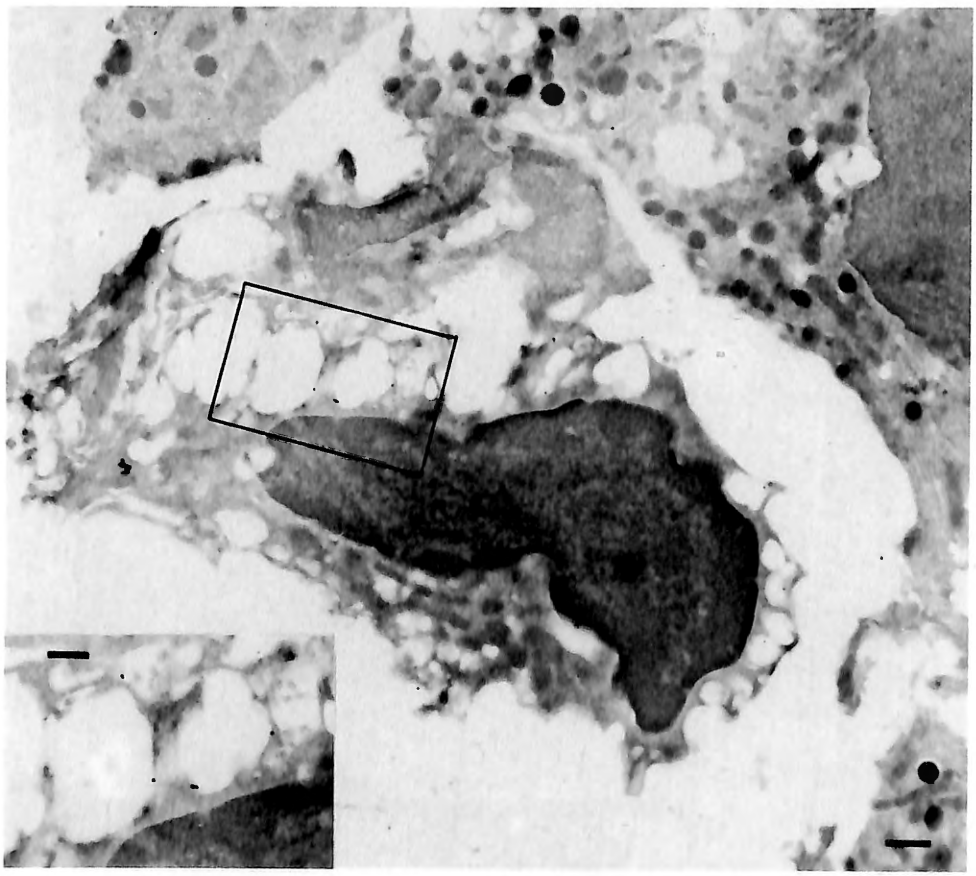


Fig. 4. — Immuno-EM of cryo-section of rat AP labelled with the OX6 moAb : example of *type III* MHC-class II-expressing cell, reminiscent of the « classical » FS cell (× 14000 ; Bar = 0.5 μm). The *type III* cell is characterized by short microvillous projections, many large intracellular vesicles and typical organelles (see Fig. 5, 6). This cell is located in the endocrine compartment and is surrounded by granulated endocrine cells. The inset shows high power magnification (× 31000 ; Bar = 0.2 μm) of some vesicles labeled with the OX6 moAb.

labellings exceeded background. This result corresponds with the LO staining pattern of cryo-sections with the S100 anti-serum (ALLAERTS *et al.*, 1995). Fig. 5 shows a cryo-section of FS cells localized in a cluster at the centre of the endocrine parenchyme compartment, and surrounding a follicle wherein microvillous protrusions are found. The ultrastructural characteristics, immunopositivity for S100 and topographical localization (Fig. 5a), indicate that this cell type corresponds to the classical FS cells, as described by VILA-PORCILE (1972). Recently, using low temperature resin (Lowicryl HM20) immunohistochemistry at the EM level, we could demonstrate $S100^+$ and $OX6^+$ cells with excellently preserved topography reminiscent of FS cells (ALLAERTS *et al.*, 1995).

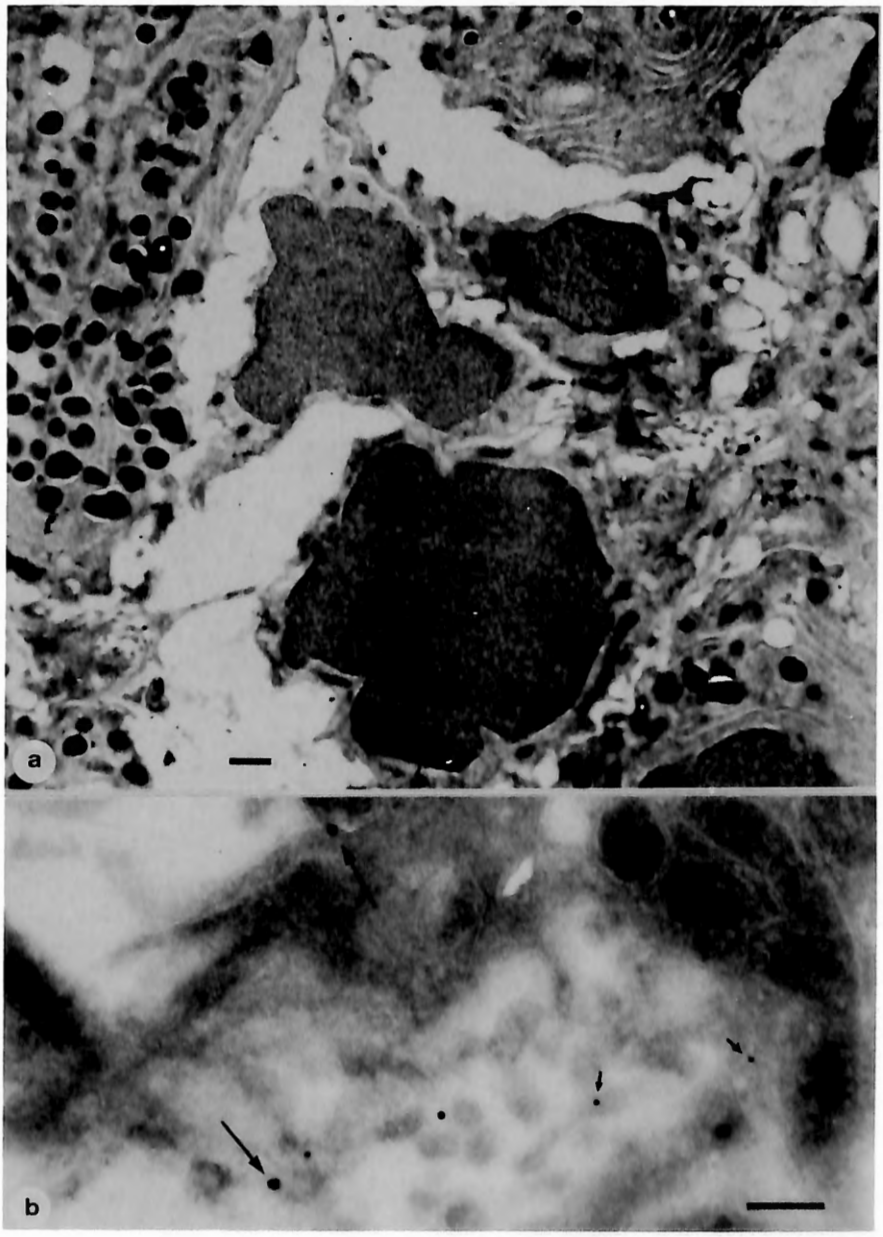


Fig. 5. — Topographical localization of FS cells at the ultrastructural level in cryo-sections of rat AP labelled with the OX6 moAb, showing a cluster of FS cells at the center of a parenchyme cord and surrounding a follicle (arrowhead) (5a) (× 14000 ; bar = 0.5 μm). Microvillous protrusions into the follicle lumen are labelled for the MHC-class II determinants (25 nm gold label ; longer arrows) and the S100 protein (15 nm gold label ; shorter arrows) (5b) (× 64000 ; bar = 0.2 μm).

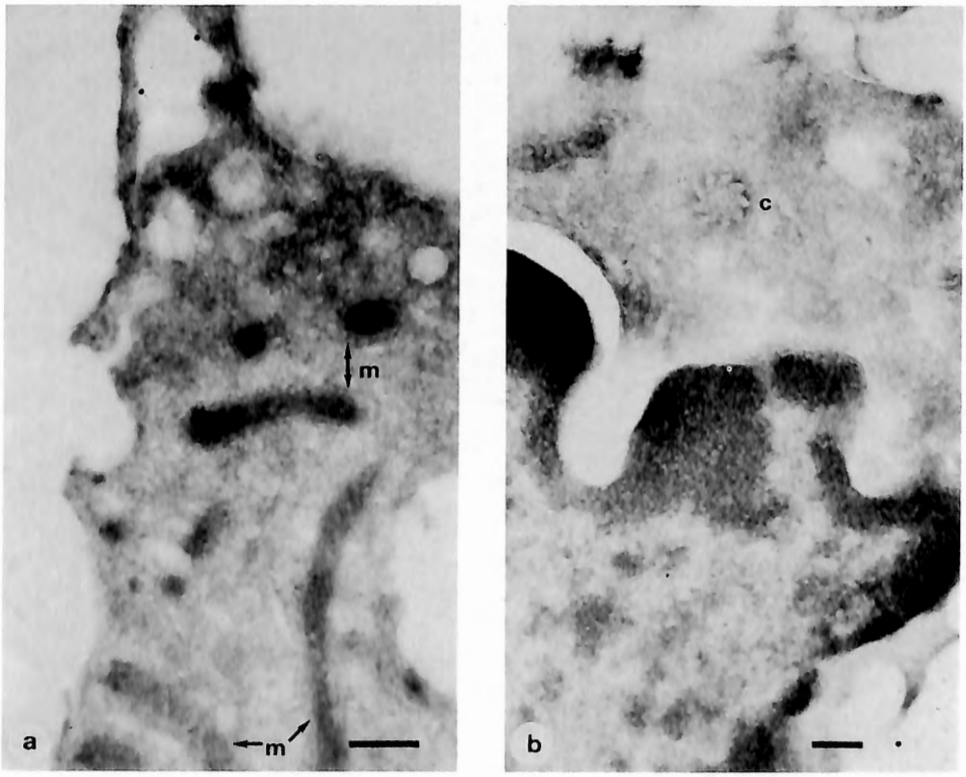


Fig. 6. — Immuno-EM image to demonstrate the ultrastructural characteristics of FS cells in cryo-sections of rat AP labeled with the OX6 moAb. The cells are labeled for the MHC-class II marker, and display long vermiform mitochondria (m) (6a) (× 58000) and a centriole (c) (6b) (× 42000) as described by VILA-PORCILE (1972) (Bars = 0.2 μm).

Further, using a double immunogold-labelling procedure with gold particles of 15 nm and 25 nm diameter respectively, we were able to identify cells with the third phenotype described above bearing both the S100 protein and the OX6 antigen (Fig. 5b, 8a). The larger gold particles labelling the MHC-class II molecules were found at the cell membrane and within the cytoplasm, whereas the smaller gold particles labelling the S100 protein were more numerous within the cell nucleus (Fig. 8a). Some protruding microvilli found within the follicular lumen of an FS cell cluster were labelled with the MHC-class II marker and the S100 protein (Fig. 5b). Occasionally, also cells with the second phenotype (cfr. above) were found immunopositive for both the S100 protein and the OX6 antigen (Fig. 8b).

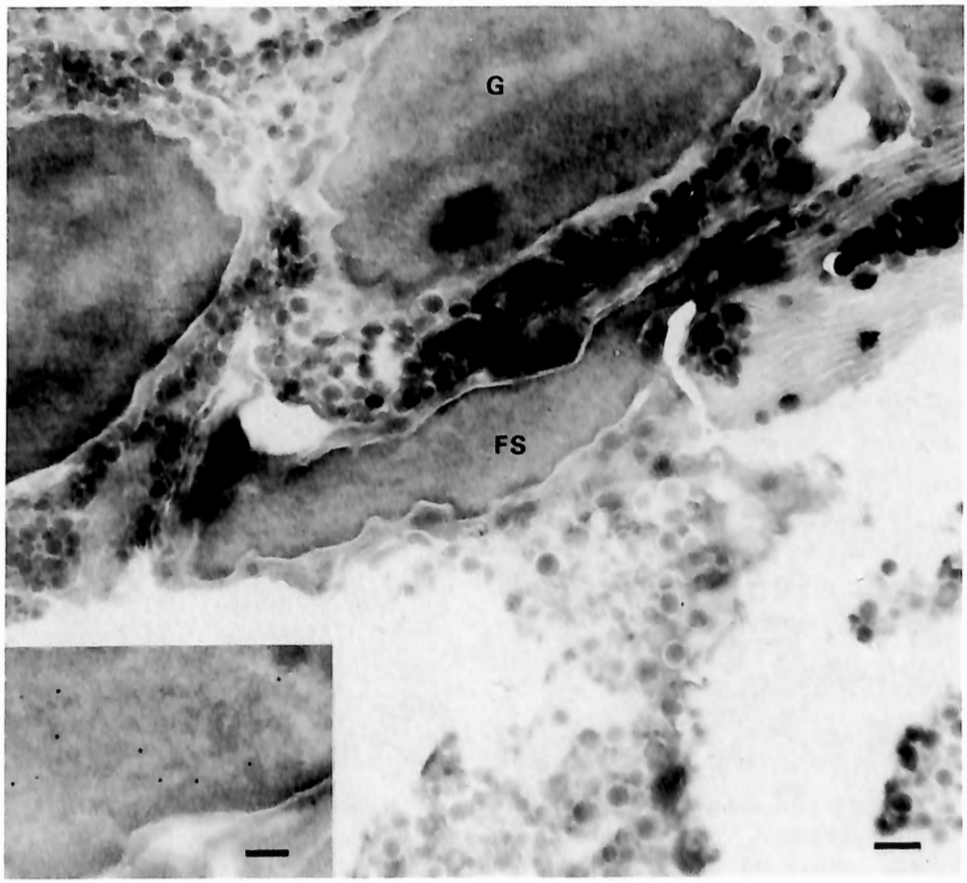


Fig. 7. — Immuno-EM of cryo-section of rat AP labelled with the polyclonal anti-S100 serum : example of FS cell (FS) surrounded by granulated endocrine cells (G) (× 14000 ; bar = 0.5 μm). The FS cell nucleus is abundantly labelled with the S100 antibodies (high power magnification in inset) (× 31000 ; bar = 0.2 μm).

Mixed leukocyte reaction assay with pituitary Dendritic Cells

In Fig. 9a the autologous stimulation of T lymphocyte proliferation by pituitary cells is shown. A cell population obtained from the interphase of the Nycodenz gradient corresponding with a DC enriched fraction of splenic cells and consisting of 34 % $S100^+$ and 25 % $OX6^+$ cells was capable of stimulating T lymphocyte proliferation for a stimulator-responder ratio as low as 1 :160. On the other hand, in the pellet phase of this Nycodenz gradient (remaining pituitary cell population with only 2 % $S100^+$ and 8 % $OX6^+$ cells) a stimulation was only observed for ratios larger than 1 :20.

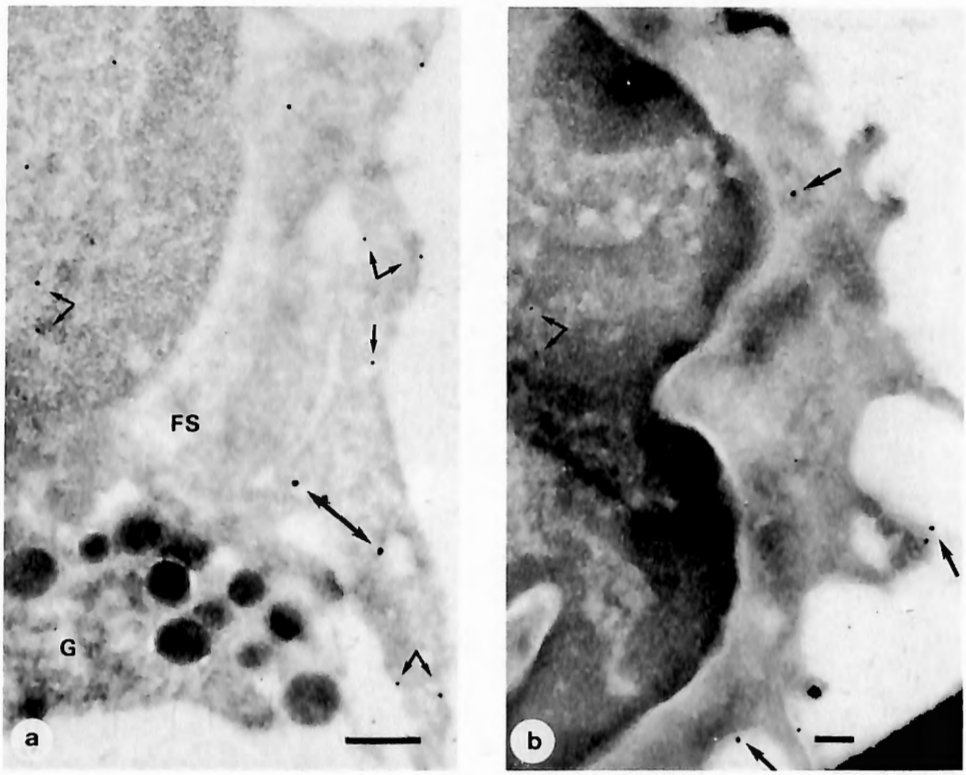
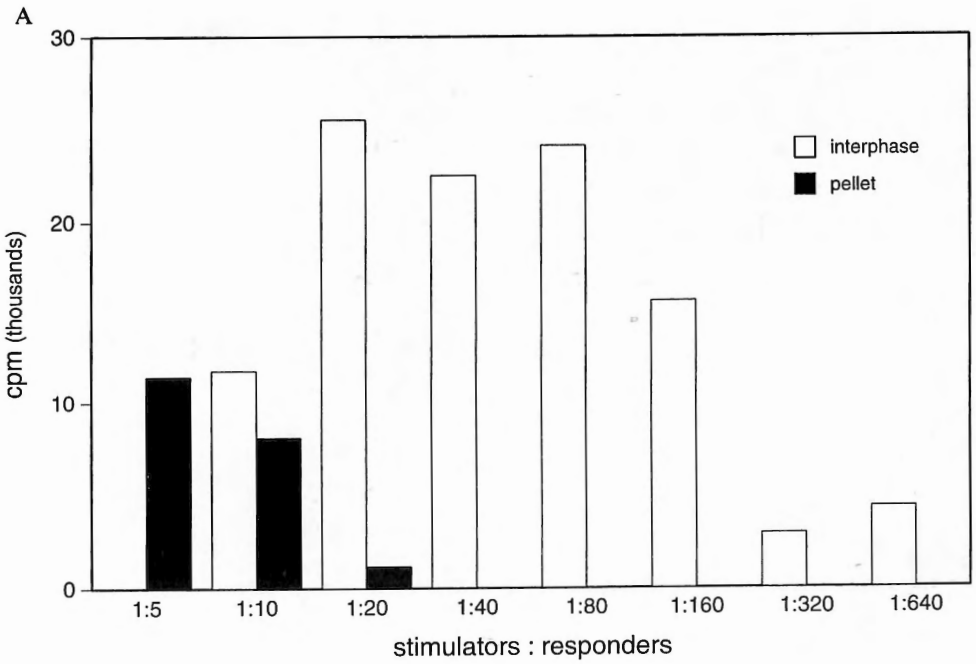


Fig. 8. — Double immuno-labelling of ultrathin cryo-sections of rat AP with the OX6 moAb (25 nm gold label; longer arrows) and the anti-S100 serum (15 nm gold label; shorter arrows). A FS cell (FS), reminiscent of the *type III* MHC-class II-expressing cell is shown in 8a : the cell is abundantly labelled for the S100 protein and weakly labelled for the MHC-class II determinants (× 60000). A *type II* cell is shown in 8b, labelled for the MHC-class II determinants, with only very little label for the S100 protein (× 32000). G : fragment of granulated endocrine cell (Bars = 0.2 μm).

The autologous stimulation of T lymphocyte proliferation by pituitary cells obtained by BSA gradient sedimentation is shown in Fig. 9b and c. Original populations of dispersed pituitary cells called « originals », and cell populations obtained by velocity sedimentation at 1 g (cfr. above) were able to stimulate T lymphocyte proliferation for stimulator-responder ratios varying from 1 :5 to 1 :320 (Fig. 9b). At the maximally effective stimulator-responder ratio, the cell populations of the sedimentation gradient were more effective in stimulating T lymphocyte proliferation as compared to the originals (Fig. 9c). The combined fractions 1 and 2 were the most potent stimulatory fraction in this assay (Fig. 9c). The magnitude of maximal T lymphocyte proliferation, moreover, is highly correlated with either the proportional number of MHC-class II expressing cells or the proportional number of $S100^+$ cells (unpublished observations).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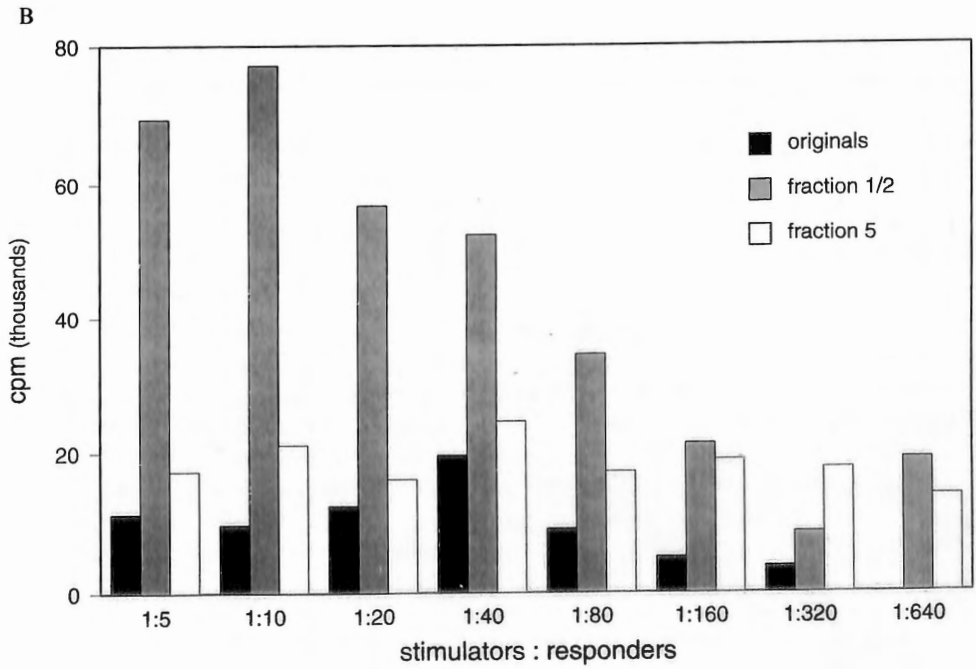
B
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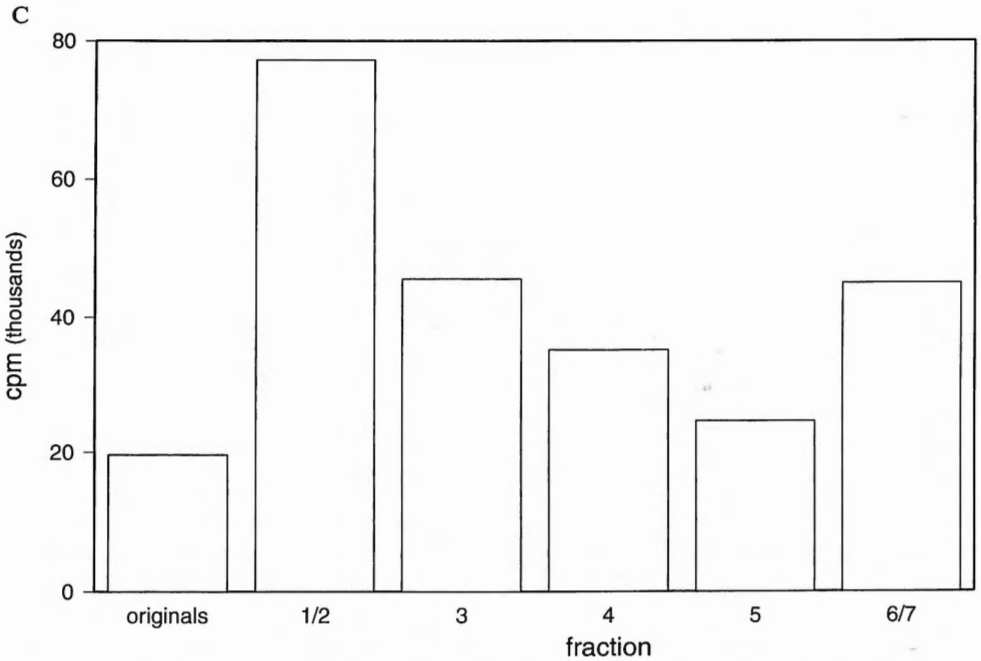

Fig. 9. — Mixed leukocyte reaction assay with pituitary dendritic cells. A. T cell proliferation induced by pituitary cells separated by Nycodenz gradient in stimulator : responder ratio between 1 :5 and 1 :640. B. T cell proliferation induced by pituitary cell populations (original population, BSA gradient fractions 1 plus 2 and BSA gradient fraction 5). C. T cell proliferation induced by pituitary cell populations at maximally effective stimulator : responder ratio (representative experiment out of 3 similar experiments).

DISCUSSION

In the present paper a review is given of the myeloid markers found in mouse, rat and human anterior pituitary as previously described (ALLAERTS *et al.*, 1991, 1993, 1995). We previously treated the problem of the (partial) overlap between the different cell populations immunopositive for markers of the monocyte-macrophage-dendritic cell lineage (ALLAERTS *et al.*, 1995). The present ultrastructural investigation of $S100^+$ and MHC-class II expressing cells in the rat AP is suggestive for the occurrence of three distinct immunopositive phenotypes within the morphological spectrum of folliculo-stellate (FS) and/or dendritic cells (DC) in the AP. Hitherto, this cell group has been regarded as either a resident pituitary cell population (folliculo-stellate cells ; VILA-PORCILE, 1972) or as a lymphoid cell group which may also occur in almost any non-lymphoid organ (dendritic cells ; STEINMAN and COHN, 1973, KLINKERT *et al.*, 1982). Moreover, no conclusive evidence has been brought forward with regard to the embryological origin of the FS cell group (ALLAERTS *et al.*, 1995), although a myeloid origin of the DC cell group is commonly accepted (KAMPERDIJK *et al.*, 1994). The heterogeneity of the FS cell group

has already been proposed, based on ultrastructural (VILA-PORCILE, 1972) or immunohistochemical findings (TACHIBANA and YAMASHIMA, 1988 ; NAKAGAWA *et al.*, 1985). The presence of DC markers in the mouse, rat and human AP, the fact that $S100^+$ FS cells and MHC class II expressing DC of the rat AP co-sediment within the upper gradient fractions of a velocity gradient (ALLAERTS *et al.*, 1993) and the occurrence of double immunolabelled cells at the EM level (ALLAERTS *et al.*, 1995 ; present data) are suggestive for a (partial) overlap between the two cell groups. Moreover, the present finding of an immune-accessory function of pituitary cells *in vitro*, which is most prominent in the S100-enriched and MHC-class II enriched cell population obtained by gradient sedimentation, demonstrates that these cells are immunocompetent cells comparable to lymphoid DC. Because of the lower numbers of DC marker expressing cells in the AP compared to the numbers of $S100^+$ FS cells, the overlap between the two cell groups is only partial, and this fact adds to the heterogeneity of the FS cell group (ALLAERTS *et al.*, 1995).

It remains to be elucidated whether the heterogeneity of the FS cell group as revealed by ultrastructural and immunohistochemical characteristics is also a functional heterogeneity. To date, no information is available concerning the stability of the immunohistochemical markers and functional states of the FS cell group during normal and pathological development. The possibility of transient differentiation stages within the FS and DC group is substantiated by the occurrence of intermediate forms between the FS and DC phenotype, that also express immunohistochemical markers of the two cell groups (present data).

The presence of DC in the AP, that moreover may act as immune-accessory cells, raises the question of the functional significance of this cell group. We previously demonstrated using an *in vitro* culture system that FS cells communicate with granulated cells of the pituitary parenchyme (BAES *et al.*, 1987 ; ALLAERTS and DENEF, 1989) and FS cells are the likely producers of IL-6 in the AP (VANKELECOM *et al.*, 1989, 1993). It was suggested that FS cells may exert an endocrine regulatory function as well as an « immune defense » function (ALLAERTS *et al.*, 1990). A remarkable synchronization in the rat AP during development of IL-6 producing (CARMELIET *et al.*, 1991) and $S100^+$ (SHIRASAWA *et al.*, 1983) and MHC-class II expressing cells was previously observed (ALLAERTS *et al.*, 1993). In the AP of an autoimmune-prone rat model, the BB rat, an increased number of $S100^+$ and MHC class II expressing cells was found, compared to the Wistar and Lewis rat AP (unpublished observations). Whereas in the thyroid (VOORBIJ *et al.*, 1990) and pancreatic islets (VOORBIJ *et al.*, 1989) of the BB rat DC play a crucial role in initializing autoimmune thyroiditis or insulitis, respectively, it is unknown whether BB rats may also develop an autoimmune adenohypophysitis. Autoimmune hypophysitis has been described in humans (MAUERHOFF *et al.*, 1987) and an animal model of adenohypophysitis resulting from injecting AP homogenates in the rat was developed by LEVINE (1967) (reviewed in ALLAERTS and DREXHAGE, 1994). Recently, it was reported that injection of the S100β specific T cell lines activated with the S100β protein in naive Lewis rats resulted in experimental allergic encephalitis (KOJIMA *et al.*, 1994). The involvement of DC in the initiation of endocrine autoimmune disease (KNIGHT *et al.*, 1988) opens a new perspective on the immune sur-

veillance function of the FS and DC cell group in the AP, apart from their role as IL-6 producing cells (VANKELECOM *et al.*, 1989). The expression of IL-6 mRNA and IL-6 receptor mRNA in the rat pituitary (SCHÖBITZ *et al.*, 1992 ; VELKENIERS *et al.*, 1994), thereby has been interpreted as part of an inflammation process with a central pituitary effect (SCHÖBITZ *et al.*, 1992 ; REICHLIN, 1993), or was interpreted as the manifestation of local paracrine or autocrine communication processes, which may dysfunction in pituitary adenomas (TSAGARIKIS *et al.*, 1992 ; VELKENIERS *et al.*, 1994).

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REFERENCES

ALLAERTS, W., D.M. FLUITSMA, E.C.M. HOEFSMIT, P.H.M. JEUCKEN, H. MORREAU, F.T. BOSMAN, and H.A. DREXHAGE (1995) — Immunohistochemical resemblance between dendritic cells and folliculo-stellate cells in normal human and rat anterior pituitaries. *Journal of Neuroendocrinology*, (in press).

ALLAERTS, W. and C. DENEF (1989) — Regulatory activity and topological distribution of folliculo-stellate cells in rat anterior pituitary cell aggregates. *Neuroendocrinology*, 49 : 409-418.

ALLAERTS, W. and H.A. DREXHAGE (1994) — Dendritic cells in autoimmune disease. *In : C.* BRUIJNZEEL-KOOMEN, E.C.M. HOEFSMIT, (Eds.) *Immunopharmacology of macrophages and other antigen-presenting cells.* Chapter 8. Academic Press Ltd., New York : 117-134.

ALLAERTS, W., P. CARMELIET and C. DENEF (1990) — New perspectives in the function of pituitary folliculo-stellate cells. *Mol. Cell Endocrinol.*, 71 : 73-81.

ALLAERTS, W., A.M.I. TIJSSEN, P.H.M. JEUCKEN, H.A. DREXHAGE and J. DE KONING (1994) — Influence of folliculo-stellate cells on biphasic luteinizing hormone secretion response to gonadotropin-releasing hormone in rat pituitary cell aggregates. *Eur. J. Endocrinol.*, 130 : 530-539.

ALLAERTS, W., P.H.M. JEUCKEN, L.J. HOFLAND and H.A. DREXHAGE (1991) — Morphological, immunohistochemical and functional homologies between pituitary folliculo-stellate cells and lymphoid dendritic cells. *Acta Endocrinol. (Copenh.)*, 125 : 92-97.

ALLAERTS, W., P.H.M. JEUCKEN, F.T. BOSMAN and H.A. DREXHAGE (1993) — Relationship between dendritic cells and folliculo-stellate cells in the pituitary : immunohistochemical comparison between mouse, rat and human pituitaries. *In :* E.W.A. KAMPERDIJK, P. NIEUWENHUIS, and E.C.M. HOEFSMIT, (Eds). *Dendritic cells in fundamental and clinical immunology*. New York, Plenum Press : 637-642.

ARKEMA, J.M.S., I.L. SCHADEE-EESTERMANS, D.M. BROEKHUIS-FLUITSMA and E.C.M. HOEFSMIT (1991a) — Localization of class II molecules in storage vesicles, endosomes and lysosomes in human dendritic cells. *Immunobiology*, 183 : 396-407.

ARKEMA, J.M.S., I.L. SCHADEE-EESTERMANS, R.H.J. BEELEN and E.C.M. HOEFSMIT (1991b) — A combined method for both endogenous myeloperoxidase and acid phosphatase cytochemistry as well as immunoperoxidase surface labeling discriminating human peripheral blood derived dendritic cells and monocytes. *Histochemistry*, 95 : 573-578.

AUDINOT, V., J. BRÉARD, L. CHEVALIER, D. CUSSAC, and C. KORDON (1992) — *Progress in NeuroEndocrinImmunology*, 5 : 1-12.

BAES, M., W. ALLAERTS and C. DENEF (1987) — Evidence for functional communication between folliculo-stellate cells and hormone-secreting cells in perifused anterior pituitary cell aggregates. *Endocrinology*, 120 : 685-691.

BIRBECK, M.S., A.S. BREATHNACH, and J.D. EVERALL (1961) — An electron microscope study of basal melanocytes and high-level clear cells (Langerhans cells) in vitiligo. *J. Invest. Dermatol.*, 37 : 51-64.

BRENAN, M. and M. PUKLAVEC (1992) — The MRC OX-62 antigen : a useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. *J. Exp. Med.*, 176 : 1457-1465.

CARMELIET, P., H. VANKELECOM, J. VAN DAMME, A. BILLIAU and C. DENEF (1991) — Release of interleukin-6 from anterior pituitary cell aggregates : developmental pattern and modulation by glucocorticoids and forskolin. *Neuroendocrinology*, 53 : 29-34.

COATES, P.J. and I. DONIACH (1988) — Development of folliculo-stellate cells in the human pituitary. *Acta Endocrinol. (Copenh.)*, 119 : 16-20.

COCCHIA, D. and N. MIANI (1980) — Immunocytochemical localization of the brain-specific S100 protein in the pituitary gland of adult rat. *J. Neurocytol.*, 9 : 771-782.

COCCHIA, D., F. MICHETTI and R. DONATO (1981) — Immunochemical and immunocytochemical localization of S-100 antigen in normal human skin. *Nature*, 294 : 85-87.

DE MEY, J., M. MOEREMANS, G. GEUENS, R. NUYDENS and M. DE BRABANDER (1981) — High resolution light and electron microscopic localization of tubulin with the IGS (immunogold staining) method. *Cell Biol. Int. Rep.*, 3 : 889.

DIJKSTRA, C.D., E.A. DÖPP, P. JOLING and G. KRAAL (1985) — The heterogeneity of mononuclear phagocytes in lymphoid organs : distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology*, 54 : 589-599.

DREXHAGE, H.A., H. MULLINK, J. DE GROOT, J. CLARKE and B.M. BALFOUR (1979) — A study of cells present in peripheral lymph of pigs with special reference to a type of cell resembling the Langerhans cell. *Cell Tissue Res.*, 202 : 407-430.

FITHIAN, E., P. KUNG, G. GOLDSTEIN, M. RUBENFELD, C. FENOGLIO and R. EDELSON (1981) — Reactivity of Langerhans cells with hybridoma antibody. *Proc. Natl. Acad. Sci. USA*, 78 : 2541-2544.

FREUDENTHAL, P.S. and R.M. STEINMAN (1990) — The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad. Sci. USA*, 87 : 7698-7702.

GON, G. (1987) — The origin of ciliated cell cysts of the anterior pituitary. An experimental study in the rat. *Virchows Arch. Abt. A. Pathol. Anat. Histopathol.*, 412 : 1-9.

GRIFFITHS, G. (1993) — *Fine structure immunocytochemistry*. Springer Verlag, Heidelberg.

HART, D.N.J. and J.W. FABRE (1981) — Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J. Exp. Med.*, 154 : 347-361.

HAVENITH, C.E.G., A.J. BREEDIJK, M.A.M. VERDAASDONK, E.W.A. KAMPERDIJK and R.H.J. BEELEN (1992) — An improved and rapid method for the isolation of rat lymph node or spleen T lymphocytes for T cell proliferation assays. *J. Immunol. Methods*, 153 : 73-79.

HOEFSMIT, E.C.M., A.M. DUIJVESTIJN, and E.W.A. KAMPERDIJK (1982) — Relation between Langerhans cells, veiled cells, and interdigitating cells. *Immunobiol.*, 161 : 255-265.

INABA, K. and R.M. STEINMAN (1987) — Monoclonal antibodies to LFA-1 and to CD4 inhibit the mixed leukocyte reaction after the antigen independent clustering of dendritic cells and lymphocytes. *J. Exp. Med.*, 165 : 1403-1417.

ISHII, Y., T. TAKAMI, Y. KOKAI, H. YUASA, J. FUJIMOTO, T. TAKEI and K. KIKUCHI (1985) — A novel human B-lymphocyte antigen shared with lymphoid dendritic cell : characterization by monoclonal antibody. *Clin. Exp. Immunol.*, 61 : 624-632.

KABEL, P.J., H.A.M. VOORBIJ, M. DE HAAN, R.D. VAN DER GAAG, and H.A. DREXHAGE (1988) — Intrathyroidal dendritic cells. *J. Clin. Endocrinol. Metab.*, 65 : 199-207.

KAMPERDIJK, E.W.A., E. VAN VUGT, C.D. RICHTERS and R.H.J. BEELEN (1994) — Morphology of dendritic cells. In : C.A.F.M. BRUIJNZEEL-KOOMEN, and E.C.M. HOEFSMIT (Eds). *Immunopharmacology of Macrophages and other antigen-presenting cells*. The handbook of Immunopharmacology Series, Academic Press, New York : 45-61.

KAMPERDIJK, E.W.A., M.L. KAPSENBERG, M. VAN DEN BERG and E.C.M. HOEFSMIT (1985) — Characterization of dendritic cells, isolated from normal and stimulated lymph nodes of the rat. *Cell Tissue Res.*, 242 : 469-474.

KLINKERT, W.E.F., J.H. LABADIE and W.E. BOWERS (1982) — Accessory and stimulating properties of dendritic cells and macrophages isolated from various tissues. *J. Exp. Med.*, 156 : 1-19.

KNIGHT, S.C., J. FARRANT, J. CHAN, A. BRYANT, P.A. BEDFORD and C. BATEMAN (1988) — Induction of autoimmunity with dendritic cells : studies on thyroiditis in mice. *Clin. Immunol. Immunopathol.*, 48 : 277-289.

KNIGHT, S.E., J. FARRANT, A.J. BRYANT, S. EDWARDS, S. BURMAN, A. LEVER, J. CLARKE and A.D. WEBSTER (1986) — Non-adherent, low-density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunology*, 57 : 595-603.

KNIGHT, S.C. (1984) — Veiled cells — « dendritic cells » of the peripheral lymph. *Immunobiol.*, 168 : 349-361.

KOJIMA, K., C. LININGTON, H. LASSMAN, and H. WEKERLE (1994) — Induction of experimental allergic encephalitis (EAE) by S100β : an autoantigen expressed in both the CNS and thymus. *J. Neuroimmunol.*, 54 : 176 (Abstract).

LEDBETTER, J.A. and L.A. HERZENBERG (1979) — Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.*, 47 : 63-90.

LEENEN, P.J.M., M. MELIS, G. KRAAL, A.T. HOOGEVEEN, and W. VAN EWIJK (1992) — Monoclonal antibody ER-BMDM1 recognizes a macrophage and dendritic cell differentiation antigen with aminopeptidase activity. *Eur. J. Immunol.*, 22 : 1567-1572.

LEVINE, S. (1967) — Allergic adenohypophysitis : new experimental disease of the pituitary gland. *Science*, 158 : 1190-1191.

MAUERHOFF, T., R. MIRAKIAN and G.F. BOTTAZZO (1987) — Autoimmunity and the pituitary. *In :* DONIACH, D. and G.F. BOTTAZZO (Eds). *Baillière's Clinical Immunology and Allergy*, Vol. 1 (1), London : Baillière Tindall : 217-235.

MCLEAN, J.W. and P.K. NAKANE (1974) — Periodate-lysine-paraformaldehyde fixative : a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.*, 22 : 1077-1083.

METLAY, J.P., M.D. WITMER-PACK, R. AGGER, M.T. CROWLEY, D. LAWLESS, and R. STEINMAN (1990) — The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.*, 171 : 1753-1771.

MOORE, B.W. (1965) — A soluble protein characteristic of the nervous system. *Biochem. Biophys. Res. Commun.*, 19 : 739-744.

NAKAGAWA, F., B.A. SCHULTE and S.A. SPICER, (1985) — Glycoconjugate localization with lectin and PA-TCH-SP cytochemistry in rat hypophysis. *Ann. J. Anat.*, 174 : 61-81.

NAKAJIMA, T., H. YAMAGUCHI and K. TAKAHASHI (1980) — S100 protein in folliculo-stellate cells of the rat pituitary anterior lobe. *Brain Res.*, 191 : 523-531.

NUSSENZWEIG, M.C., R.M. STEINMAN, J.C. UNKELESS, M.D. WITMER, B. GUTCHINOV, and Z.A. COHN (1981) — Studies of the cell surface of mouse dendritic cells and other leukocytes. *J. Exp. Med.*, 154 : 168-187.

POULTER, L.W., D.A. CAMPBELL, C. MUNROC and G. JANOSSY (1986) — Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scand. J. Immunol.*, 24 : 351-357.

REICHLIN, S. (1993) — Neuroendocrine-immune interactions. *N. Engl. J. Med.*, 329 : 1246-1253.

REINHERZ, E.L., P.C. KUNG, J.M. PESANDO, J. RITZ, G. GOLDSTEIN, and S.F. SCHLOSSMANN (1979) — Ia determinants on human T-cell subsets defined by monoclonal antibody. Activation stimuli required for expression. *J. Exp. Med.*, 150 : 1472-1482.

SANTIAGO-SCHWARZ, F., E. BELILOS, B. DIAMOND, and S.E. CARSONS (1992) — TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J. Leukoc. Biol.*, 52 : 274-281.

SCHECHTER, J., N. AHMAD and R. WEINER (1988) — Activation of anterior pituitary folliculo-stellate cells in the formation of estrogen-induced prolactin-secreting tumors. *Neuroendocrinology*, 48 : 569-576.

SCHÖBITZ, B., D.A.M. VOORHUIS and E.R. DE KLOET (1992) — Localization of interleukin-6 mRNA and interleukin-6 receptor mRNA in rat brain. *Neurosci. Letters*, 136 : 189-192.

SHIRASAWA, N., H. KIHARA, S. YAMAGUCHI and F. YOSHIMURA (1983) — Pituitary folliculo-stellate cells immunostained with S-100 protein antiserum in postnatal, castrated and thyroidectomized rats. *Cell Tissue Res.*, 231 : 235-249.

STEFANSSON, K., R.L. WOLLMANN, B.W. MOORE and B.G.W. ARNASON (1982) — S-100 protein in human chondrocytes. *Nature*, 295 : 63-64.

STEINMAN, R.M., D.S. LUSTIG and Z.A. COHN (1974) — Identification of a novel cell type in peripheral lymphoid organs of mice. III. Functional properties *in vivo*. *J. Exp. Med.*, 139 : 1431-1445.

STEINMAN, R.M. and M.C. NUSSENZWEIG (1980) — Dendritic cells : features and functions. *Immunol. Rev.*, 53 : 127-147.

STEINMAN, R.M. and Z.A. COHN (1973) — Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.*, 137 : 1142-1162.

TACHIBANA, O. and T. YAMASHIMA (1988) — Immunohistochemical study of folliculo-stellate cells in human pituitary adenomas. *Acta Neuropathol.*, 76 : 458-464.

TAKAHASHI, K., T. ISOBE, Y. OHTSUKI, T. AKAGI, H. SONOBE and T. OKUYAMA (1984) — Immunohistochemical study of the distribution of α and β of S-100 protein in human neoplasms and normal tissues. *Virchows Arch. [Cell. Pathol.]*, 45 : 385-396.

TEW, J., M.H. KOSCO, G.F. BURTON and A.K. SZAKAL (1990) — Follicular dendritic cells as accessory cells. *Immunol. Rev.*, 117 : 185-211.

THOMAS, R., L.S. DAVIS and P.E. LIPSKY (1993) — Isolation and characterization of human peripheral blood dendritic cells. *J. Immunol.*, 150 : 821-843.

TOKUYASU, K.T. (1973) — A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.*, 57 : 551-565.

TOKUYASU, K.T. (1978) — A study of positive staining of ultrathin frozen sections. *J. Ultrastruct. Res.*, 63 : 287-307.

TSAGARIKIS, S., G. KONTOGEORGOS, P. GIANNOU, N. THALASSINOS, J. WOOLLEY, G.M. BESSER and A. GROSSMAN (1992) — Interleukin-6, a growth promoting cytokine, is present in human pituitary adenomas : an immunocytochemical study. *Clinical Endocrinology*, 37 : 163-167.

UCCINI, S., D. VITOLO, A. STOPPACCIARO, D. PALIOTTA, A.M. CASSANO, P. BARSOTTI, L.P. RUCO and C.D. BARONI (1986) — Immunoreactivity for S100 protein in dendritic and in lymphocyte-like cells in human lymphoid tissues. *Virchows Arch. Abt. B. Cell Pathol.*, 52 : 129-141.

VANKELECOM, H., P. MATTHYS, J. VAN DAMME, H. HEREMANS, A. BILLIAU and C. DENEF (1993) — Immunocytochemical evidence that S-100-positive cells of the mouse anterior pituitary contain interleukin-6 immunoreactivity. *J. Histochem. Cytochem.*, 41 : 151-156.

VANKELECOM, H., P. CARMELIET, J. VAN DAMME, A. BILLIAU and C. DENEF (1989) — Production of interleukin-6 by folliculo-stellate cells of the anterior pituitary gland in a histiotypic cell aggregate culture system. *Neuroendocrinology*, 49 : 102-106.

VELKENIERS, B., P. VERGANI, J. TROUILLAS, J. D'HAENS, R.J. HOOGHE, and E.L. HOOGHE-PETERS (1994) — Expression of IL-6 mRNA in normal rat and human pituitaries and in human pituitary adenomas. *J. Histochem. Cytochem.*, 42 : 67-76.

VILA-PORCILE, E. (1972) — Le réseau des cellules folliculo-stellaires et les follicules de l'adénohypophyse du rat (pars distalis). *Z. Zellforsch*, 129 : 328-369.

VOORBIJ, H.A.M., P.H.M. JEUCKEN, P.J. KABEL, M. DE HAAN and H.A. DREXHAGE (1989) — Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. *Diabetes*, 38 : 1623-1629.

VOORBIJ, H.A.M., P.J. KABEL, M. DE HAAN, P.H.M. JEUCKEN, R.D. VAN DER GAAG, M.H. DE BAETS and H.A. DREXHAGE (1990) — Dendritic cells and class II MHC expression on thyrocytes during the autoimmune thyroid disease of the BB rat. *Clin. Immunol. Immunopathol.*, 55 : 9-22.

ZEID, N.A. and H.K. MULLER (1993) — S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. *Pathology*, 25 : 338-343.

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METHOD FOR CULTURE OF A BOVINE PULMONARY ENDOTHELIAL CELL STRAIN (CPAE) IN A SERUM-FREE MEDIUM AND ON MICROPOROUS MEMBRANES COATED WITH MATRIGEL™

by

SARAH BAATOUT (1), FABIENNE KINARD **, CLAUDE REMACLE **
and YVES-JACQUES SCHNEIDER *
* Laboratory of Cellular Biochemistry and ** Laboratory of Cell Biology,
University of Louvain, B-1348 Louvain-la-Neuve, Belgium

SUMMARY

In order to optimize culture conditions of bovine pulmonary endothelial cells (CPAE), we have compared different culture media, supports and extracellular matrices. Cell biomass was estimated by protein assay using Lowry's method. Different constituents including albumin, hydrocortisone, insulin, transferrin, triiodotyronine, Epidermal Growth Factor (EGF) and Fibroblast Growth Factor were separately added to Basal Defined Medium (BDM). Among them, BDM supplemented with EGF (1 ng/ml), hydrocortisone (100 nM), insulin (1 µg/ml), triiodotyronine (2 nM) and linoleic acid complexed to albumin (10 µg/ml) also named 'synthetic BDM' appeared to be the best serum-free nutritive medium and showed similar results as compared to DMEM supplemented with 10 % Fetal Bovine Serum (FBS).

Several supports have then been tested including tissue culture polystyrene (as a reference), teflon, polycarbonate or poly(ethylene terephthalate) track-etched membranes. Among them, cells cultivated on surface treated membranes in poly(ethylene terephthalate) exhibited the highest protein content with a significant increase in comparison to tissue culture polystyrene, probably because cells are fed on the two faces instead of one.

On treated poly(ethylene terephthalate) membranes, cells kept their endothelial morphology and ultrastructure.

Finally, cell biomass on several exogenous extracellular matrices was studied. Cells were cultivated in 'synthetic BDM' or DMEM supplemented with 10 % FBS and on poly(ethylene terephthalate) membranes. Among fibronectin, matrigel™ (solubilized tissue basement membrane), laminin, collagen (type I) and polylysine; matrigel™ appeared to be the optimal extracellular matrix.

(1) Correspondence to S. BAATOUT, Laboratory of Experimental Hematology and Oncology, Catholic University of Louvain, 1, rue G. Thérasse, B-5530 Mont-Godinne, Belgium.

In conclusion, we demonstrated herewith that bovine pulmonary endothelial cell cultures can be optimized in a serum-free medium and on microporous membranes with matrigel™ as an extracellular matrix without alterations of cell morphology.

Key words : CPAE cells, in vitro model, serum-free medium, microporous membranes, poly(ethylene terephthalate), extracellular matrix, matrigel.

INTRODUCTION

Alterations in the function of endothelial cells play an important role in the development of atherosclerosis (Ross *et al.*, 1974 ; Ross and Glomset, 1976). Cultivated endothelial cells provide a powerful approach with which to study the characteristics and function of the endothelium. However, the presence of serum in the nutritive medium hampers the precise determination of the effect of various components on their function. We have developed a new serum-free, chemically defined, Basal Defined Medium (BDM) which considerably increases the secretion of monoclonal antibody by hybridoma cells (Schneider, 1989 ; Schneider and Lavoix, 1990) ; with a low protein content it also allows the long-term (3 to 4 weeks) culture of rat hepatocytes (Jin *et al.*, 1989) or intestinal epithelial cells (Halleux and Schneider, 1991). Equally important is the nature of the support upon which cells rest. We have recently reported that the use of poly(ethylene terephthalate) microporous membranes improves the culture of various mammalian cells (Sergent-Engelen *et al.*, 1990). Moreover, the deposition of an extracellular matrix on the surface of the supports is necessary for the optimal growth of many adherent cells. Several kinds of coating have been proposed such as laminin, fibronectin, collagen (type I), polylysine or a solubilized tissue basement membrane gel matrigel™. These components have been shown to affect the ability of cells to proliferate (Timpl *et al.*, 1979 ; Jaffe, 1980 ; Kleinman *et al.*, 1981 ; Chazou *et al.*, 1981 ; Macarak and Howard, 1983).

Herewith, in order to optimize culture conditions of bovine pulmonary endothelial cells (CPAE), we present data about the effects of several components of the medium, different supports and several exogenous extracellular matrices on the biomass of bovine pulmonary artery endothelial (CPAE) cells. Based on these observations, we have developed a cell culture system using serum-free medium, poly(ethylene terephthalate) microporous membranes and matrigel™ as an exogenous extracellular matrix to grow and maintain endothelial cells under defined conditions.

Abbreviations used : BDM, Basal Defined Medium ; CPAE, pulmonary artery endothelial cells ; DMEM, Dulbecco's Modified Eagle's Medium ; EGF, Epidermal Growth Factor ; FBS, Fetal Bovine Serum ; FGF, Fibroblast Growth Factor ; Hydrocortis, Hydrocortisone ; Lin ac : alb, linoleic acid complexed to albumin ; mPET, membranes in Poly(ethylene terephthalate) ; nPC, native Polycarbonate ; nPET, native Poly(ethylene terephthalate) ; PET, Poly(ethylene terephthalate) ; sBDM, synthetic Basal Defined Medium ; T3, Triiodotyronine ; TCPS, Tissue Culture Polystyrene ; tmPET, treated membranes in Poly(ethylene terephthalate).

MATERIAL AND METHODS

Cell culture and subculture

The bovine pulmonary artery endothelial cell strain (CPAE) was obtained from the American Type Culture Collection (Rockville, Md). This cell strain has been characterized and shown to be endothelial in origin by angiotensin-converting enzyme activity, factor VIII related antigen and the presence of Weibel-Palade bodies (DEL VECCHIO and SMITH, 1981 ; DEL VECCHIO and LINCOLN, 1983). CPAE cells were maintained in 175-cm^2 flasks. For all experiments, cells were used between passages 11 and 20 and cultivated under water saturation and 5 % CO_2 (v/v) in air. CPAE cultures have been passed at a split ratio of 1:3 or 1:2 onto tissue culture polystyrene (Nunc, Intermed, Denmark). Daily phase contrast observations of morphology were used for assessing the culture state. Cell viability was assessed using trypan blue exclusion.

Culture medium

Basal Defined Medium (BDM ; SCHNEIDER, 1989) consists of a 5:5:1 (v/v/v) mixture of Iscove's Dulbecco's modified Eagle's, Ham's F12 and NTCT 135 media. To this, 25 mM glucose, 6 mM glutamine, 0.01 % (w/v) Pluronic F68, 50 µM ethanolamine, 25 mM HEPES, 3 g/l $NaHCO_3$ and 30 µg/ml penicillin and 70 µg/ml streptomycin were added. Routine culture was carried out in BDM supplemented with 1 % Fetal Bovine Serum (Gibco).

For some experiments, BDM was further supplemented with different substances : linoleic acid complexed to albumin I at two different concentrations (10 and 100 µg/ml, Gibco), insulin (1 µg/ml, Gibco), EGF (10 ng/ml, Gibco), FGF (50 ng/ml, Gibco), transferrin (10 µg/ml, Gibco), triiodotyronine or T3 (2 nM, Gibco), hydrocortisone (100 nM, Gibco), trace elements (Mn, Mo, Ni, Si, Sn and V) at the concentrations described by MCKEEHAN *et al.*, (1976). 'Synthetic BDM' (sBDM) corresponds to BDM supplemented with insulin, EGF, trace elements, ascorbic acid, alpha tocopherol, glucagon, prolactin, somatotropin, dexamethasone and linoleic acid complexed to albumin as previously used for rat hepatocytes (JIN *et al.*, 1989). Dulbecco's Modified Essential Medium (DMEM) supplemented with 10 % FBS was used as reference.

Biomass study by protein assay

Cells were harvested with trypsin (2.5 g/l) and EDTA (1.1 g/l) and seeded in each culture condition at an initial concentration of 50,000 living cells/cm^2. After different incubation periods at 37° C, cells were washed 3 times with PBS and dissolved twice in 1 ml of Na deoxycholate 1 % (w/v) previously adjusted to pH 11.3 with NaOH. Cell protein content was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard. Results are expressed in micrograms per cm^2 of growth area.

Preparation and coating of flasks

Cell cultures were carried out on different supports. For tissue culture polystyrene (TCPS), 6-well tissue culture plates (Nunc, Intermed, Denmark; growth area of 9.9 cm^2) were used. Polycarbonate and poly(ethylene terephthalate) exist as films (native polycarbonate nPC and native poly(ethylene terephthalate) nPET), as microporous (poly(ethylene terephthalate) membranes mPET or as treated poly(ethylene terephthalate) membranes tmPET. m and tmPET membranes were kindly provided by Whatman s.a. (Louvain-la-Neuve, Belgium). Inserts were prepared as previously described (HALLEUX and SCHNEIDER, 1991). Teflon flasks (Petriperm) were provided by Heraeus (Danau, FRG).

To assess the effect of exogenous extracellular matrices, culture substrata were precoated for 2 h at 37°C with laminin (10 µg/ml, Sigma), fibronectin (10 µg/ml, Gibco), polylysine (10 µg/ml, Sigma), type I collagen (30 µg/ml, Sigma) or matrigel™ (5 µl diluted 10 times in BDM, Flow Lab., Brussels, Belgium) and rinsed with PBS just before use.

Scanning electron microscopy

For ultrastructural studies, monolayers growing on microporous membranes were washed twice in PBS, fixed *in situ* with 2.5 % (w/v) glutaraldehyde in PBS for 1 h and rinsed twice with PBS, postfixed with osmium tetroxide (2 % w/v in PBS) for 1 h and rinsed 3 times with PBS. Samples were dehydrated with acetone in a critical point dryer (Balzers Union, Lichtenstein) and sprayed with gold (Balzers SCD040) before examination with a Hitachi S570 scanning electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

ANOVA 2 analysis and Newman-Keuls test were used. Results are expressed as the mean of 6 experiments ± SEM.

RESULTS

Effect of different medium components on CPAE biomass

The effect of different components classically added to nutritive medium on CPAE biomass was assessed by determining the protein content and compared to BDM, on one hand, and to DMEM supplemented with 10 % FBS, on the other hand (Fig. 1). When cells were cultivated in BDM supplemented with linoleic acid complexed to albumin, biomass was in the same order of magnitude as cells grown in the presence of BDM alone. Insulin and transferrin were found to be slightly mitogenic since biomass was significantly increased in their presence ($p < 0.05$). The addition of EGF or FGF to BDM augmented significantly CPAE growth in comparison to cells cultivated in the presence of BDM supplemented with trans-

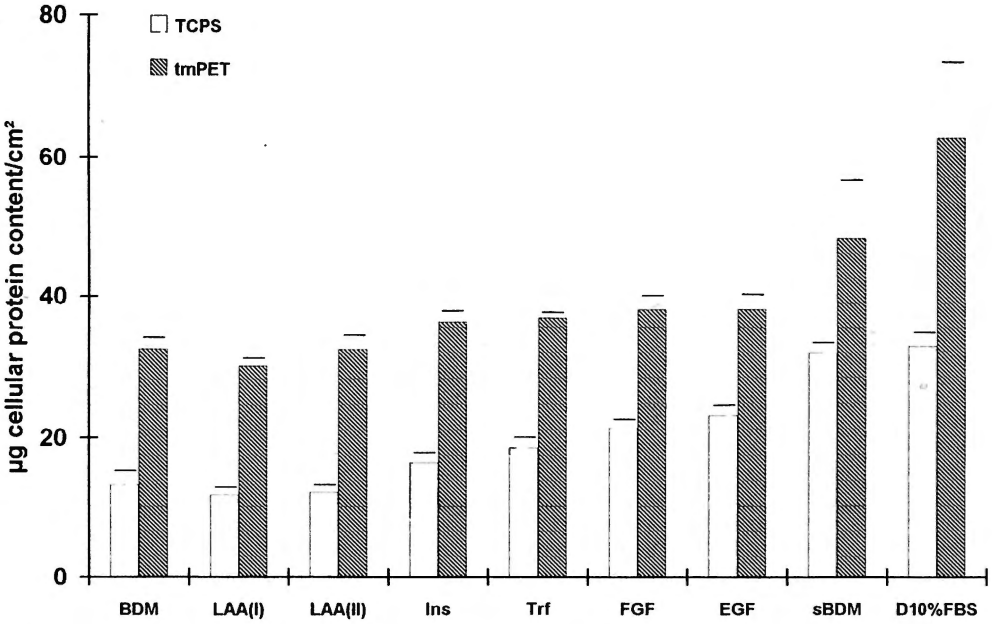

Fig. 1. — Influence of different components of the nutritive medium on the biomass of CPAE cells cultivated either on tissue culture TCPS or on tmPET. The final concentrations were linoleic acid complexed to albumin (10 µg/ml : LAAI; 100 µg/ml : LAAII), insulin (Ins : 1 µg/ml), transferrin (Trf : 10 µg/ml), FGF (50 ng/ml), EGF (10 ng/ml). Protein content (µg/cm^2) was measured after 36 h of culture. Synthetic BDM and DMEM 10 % FBS are abbreviated as sBDM and D10 % FBS, respectively.

ferrin or insulin ($p < 0.05$). The highest protein contents were obtained when the medium was either sBDM or DMEM supplemented with 10 % FBS ($p < 0.05$). DMEM was used as a control because this medium is known to be adequate for CPAE culture (GARCIA *et al.*, 1986).

In a second set of experiments, CPAE cells were cultivated in BDM but for each individual test, different components including some of the sBDM medium were added and a comparison was made with DMEM supplemented with 10 % FBS (Fig. 2). Results indicated that there was a significant difference between BDM alone and BDM supplemented with either triiodotyronine, trace elements, hydrocortisone or linoleic acid complexed to albumin ($p < 0.05$). EGF and insulin gave quantitatively similar results and were shown to be more effective than BDM supplemented with either triiodotyronine, trace elements, hydrocortisone or linoleic acid complexed to albumin ($p < 0.05$). Although there was a more important difference than in Fig. 1, the difference between sBDM and DMEM 10 % FBS was not significant. This difference can be explained by the fact that in Fig. 1, cells were at passage 12 and in Fig. 2, they were at passage 15.

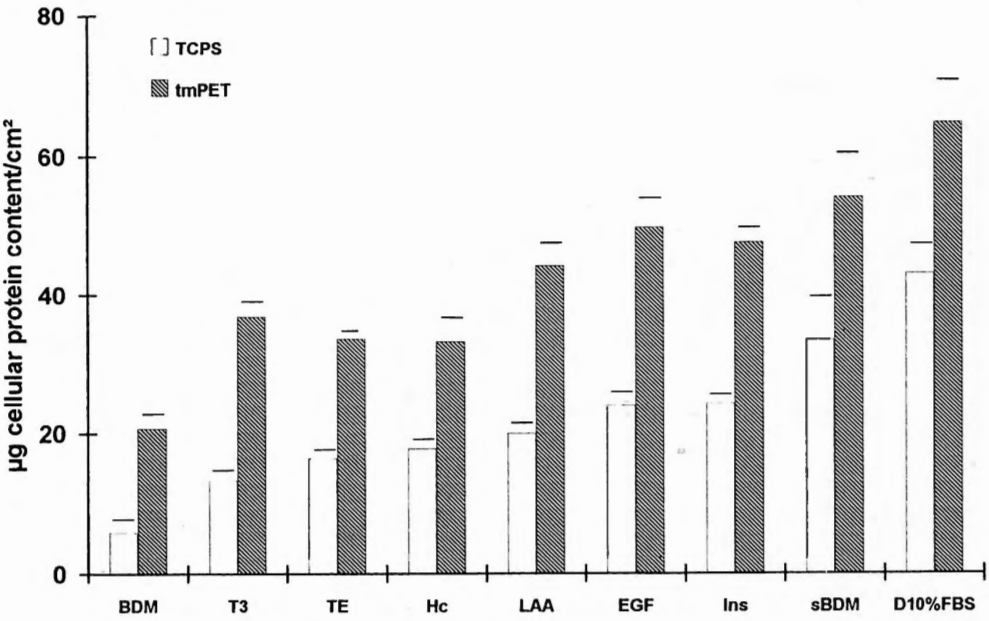

Fig. 2. — Influence of some components including sBDM constituents on the biomass of CPAE cells cultivated on TCPS or on tmPET. The final concentrations were hydrocortisone (Hc : 100 nM), linoleic acid complexed to albumin (LAA : 10 µg/ml), triiodotyronine or T3 (2 nM), insulin (Ins : 1 µg/ml), EGF (1 ng/ml). Protein content (µg/cm^2) was measured after 36 h of culture. Synthetic BDM and DMEM 10 % FBS are abreviated as sBDM and D10 %FBS, respectively.

Effect of different supports on CPAE growth

Figs 1 and 2 show that in every situation the cell yield was higher on microporous PET membranes than on TCPS dishes. Selective advantages of other substrata were investigated (Fig. 3) on CPAE cells at passage 17. Tissue culture polystyrene (TCPS) was used as reference. Teflon, polycarbonate (PC) or poly(ethylene terephthalate) (PET) in the form of film or microporous membranes were tested. Teflon gave the lowest protein content. No significant difference was detected when cells were cultivated either on TCPS, PC films or PET membranes. However, the PET films gave a significant higher protein content ($p < 0.05$). TmPET produced the highest CPAE biomass ($p < 0.05$). These results indicate that the use of tmPET considerably increased the protein content. Comparable results were observed for sBDM and DMEM supplemented with 10 % FBS.

Effect of different extracellular matrices on CPAE growth

To characterize the effect of different extracellular matrix proteins on the biomass of CPAE cells, protein assay measurements were performed on cells cultivated on fibronectin, matrigel™, laminin, type I collagen or polylysine. These

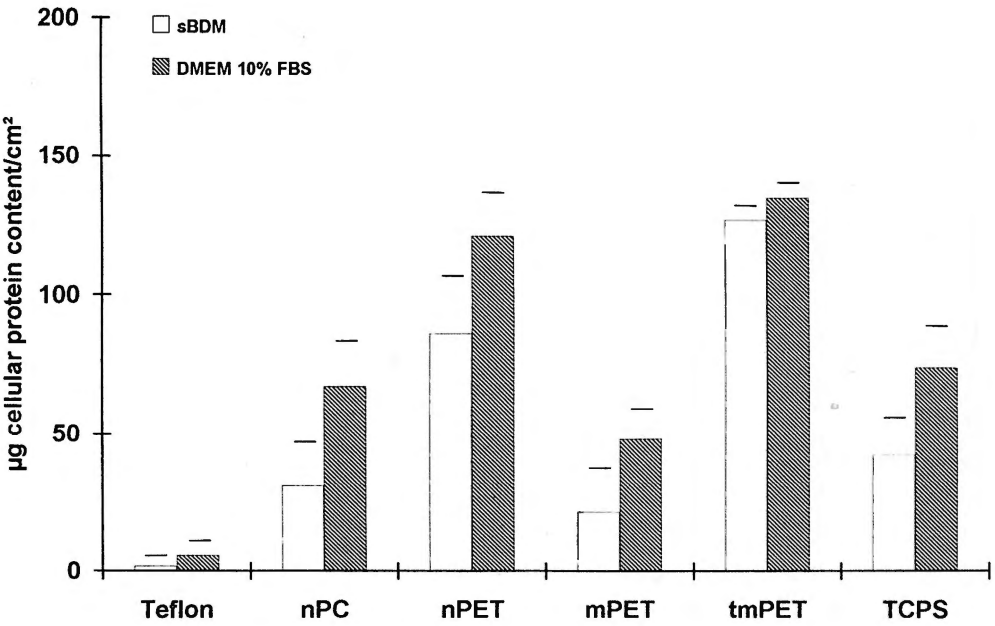

Fig. 3. — Comparison of CPAE cells biomass after 4 days of culture on different supports and either in sBDM or in DMEM supplemented with 10 % FBS. The supports were teflon membranes, polycarbonate (nPC) or PET (nPET) films, treated and nontreated PET membranes (tmPET or mPET, respectively) and tissue culture polystyrene (TCPS).

experiments were performed on TCPS (Fig. 4) and on tmPET (Fig. 5) and involved both sBDM and DMEM containing 10 % FBS. Extracellular matrices without cells were shown to exhibit a protein content which never exceeded 5 % of the total protein content.

When cells were cultivated on TCPS (Fig. 4) coated with laminin or matrigel™, no significant increase in protein content was detected in comparison to TCPS without precoating. A significantly enhanced protein content ($p < 0.05$) was detected with polylysine, fibronectin and collagen as compared to TCPS without precoating.

In comparison with noncoated tmPET, no significant increase was observed with fibronectin or laminin. In contrast, after precoating of tmPET with collagen or polylysine, a significant increase in protein content was observed as compared to noncoated tmPET ($p < 0.05$). The highest yield was recorded with matrigel™ as an exogenous extracellular matrix ($p < 0.05$).

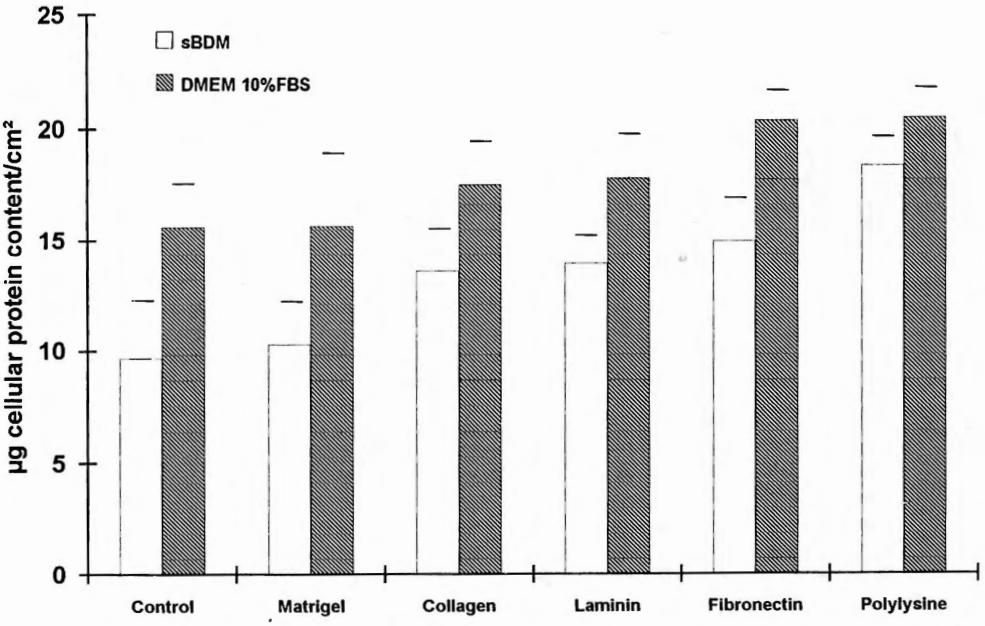

Fig. 4. — Comparison of CPAE cells biomass after a 36 h culture on TCPS precoated with different extracellular matrices and either in sBDM or DMEM supplemented with 10 % FBS. The used extracellular matrices were fibronectin (10 µg/ml), laminin (10 µg/ml), type I collagen (30 µg/ml), polylysine (10 µg/ml) and matrigel™ (dilution : 10 ×).

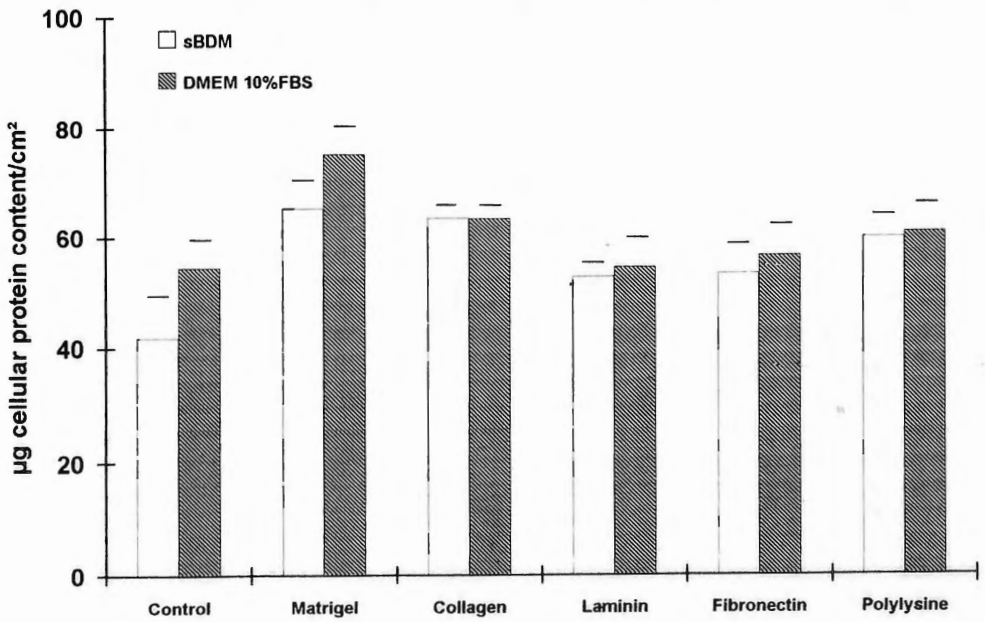

Fig. 5. — Comparison of CPAE cells biomass after a 36 h culture on tmPET precoated with different extracellular matrices, in either sBDM or DMEM supplemented with 10 % FBS. The used extracellular matrices were fibronectin (10 µg/ml), laminin (10 µg/ml), type I collagen (30 µg/ml), polylysine (10 µg/ml) and matrigel™ (dilution : 10 ×).

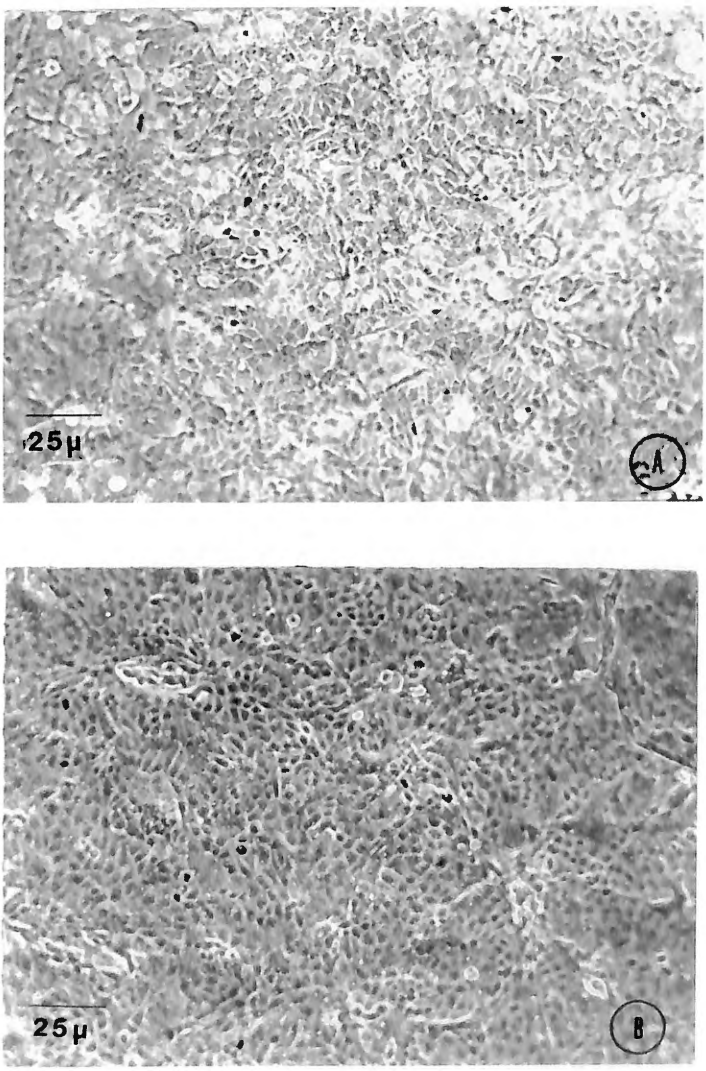

Fig. 6. — Phase contrast micrographs of CPAE cells cultivated for 5 days in BDM supplemented with 1 % FBS on TCPS (A) or tmPET (B).

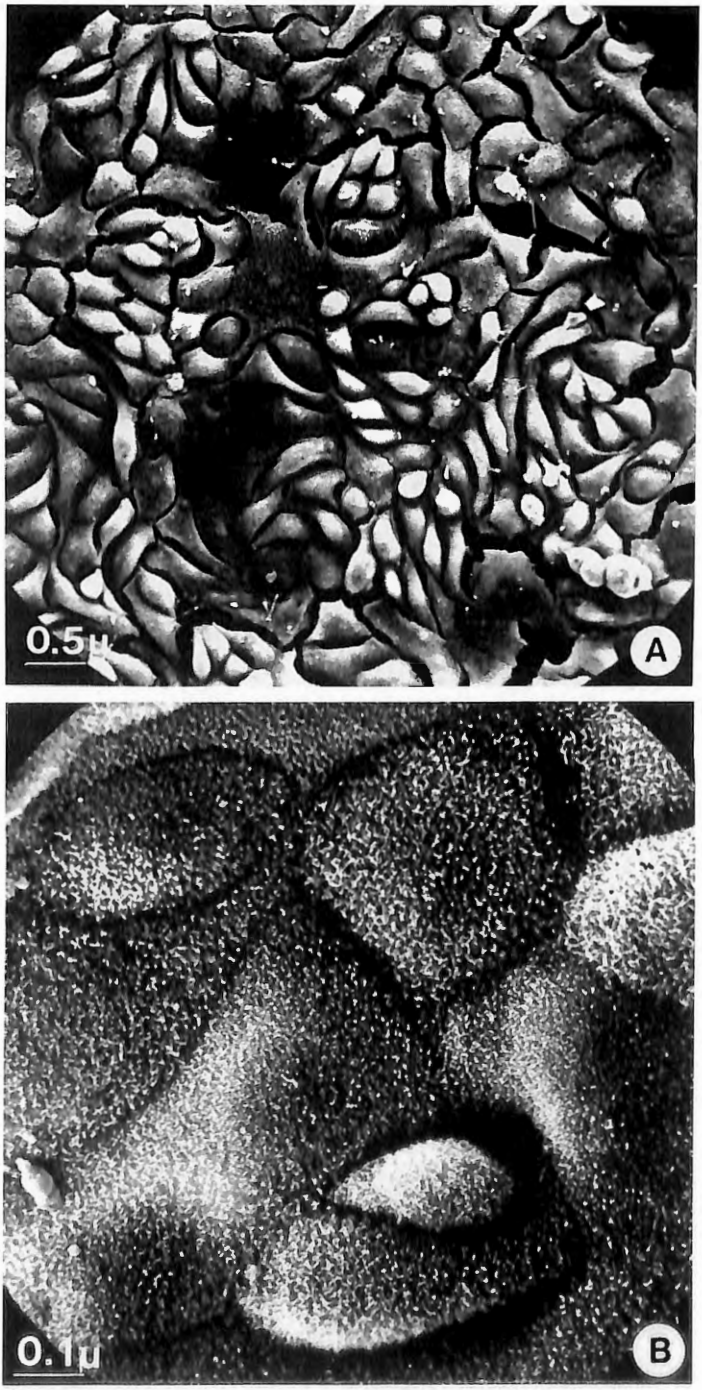

Fig. 7. — Scanning electron micrographs of CPAE cells cultivated for 5 days in BDM supplemented with 1 % FBS on tmPET.

Morphological and ultrastructural characterizations of CPAE cells on TCPS and on treated membranes of poly(ethylene terephthalate)

Fig. 6 gives representative pictures of CPAE cells after five days of culture in BDM containing 1 % FBS on TCPS (Fig. 6a) or tmPET (Fig. 6b). CPAE cells covered each substratum showing a confluent layer of cells with a characteristic cuboidal configuration.

Scanning electron microscopy of the surface of a complete CPAE cell monolayer grown on tmPET revealed an intimate association of the cells with the typical cobblestone appearance after five days of culture (Figs 7a and b), despite some diversity in the cell configuration.

DISCUSSION

The use of a cell culture system as an *in vitro* model of the vascular endothelium has a number of advantages, such as offering direct access to luminal and abluminal fluid for analysis, being highly simplified and limited to a single cell type and having an experimental medium that can be defined in terms of the chemical and biological composition. However, the factors that simplify the system also raise questions about its relevance to the real *in vivo* situation.

We varied several aspects of the culture conditions in an attempt to optimize CPAE culture. This cell strain is a well-characterized strain of bovine pulmonary endothelial cells. The effects of different conditions on the biomass of CPAE cells were measured by protein quantification (LOWRY *et al.*, 1951).

In order to optimize culture conditions of bovine pulmonary endothelial cells (CPAE), as a first step, the effect of different components of the nutritive medium on the biomass of CPAE was studied. Among all the media tested, albumin had only a minor effect, which agrees with the observations of DE GROOT *et al.* (1983). Albumin acts as a binding protein for fatty acid and also as a detoxifying agent in a manner similar to that postulated for transferrin (GUILBERT and ISCOVE, 1976). Hydrocortisone and triodotyronine displayed a slight effect on CPAE cell biomass. Hydrocortisone is known to improve the morphology of RF1 cells (a line of rat ovarian follicular cells) but does not affect their growth rate while it stimulates the epithelial cell line TM4 (HAYASHI *et al.*, 1987). The concentration of insulin required for stimulation of CPAE growth is high to reach an IGF-1 effect (HAYASHI *et al.*, 1987). Most of the stimulatory activity of transferrin is presumably related to its iron binding property. Insulin and transferrin are known to stimulate several cell types (BARNES and SATO, 1980). EGF and FGF have been widely used and are reported to stimulate growth of a variety of cell lines including endothelial cells, HeLa and BALB 3T3 cells.

CPAE cells grow well in a serum-free synthetic medium. sBDM medium is a synthetic nutritive medium originally developed for optimal growth of hybridoma cells and monoclonal antibody secretion (SCHNEIDER, 1989 ; SCHNEIDER and LAVOIX, 1990) ; it contains mineral cations, vitamins, coenzymes, nucleotide precursors, high

concentrations of glucose, amino acids, and antioxidant substances. After addition of appropriate hormones and growth factors, it also sustains growth and differentiation of anchorage dependent, polarized mammalian cells such as hepatocytes, MDCK, Hep G2 or CHO cells (JIN *et al.*, 1989).

The use of surface treated microporous membranes in PET as support significantly improves the biomass of CPAE cells. Culture on these membranes gives better biomass than on tissue culture polystyrene or polycarbonate (SERGENT-ENGELEN *et al.*, 1990). These results suggest that the improvement is due, on the one hand, to the physicochemical nature of the substratum surface since cell adhesion and biomass are enhanced on surface-treated PET as compared to the native polymer and, on the other hand, to the microporosity since for a same surface treatment, culture is improved on a microporous membrane as compared to the nonporous substratum. The PET membranes are also chemically inert and do not adsorb lipophilic solutes. In these culture conditions, CPAE cells expose their apical pole to the upper compartment of the insert, whereas their basal pole adheres to the PET membrane and is, therefore, in contact with the lower compartment. Such a model could therefore be appropriate for permeability studies of substances through the endothelial layer.

The substrate upon which cells rest when maintained in tissue culture is important for their biomass. The extracellular matrix provides a natural substrate upon which cells can undergo their characteristic changes in morphology occuring at mitosis as well as their differentiation. The endothelial cell is normally adherent to a complex basement membrane extracellular matrix consisting of type IV collagen, laminin, fibronectin, vitronectin, heparan sulfate proteoglycan, entactin, thrombospondin and von Willebrand factor (KRAMER *et al.*, 1987). We found that CPAE were able to proliferate on diverse extracellular matrices. Fibronectin is abundant in both vascular basement membrane and the surrounding interstitium and is a major extracellular connective tissue component. The protein content of CPAE cells was enhanced when culture occurs on TCPS precoated with fibronectin (10 μg/ml) and suggests that fibronectin is an important cell spreading factor. This substrate requirement of CPAE has also been found by others (GOLD and PEARLSTEIN, 1980). Either on surface treated PET membranes or on TCPS, we found an advantage in using collagen. Among the various components of the extracellular matrix, collagen is known to play an important role in promoting cell attachment, cell migration and cell biomass (GOSPODAROWICZ and LUI, 1981). Binding proteins for type I collagen and laminin have been observed in a bovine aortic endothelial cell-derived line. A laminin precoating either on TCPS or tmPET did not improve CPAE cells biomass. Polylysine has been found to improve attachment and clonal growth of human and chicken fibroblasts (MCKEEHAN *et al.*, 1976). In our experiments, polylysine gave a significant increase in the total protein content of CPAE cells. Matrigel™ is a solubilized basement membrane gel extracted from the mouse Engelbreth-Holm-Swarm sarcoma. Cultures of human endothelial cells can be induced to undergo extremely rapid morphological differentiation into capillary-like structures by matrigel™. When cells were cultivated on treated PET membranes, matrigel™

gave the highest protein content whereas precoating of TCPS did not affect CPAE cell biomass.

In light microscopy, the cells formed a continuous monolayer on TCPS and on surface treated PET membranes. The ultrastucture was similar on TCPS and on treated PET membranes.

In conclusion, in a synthetic hormono-defined nutritive medium, on treated PET membranes precoated with matrigel™, culture of CPAE cells gave excellent results. Moreover, under these conditions, cells keep their endothelial morphology and ultrastructure.

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REFERENCES

BARNES D. and G. SATO (1980) — Methods for growth of cultivated cells in serum-free medium. *Anal. Bioch.*, **102** : 255-270.

CHAZOU E.I., A.V. ALEXEEV, A.S. ANTONOV, V.E. KOTELIANSKY, V.L. LEYTIN, E.V. LYUBIMOVA, V.S. REPIN, D.D. SVIRIDOV, V.P. SVIRIDOV, V.P. TORCHILIN and V.N. SMIRNOV (1981) — Endothelial cell culture on fibrillar collagen : model to study platelet adhesion and liposome targeting to intracellular collagen matrix. *Proc. Natl. Acad. Sci. USA*, **78** : 5603-5607.

DE GROOT P.G, C. WILLEMS, M.D. GONSALVES., W.G. VAN AKEN and J.A. VAN MOURIK (1983) — The proliferation of human umbilical vein endothelial cells in serum-free medium. *Thromb. Res.*, **31** : 623-634.

DEL VECCHIO P.J. and J.R. SMITH (1981) — Expression of angiotensin converting enzyme in cultivated pulmonary artery endothelial cells. *J. Cell Physiol.*, **108** : 337-345.

DEL VECCHIO P.J. and D.W. LINCOLN (1983) — Phenotypes of cultivated endothelium expression of angiotensin-converting enzyme activity. In : *The endothelial cell — A pluripotent control cell of the vessel wall. D.G.S. Thilo-Korner and R.I. Freshney, eds. S. Karger Publishers, Basel* : 67-83.

GARCIA J.G.N., A. SIFLINGER-BIRNBOIM, R. BIZIOS, P.J. DEL VECCHIO, J.W. FENTON and A.B. MALIK (1986) — Thrombin-induced increase in albumin permeability across the endothelium. *J. Cell Physiol.*, **128** : 96-104.

GOLD L.I. and E. PEARLSTEIN (1980) — Fibronectin-collagen binding and requirement during cellular adhesion. *Biochem. J.*, **186** : 551-559.

GOSPODORAWICZ D. and G.M. LUI (1981) — Effect of substrata and fibroblast growth factor on the proliferation in vitro of bovine aortic endothelial cells. *J. Cell. Physiol.*, **109** : 69-81.

GUILBERT L.J. and N.N. ISCOVE (1976) — Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell culture. *Nature.*, **263** : 594-595.

HALLEUX C. and Y.J. SCHNEIDER (1991) — Iron absorption by intestinal epithelial cells : 1. $CaCo_2$ cells cultivated in serum-free medium, on poly(ethylene terephthalate) microporous membranes, as an in vitro model. *In vitro Cell. Dev. Biol.*, **27a(4)** : 293-302.

HAYASHI I., J. LARNER and G. SATO (1987) — Method for growth of TM4 cells in a serum-free medium. *In vitro*, **34** : 23-30.

JAFFE E.A. (1980) — Culture of human endothelial cells. *Transplant. Proc.*, **88** : 473-485.

JIN Y., A. BACQUET, A. FLORENCE and Y.J. SCHNEIDER (1989) — Desferrithiocin and desferrioxamine B : cellular pharmacology and storage iron immobilisation. *Biochem. Pharmacol.*, **38** : 3233-3240.

KLEINMAN H.K., R.J. KLEBE and G.R. MARTIN (1981) — Role of collagenous matrices in the adhesion and growth of cells. *J. Cell. Biol.*, **88** : 473-485.

KRAMER R.H., K.G. BENSCH and M.A. KARASEK (1987) — Isolation and characterization of human vascular endothelial cells with application to studies of the subendothelial matrix. In : *Cell separation : Methods and selected applications*, vol. 5. T.G. Pretlow II and T.P. Pretlow, eds Academic Press, Orlando, FL : 1-27.

LOWRY O.H., N.J. ROSEBROUGH and A.L. FARR (1951) — Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193** : 265-275.

MACARAK E.J. and P.S. HOWARD (1983) — Adhesion of endothelial cells to extracellular matrix proteins. *J. Cell. Physiol.*, **116** : 76-86.

MCKEEHAN W.L., W.G. HAMILTON and R.G. HAM (1976) — Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **73** : 2023-2026.

ROSS R., J.A. GLOMSET, B. KARIYA and L. HARKER (1974) — A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc. Natl. Acad. Sci. USA*, **71** : 1207-1210.

ROSS R. and J.A. GLOMSET (1976) — The pathogenesis of atherosclerosis (part one and part two). *N. Engl. J.* Med. **295,** 369-377 and 420-427.

SCHNEIDER Y.-J. (1989) — Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium. *J. Immunol. Methods*, **116** : 65-77.

SCHNEIDER Y.-J. and A. LAVOIX (1990) — Monoclonal antibody production in semi-continuous serum- and protein-free culture : effect of glutamine concentration and culture conditions on cell growth and antibody secretion. *J. Immunol.*, **129** : 251-268.

SERGENT-ENGELEN T., C. HALLEUX, E. FERRAIN, *et al.* (1990) — Improved cultivation of polarized animal cells on culture inserts with new transparent polyethylene terephthalate or polycarbonate microporous membranes. *Biotechnol. Techniques*, **4** : 89-94.

TIMPL R., H. ROHDE, P. GEHRAN-ROBEY, S.I. RENNARD, J.M. FOIDARD and G.R. MARTIN (1979) — Laminin a glycoprotein from basement membrane. *J. Biol. Chem.*, **254** : 9333-9937.

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DAPHNIA MAGNA STRAUS LIVING IN AN AERATED SEWAGE LAGOON AS A SOURCE OF CHITIN : ECOLOGICAL ASPECTS

by

HENRY-MICHEL CAUCHIE (1), LUCIEN HOFFMANN (2,3), MARIE-FRANCE JASPAR-VERSALI (1), MERCÈDES SALVIA (3) and JEAN-PIERRE THOME (1)

(1) Université de Liège, Institut de Zoologie, Service de Morphologie, Systématique et Ecologie Animales (Unité d'Ecotoxicologie), 22, Quai Van Beneden — B-4020 Liège

(2) Université de Liège, Département de Botanique, Laboratoire d'Algologie, de Mycologie et de Systématique Expérimentale, B22, Sart Tilman — B-4000 Liège

(3) Cellule d'Hydrobiologie, CRP — Centre Universitaire, 162a, Avenue de la Faïencerie, L-1511 Luxembourg

SUMMARY

As a means of determining the suitability of microcrustaceans living in aerated lagoons as a source of chitin, the biomass of these animals was measured twice monthly from October 1993 to November 1994 in a lagoon receiving the waste waters of the town of Differdange (G.D. of Luxembourg). The production and chitin content of *Daphnia magna* Straus, the dominant species of the zooplanktonic community (by number and biomass), were assessed over the same period. From December to February, the microcrustacean biomass was low, varying from 0.035 to 0.440 $g.m^{-2}$. High biomass values were observed in April and June (about 4.3 $g.m^{-2}$) as a consequence of phytoplankton blooms and an increase in temperature. In November, despite the scarcity of algal food, the *Daphnia magna* biomass peaked up to 5.5 $g.m^{-2}$ due to the ability of these organisms to use bacteria and detritus for food. Throughout the sampling period, the *Daphnia magna* biomass far exceeded the *Cyclops* spp biomass.

Fluctuations in the daily chitin production of *Daphnia magna* paralleled the biomass fluctuations of the species, with maximum values measured in April (1.9 $g.m^{-2}.day^{-1}$) and June (4.8 $g.m^{-2}.day^{-1}$). The chitin content of *Daphnia magna* ranged from 2.9 to 7.0 % of the total body dry weight. By the summation of the daily chitin productions of the species, the annual chitin production of *Daphnia magna* was estimated at 11.5 $g.m^{-2}.year^{-1}$. This figure is compared with the chitin production of other crustaceans in the natural environment.

Prospects for using *Daphnia magna* living in the studied aerated sewage lagoon as a source of chitin are discussed.

Key words : *Daphnia magna*, sewage lagoon, chitin, production.

INTRODUCTION

Chitin is the second most abundant biopolymer in the world after cellulose (SANDFORD, 1988). Chitin and its derivative chitosan have found numerous applications in agriculture and industry, principally in the cosmetic, pharmaceutical, and biomedical sectors (SANDFORD, 1988). Consequently there is an increasing interest in chitin in all forms and of all degrees of purity. The current sources of chitin for industry are crab shells (*Cancer magister*, *Chionectes japonica*, and *Paralithodes camschatica*) and shrimp shells (*Pandalus borealis*) (HIRANO, 1988 ; SANDFORD, 1988). This kind of raw material presents, however, some disadvantages, such as (1) a high degree of mineralization which necessitates drastic acid treatment (often resulting in partial hydrolysis of the chitin) (BRINE and AUSTIN, 1981) and (2) variations in the chemical composition according to the sex, moulting stage, or age. Because of these disadvantages, investigators seek alternative chitin sources, such as less calcified crustaceans. In this context, JEUNIAUX *et al.* (1993) have estimated at 2.3×10^9 T the total amount of chitin produced by crustaceans each year. This estimate, however, does not take into account the amount produced in artificial ecosystems such as aerated sewage lagoons, believed to be adequate sources of chitinous raw material because they generally support large biomasses of planktonic crustaceans such as copepods and cladocerans (DINGES, 1976 ; SEVRIN-REYSSAC, 1993 ; ULHMANN *et al.*, 1994). Furthermore, the cuticle mineralization rate of such zooplankters is low (BAUDOUIN and RAVERA, 1972). The commonly acknowledged major steps in lagoon treatment are (1) aerobic bacterial decomposition of organic wastes resulting in the release of nutrients, (2) algal utilization of the nutrients (DINGES, 1976). Filter feeding zooplanktonic organisms can develop in these lagoons thanks to a sufficient oxygen concentration (> 1 mg/l — SHAPIRO, 1990) and to abundant algal (LAMPERT, 1974 ; GOPHEN, 1977) and bacterial (MCMAHON and RIGLER, 1965 ; PETERSON *et al.*, 1978) food. Zooplankton standing crops are often considerable and are mainly composed of *Daphnia magna*, which is generally the dominant species in lagoons (SEVRIN-REYSSAC, 1993). At the present time, these zooplankters are used as fertilizers in aquaculture, baits for fishing and animal food, mainly in aquaculture (SEVRIN-REYSSAC, 1994). Therefore, their use as a source of chitin for special chemical or medical applications would greatly enhance their commercial value.

In this perspective, the aim of this study was to evaluate the advantages of exploiting sewage lagoon microcrustaceans as a chitin source. The following criteria were examined : (1) the seasonal availability of microcrustaceans, especially *Daphnia magna*, and some limnological factors affecting its production, (2) the production and chitin content of *Daphnia magna*. The annual chitin production of this species was evaluated and compared with the chitin production in different natural environments.

DESCRIPTION OF THE SITE

The studied lagoon (60,000 m^2; 2.5 m mean depth) is located at Differdange (Grand-Duchy of Luxembourg). It collects the waste waters of the town after primary treatment. The mean resident time is approximately 50 days. The lagoon is continuously aerated. The planktonic crustacean community is composed of the cladoceran *Daphnia magna* Straus and of cyclopoid copepods of the genus *Cyclops* Müller. Rotifers are seldom abundant during the year. The zooplankton is harvested and sold as aquarium food by the company Bioplancton.

MATERIAL AND METHODS

Temperature, oxygen concentration and chlorophyll a concentration measurements

The temperature and oxygen concentration of the water were measured with a probe (WTW Oxi 196, Germany) on each sampling date. The chlorophyll a concentration was determined spectrophotometrically at 663 nm after extraction with 90 % acetone (APHA, 1985).

Sampling, biomass, and daily production of zooplankton

From October 1993 to November 1994, water samples were collected twice monthly at nine evenly spaced stations with a 3-liter van Dorn bottle at a depth of 1.5 m. The samples for zooplankton counts were filtered through a net with a mesh size of 60 µm and fixed in 4 % formalin. The individuals of the various taxon were counted in each sample in order to estimate a mean population density as described by BOTTRELL *et al.* (1976). The biomass was calculated from the estimated population density and the length-weight relationship of the studied taxon as developed by Dumont et al. (1975). The daily production of *Daphnia magna* was calculated on the basis of the formula of WINBERG *et al.* (1971) including 15 size classes (250µm wide) :

$$P = \Sigma(N_x.\Delta W_x) / D_x$$

where P is the daily production (g dry weight. $m^{-2}.day^{-1}$), N_x the number of individuals in size class x (m^{-2}), ΔW_x the weight increase of an individual growing from the size class x to the next size class (g dry weight), and D_x the development time of size class x (in days), determined from a growth curve established by MITCHELL *et al.* (1992). The daily secondary production values are expressed in $g.m^{-2}.day^{-1}$ rather than in $g.m^{-3}.day^{-1}$ because of their dependence on primary production which is a surface process. To standardize our results, we express biomass values also on the basis of area. The biomass values per cubic metre can be obtained by dividing the biomass value expressed per square metre by the mean depth (2.5 metres).

Daphnia magna chitin production

On each sampling date, the daily chitin production was evaluated on the basis of the daily production value and the chitin content of the specimens. The annual chitin production of *Daphnia magna* was estimated by summing the daily chitin productions. Between sampling dates, the daily chitin production was estimated by linear interpolation of the results obtained for sampling dates.

To determine the chitin content, we first freeze-dried the raw material (daphnids 1 mm long). Demineralization was performed with 0.5 N HCl at room temperature and proteins were extracted with 1 N NaOH at 100 ° C. The chitin content was assayed by a colorimetric method based on measuring the amount of N-Acetyl-D-Glucosamine monomers liberated after complete enzymatic hydrolysis by purified chitinases (Reissig *et al.*,1955 ; Jeuniaux, 1963, 1965). Assays were carried out with commercial chitinases (E.C. 3.2.1.14) and N-Acetyl-D-Glucosaminidase (diluted lobster serum).

RESULTS

Water temperature and oxygen concentration

In the course of a year, the water temperature varied in sinusoidal fashion (Fig. 1). The temperature was below 10° C from November to May and above 10° C from May to October. The extreme values were recorded in December (1.7° C) and July (25° C).

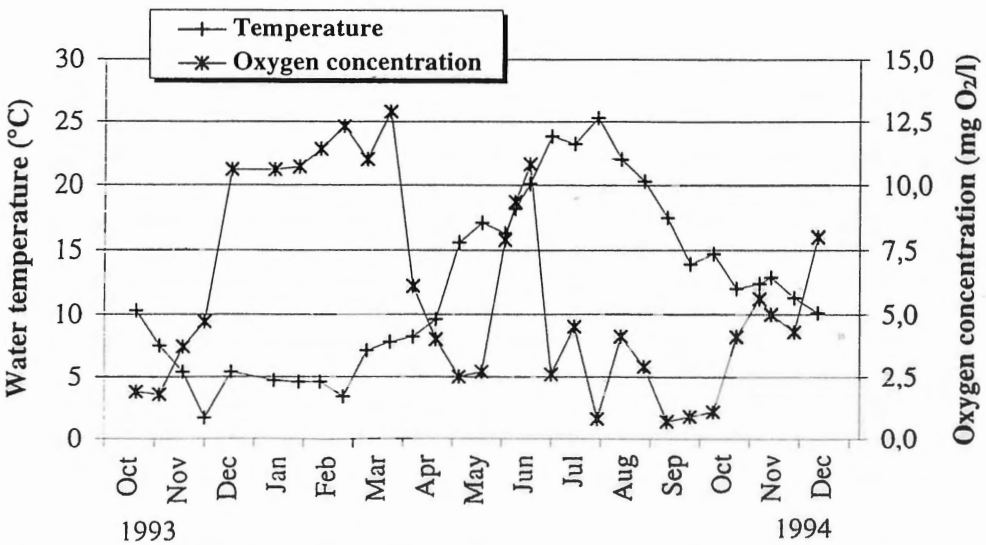

Fig. 1. — Seasonal changes in temperature and oxygen concentration of the water in the sewage lagoon of Differdange from October 1993 to December 1994.

From October to April, the oxygen concentration increased from 1.8 to 12.9 mg $0_2.l^{-1}$ (Fig. 1). Then it dropped to 2.5 mg $O_2.l^{-1}$ at the beginning of May. The oxygen concentration peaked a second time in June at 10.8 mg $O_2.l^{-1}$. From June to November, the oxygen concentration fluctuated between 0.8 and 4.5 mg $O2.l^{-1}$.

Chlorophyll a concentration and crustacean biomass

The chlorophyll a concentration remained generally low (< 1 $\mu g.l^{-1}$); two chlorophyll a peaks were observed, however, one in March (330 $\mu g.l^{-1}$) and one in June (250 $\mu g.l^{-1}$) (Fig. 2). From July to December, the chlorophyll a concentration varied between 2 and 45 $\mu g.l^{-1}$.

The crustacean biomass was low during the winter, varying from 0.035 to 0.440 $g.m^{-2}$ in terms of dry weight (Fig. 2). From March to July, biomass peaks were observed at the beginning of April (maximal value : 4.3 $g.m^{-2}$) and at the beginning of June (maximal value : 4.3 $g.m^{-2}$). Through July and August, the biomass fluctuated sharply between 0.4 and 4.7 $g.m^{-2}$. From September to November, the crustacean biomass increased and reached the highest value measured over the entire year (6.7 $g.m^{-2}$).

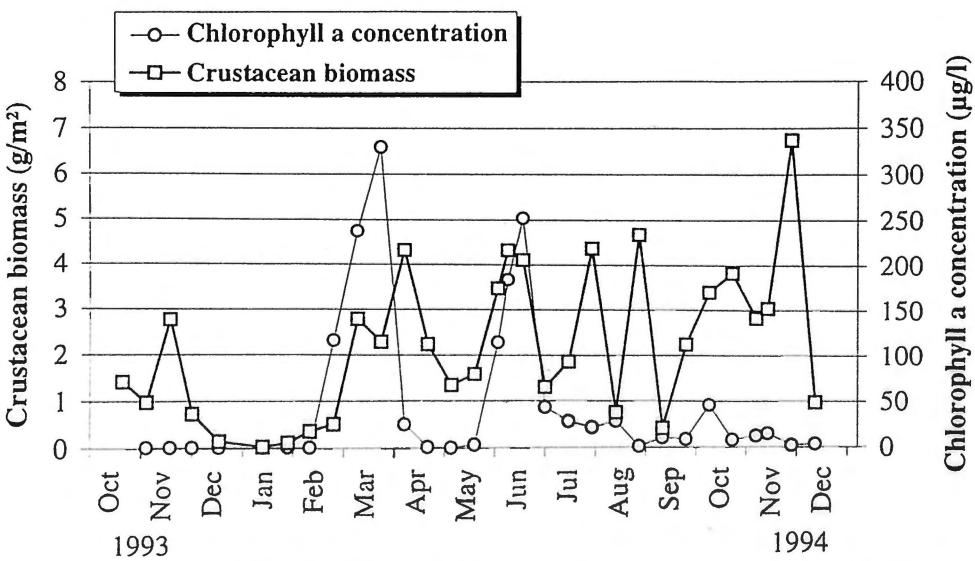

Fig. 2. — Seasonal changes in chlorophyll a concentration and microcrustacean biomass in the lagoon of Differdange from October 1993 to December 1994.

On the whole, the species-related results show that the *Daphnia magna* biomass far exceeded the *Cyclops* spp. biomass (Fig. 3). In winter, however, the biomass of *Cyclops* spp. increased earlier than that of *Daphnia magna*, probably owing to the revival from diapause of copepodits IV overwintering in the sediment (GEORGE,

1973). The observation that adults composed the majority of the population in January and February supports this explanation. Peaks of *Daphnia magna* biomass were recorded in April (3.7 $g.m^{-2}$), June (3.7 $g.m^{-2}$), July (4.3 $g.m^{-2}$), August (4.4 $g.m^{-2}$), and November (5.5 $g.m^{-2}$). *Cyclops* spp. biomass exhibited three peaks with values exceeding 0.8 $g.m^{-2}$: March (maximal value : 1.2 $g.m^{-2}$), June (maximal value : 1.0 $g.m^{-2}$), and from mid-September to the end of October (maximal value : 1.4 $g.m^{-2}$). The lowest biomasses were observed in January (0.035 $g.m^{-2}$), May (0.070 $g.m^{-2}$), and August (0.067 $g.m^{-2}$).

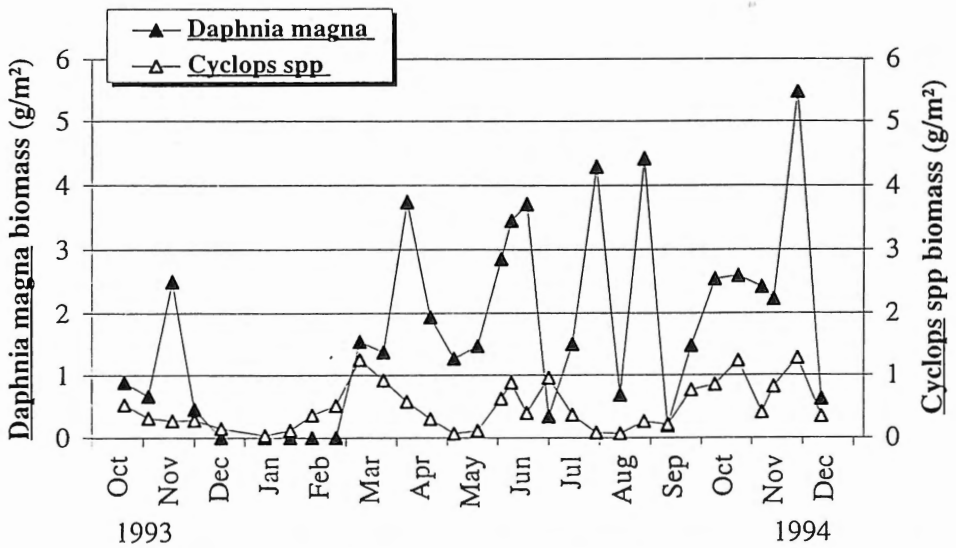

Fig. 3. — Seasonal changes in *Daphnia magna* biomass and *Cyclops* spp. biomass in the sewage lagoon of Differdange from October 1993 to December 1994.

Daphnia magna chitin production

To evaluate *Daphnia magna* chitin production, we first calculated the daily production of *Daphnia magna*, which fluctuated in the same manner as the biomass of the species (Fig. 4). In winter, the daily production was virtually nil. The highest values were recorded in April (maximal value : 1.9 $g.m^{-2}.day^{-1}$), June (maximal value : 4.8 $g.m^{-2}.day^{-1}$), and October (maximal value : 1.7 $g.m^{-2}.day^{-1}$). The calculated annual production of *Daphnia magna* was 234 $g.m^{-2}.year^{-1}$, *i.e.* 94 $g.m^{-3}.year^{-1}$. The highest ratio of daily production (P) to biomass (B), i.e. the highest productivity, was recorded in June (P/B = 1.4). Moreover, 35 % of the annual production occurred during this month.

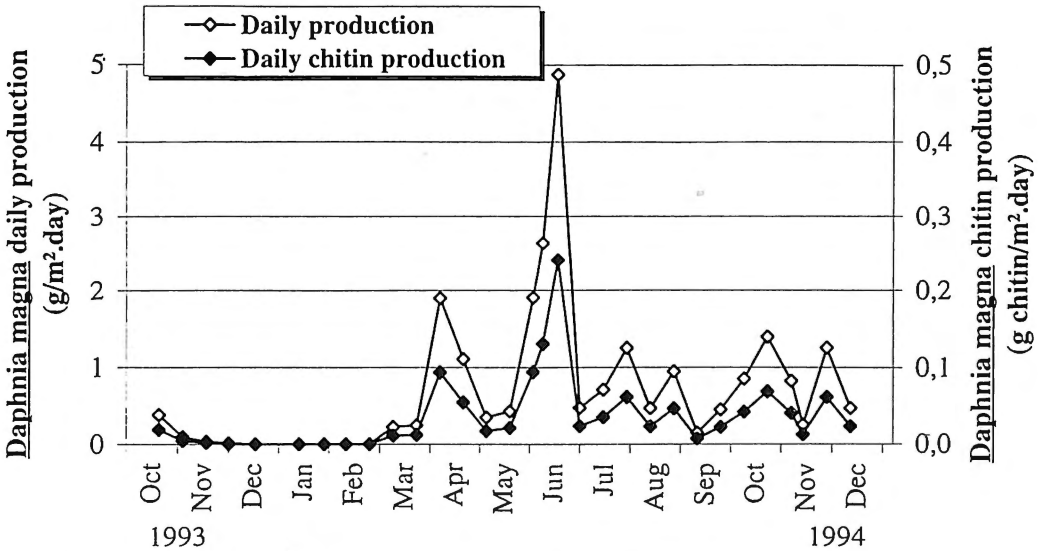

Fig. 4. — Seasonal changes in the daily production and the chitin daily production of *Daphnia magna* in the sewage lagoon of Differdange from October 1993 to December 1994.

The chitin content of *Daphnia magna* varied from 29 to 70 mg chitin per gram whole body dry weight, *i.e.* from 2.9 to 7.0 % of the total dry weight. Therefore, as the chitin content of *Daphnia magna* appears to be relatively constant over the year, a mean value of 4.9 % was used to calculate the daily chitin production of *Daphnia magna*. The daily chitin production is thus a constant part of the daily production. Consequently, daily chitin production and total daily production vary similarly (Fig. 4). Maximal values were recorded in April (94 mg chitin.m^{-2}.day^{-1}), June (241 mg chitin.m^{-2}.day^{-1}), and October (82 mg chitin.m^{-2}.day^{-1}). The annual chitin production of *Daphnia magna* reached 11.5 g chitin.m^{-2}.$year^{-1}$, *i.e.* 4.6 g chitin.m^{-3}.$year^{-1}$. The annual chitin production for the whole lagoon was thus estimated at 690 kg.

The annual chitin production of *Daphnia magna* at Differdange is compared in Table 1 with the chitin production of crustaceans in both fresh and salt water (JEUNIAUX *et al.*, 1993). The figure obtained in this study is four to eighty times higher than the annual chitin production of other Daphnia species living in eutrophic lakes, whatever the unit used (g.m^{-2}.$year^{-1}$ or g.m^{-3}.$year^{-1}$). On the whole, the amount of chitin produced annually by marine crustacean communities, expressed per square metre, is on the average ten times lower than the amount produced by *Daphnia magna* at Differdange. The differences are greater when the data are expressed per cubic metre.

TABLE 1

A comparison of the chitin production of some Crustacean community in freshwater and in marine ecosystems

Community	*Annual chitin production* ($g.m.^{-2}.year^{-1}$)	*Annual chitin production* ($g.m.^{-3}.year^{-1}$)
Fresh water		
Daphnia hyalina and *D. cucullata* (Tjeukermeer — Holland) (1)	0.14-0.30	0.092-0.204
Daphnia galeata (Lake Esrom — Denmark) (2)	3.2	0.160
Daphnia magna (sewage lagoon) (3)	11.5	4.6
Salt water		
Surface zooplankton (Calvi Bay — Corsica) (4) (Copepods, Cladocerans)	1.0	0.010
Euphausiacea (South Pacific) (5)	5.3	0.021
Euphausiacea (North Atlantic) (6)	0.01-0.04	0.00024-0.00085
Large benthic Decapods (7)	1.5	-

(1) Calculated by GERVASI *et al.* after VIJVERBERG (1981).
(2) Calculated by GERVASI *et al.* (1988) after PETERSEN (1983).
(3) The present study.
(4) GERVASI *et al.* (1988).
(5) Calculated by JEUNIAUX *et al.* (1933) after RITZ and HOZIE (1982).
(6) Calculated by JEUNIAUX *et al.* (1933) after LINDLEY and (1982).
(7) After JEUNIAUX *et al.* (1993).

DISCUSSION

In the absence of fish or invertebrate predators, the biomass and production of microcrustaceans depend mainly on the temperature and food availability (*e.g.* WEGLENSKA, 1971; WATTIEZ, 1979; HART, 1990). In spring, the two microcrustacean biomass peaks are clearly related to the chlorophyll a peaks. Yet as the algal resources decline, strong competition is liable to take place between daphnids and the phytophagous juvenile stages of *Cyclops* spp. This might be the cause of the drop in the biomass of *Daphnia magna* observed at the end of June.

From July to December, the microcrustacean biomass peaks did not correlate with the chlorophyll a concentration. Over the summer, the low availability of algae may have prevented the biomass of copepods from reaching high values, but despite the lack of algal food during the autumn of 1994, the biomass of microcrustaceans reached its highest value of the year (6.7 $g.m^{-2}$). This may be due to the ability of cyclopoid copepods and daphnids to feed on bacteria and suspended organic matter

(*e.g.* McMahon and Rigler, 1965 ; Porter, 1977 ; Nilssen, 1978 ; Peterson *et al.*, 1978).

The effect of temperature on microcrustacean biomass is highlighted by the differences observed between the maximum biomass values recorded in autumn 1993 and autumn 1994. In November 1993, the mean temperature was 6.6° C lower than in November 1994 and the biomass value (2.8 $g.m^{-2}$) was less than half that recorded in November 1994 (6.7 $g.m^{-2}$).

In order to estimate accurately the daily production of *Daphnia magna*, it is necessary to take into account how the development rate of this organism varies in relation to environmental factors. Our study being limited in time and scope, however, we used a growth curve (Mitchell *et al.*, 1992) established under laboratory conditions at constant temperature (20° C) and with a constant food supply (*Chlamydomonas reinhardii*, 0.5 $mg.l^{-1}$). Consequently, our estimate of the production may differ somewhat from the actual value because of the deviation of laboratory conditions from the prevailing natural conditions.

The maximum daily production was higher in June (4.8 $g.m^{-2}.day^{-1}$) than in April (1.9 $g.m^{-2}.day^{-1}$). This is due to the faster embryonic development and faster growth of juveniles at higher temperatures. In June, the mean temperature of the water was 20° C and embryonic development would take 2 to 3 days (Bottrell, 1975). Several generations could thus benefit from the temporary high availability of algae. In March and April, on the other hand, the mean temperature was only 7.5° C. Embryonic development would take 11 to 12 days. The daphnid offspring thus did not appear until April, when algae were scarce. This reduced their production.

As expected, winter was the least productive period. Only 2.1 % of the total production occurred during December, January, and February. This is due to the low availability of algal food and to the low water temperature. Daphnids can survive this period by producing long-lasting eggs (ephippia) that hatch in spring when the temperature and photoperiod increase (Larsson, 1991).

The annual chitin production calculated for the *Daphnia magna* population living in the sewage lagoon of Differdange is higher than that of any of the freshwater and marine communities with which it was compared (Table 1). This is probably due mainly (1) to the small number of competing species in the zooplankton community of this kind of sewage lagoon (at Differdange, the zooplankton community is composed solely of *Daphnia magna*, *Cyclops* spp., and *Brachionus* spp.), (2) to the high availability of food in the lagoon (algae in spring and summer, bacteria and suspended matter throughout the year), and (3) to the absence of fish and macroinvertebrate predators of zooplankton (Shapiro *et al.*, 1975). When the annual chitin production is expressed per cubic metre, large differences appear between *Daphnia magna* and the marine zooplankters. The reason for this is that marine zooplankters are distributed over a deeper water column than freshwater zooplankters. The low annual chitin production values reported for North Sea euphausids probably reflects the fact that Lindley (1982) did not evaluate the production of exuviae for these organisms.

CONCLUSION

In choosing the raw material for chitin production, one must consider four main criteria : (1) the seasonal and geographic availability of the source, (2) the chitin content of the organism, (3) the accessibility of the chitin in this organism, (4) the quality of the extracted chitin. Our aim is to assess the advantages of using cladocerans and copepods living in a sewage lagoon as a chitin source. So far, we have examined the first two criteria.

In terms of biomass, *Daphnia magna* appears as the dominant species of the lagoon. Because this organism is omnivorous and the food availability high (algae, bacteria, and suspended organic matter), chitin production by this species appears, in the limits of the accuracy of our estimation, very high as compared to other potential sources. The daily production, however, does depend on the temperature and food supply and is therefore subject to major seasonal variations. Winter, especially, is a critical period when the daily production is very low.

Another advantage of lagoon crustaceans is that, unlike pelagic marine *crustaceans* which are distributed over a deep water column, they are easy to harvest (with a pump-and-net system, for instance). These preliminary results suggest that microcrustaceans living in lagoons, especially *Daphnia magna*, are an excellent raw material for producing chitin.

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REFERENCES

APHA (1985) — *Standard methods for the examination of water and wastewater.* American Public Health Association, Washington (1268 pp).

BAUDOIN, M.F. and O. RAVERA (1972) — Weight, size and chemical composition of some freshwater zooplankters : *Daphnia hyalina. Limnol. Oceanogr.*, **17** : 645-649.

BOTTRELL, H.H. (1975) — Generation time, length of life, instar duration and frequency of moulting and their relationship to temperature in eight species of Cladocera from the river Thames. *Oecologia (Berlin)*, **19** : 129-140.

BOTTRELL, H.H., A. DUNCAN, Z.M. GLIWICZ, E. GRYGIEREK, A. HERZIG, H. KURAZAWA, P. LARSSON and T. WEGLENSKA (1976) — A review of some problems in zooplankton studies. *Norw. J. Zool.*, **24** : 416-456.

BRINE, C.J. and P.R. AUSTIN (1981) — Chitin variability with species and method of preparation. *Comp. Biochem. Physiol.*, **69B** : 283-286.

DINGES, R. (1976) — A proposed integrated biological wastewater treatment system. In : *Biological control of water pollution.* TOURBIER, J. , R. PIERSON and E. FURIA (Eds). University of Pennsylvania Press, Philadelphia : 225-230.

DUMONT, H.J., I. VAN DE VELDE and S. DUMONT (1975) — The dry weight estimate of biomass in a selection of Cladocera, Copepoda and rotifera from plankton, periphyton and benthos of continental waters. *Oecologia (Berlin)*, **19** : 75-97.

GEORGE, D.G. (1973) — Diapause in *Cyclops vicinus*. *Oïkos*, **24** : 136-142.

GERVASI, E., C. JEUNIAUX and P. DAUBY (1988) — Production de chitine par les Crustacés du zooplancton de la Baie de Calvi (Corse). In : *Aspects récents de la biologie des Crustacés.* IFREMER (Ed.), Brest, France : 33-38.

GOPHEN, M. (1977) — Feeding of *Daphnia* on *Chlamydomonas* and *Chlorobium*. *Nature*, **265** : 271-273.

HART, R.C. (1990) — Copepod post-embryonic durations : pattern, conformity and predictability. The realities of isochronal and equiproportional development, and trends in the copepodit nauplii duration ratio. *Hydrobiologia*, **206** : 175-206.

HIRANO, S. (1988) — Production and application of chitin and chitosan in Japan. In : *Proc. 4th Int. Conf. On Chitin and Chitosan.* SKJAK-BRAEK, G., T. ANTHONSEN and P. SANDFORD (Eds) — Elsevier Applied Science, London and New-York : 37-43.

JEUNIAUX, C. (1963) — *Chitine et chitinolyse : un chapitre de biologie moléculaire*. Masson, Paris (181 pp).

JEUNIAUX, C. (1965) — Chitine et phylogénie : application d'une méthode enzymatique de dosage de la chitine. *Bull. Soc. Chim. Biol.*, **47** : 2267-2278.

JEUNIAUX, C., M.F. VOSS-FOUCART and J.C. BUSSERS (1993) — La production de chitine par les Crustacés dans les écosystèmes marins. *Aquat. Living Ressourc.*, **6** : 331-341.

LAMPERT, W. (1974) — A method for determining food selection by zooplankton. *Limnol. Oceanogr.*, **19** : 995-998.

LARSSON, P. (1991) — Intraspecific variability in response to stimuli for male and epphipia formation in *Daphnia pulex*. *Hydrobiologia*, **225** : 281-290.

LINDLEY, J.A. (1982) — Continuous plankton records : geographical variations in numerical abundance, biomass and production of Euphausids in the North Atlantic Ocean and in the North Sea. *Mar. Biol.*, **71** : 7-10.

MCMAHON, J.W. and F.H. RIGLER (1965) — Feeding rate of *Daphnia magna* Straus on different foods labeled with radioactive phosphorus. *Limnol. Oceanogr.*, **10** : 105-113.

MITCHELL, S.F., F.R. TRAINOR, P.H. RICH and C.E. GOULDEN (1992) — Growth of *Daphnia magna* in the laboratory in relation to the nutritional state of its food species, *Chlamydomonas reinhardtii*. *J. Plankton Res.*, **14** : 379-391.

NILSSEN, J.P. (1978) — On the evolution of life histories of limnetic cyclopoid copepods. *Mem. Ist. Ital. Idrobiol.*, **36** : 193-214.

PETERSEN, F. (1983) — Population dynamics and production of *Daphnia galeata* (Crustacea : Cladocera) in Lake Esrom. *Holarctic Ecology*, *6* : 285-294.

PETERSON, B.J., J.E. HOBBIE and J.F. HANEY (1978) — *Daphnia* grazing on natural bacteria. *Limnol. Oceanogr.*, **23** : 1039-1044.

PORTER, K.G. (1977) — The plant-animal interface in freshwater ecosystems. *Amer. Sci.*, **65** : 159-170.

REISSIG, J.L., J.L. STOMINGER and L.F. LELOIR (1955) — A modified colorimetric method for the estimation of N-acetamino sugars. *J. Biol. Chem.*, **217** : 956-966.

RITZ, D.A. and G.W. HOZIE (1982) — Production of the Euphausiid *Nyctiphanes australis* in Stron Bay, South-Eastern Tasmania. *Mar. Biol.*, **68** : 103-108.

SANDFORD P.A. (1988) — Chitosan : commercial uses and potential applications. In : *Proc.4th Int.Conf.on Chitin and Chitosan.* SKJAK-BRAEK, G., T. ANTHONSEN and P. SANDFORD (Eds) — Elsevier Applied Science, London and New-York : 51-69.

SEVRIN-REYSSAC, J. (1993) — Performances et contraintes des élevages intensifs du Cladocère *Daphnia magna* Straus, utilisations des biomasses produites. *Crustaceana*, **64(3)** : 356-366.

SEVRIN-REYSSAC, J. (1994) — Le lagunage écologique. *Aqua revue*, **54** : 14-32.

SHAPIRO, J. (1990) — Biomanipulation : the next phase — making it stable. *Hydrobiologia*, **200-201** : 13-27.

SHAPIRO, J., V. LAMARRA and M. LYNCH (1975) — Biomanipulation : an ecosystem approach to lake restoration. In : *Proc. Symp. On Water Quality Management Through Biological Control. Brezonic, P.L.* and J.L. FOX (Eds) University of Florida Press, Gainesville : 85-96.

UHLMANN, D., I. RÖSKE and H.A. BRÜCKER (1994) — Process reservoirs for biofiltration and xenobiotics degradation as components of integrated systems for advanced biological water treatment. In : *Proceedings 4th Int. Conf. On Wetland systems for water pollution control. ICWS (ed.). Pergammon Press, London :* 458-471.

VIJVERBERG, J. (1981) — Populatiedynamika en produktie van zooplankton in het Tjeukemeer. Ph.D. Thesis — Rijksuniversiteit te Leiden (unpublished).

WATTIEZ, C. (1979) — Développement, croissance et fécondité de cladocères en fonction de la température et du niveau trophique. Elevage expérimental *in situ* dans deux étangs. *Hydrobiologia*, **63** : 263-283.

WEGLENSKA, T. (1971) — The influence of various concentrations of natural food on the development, fecundity and production of planktonic crustacean filtrators. *Ekol. Pol.*, **19** : 427-473.

WINBERG, G.G., K. PATALAS, J.C. WRIGHT, A. HILLBRICHT-ILKOWSKA, W.E. COOPER and K.H. MANN (1971) — Methods for calculating productivity. In : *Secondary productivity in freshwaters.* EDMONDSON, W.T. and G.G. WINBERG (Eds) — Blackwell Scientific Publication, IBP Handbook 17, Oxford and Edinburg : 303-317.

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BITE AND JOINT FORCE ANALYSIS IN *CAIMAN CROCODILUS*

by

JOHAN CLEUREN, PETER AERTS and FRITS DE VREE
University of Antwerp (U.I.A.), Department of Biology
Universiteitsplein 1, B-2610 Antwerp (Belgium)

SUMMARY

The equilibrium of forces acting on the lower jaw during biting can be assessed by static modelling. Usually, no account is taken for the actual recruitment level of the involved jaw adductors and only one fixed orientation of the food reaction forces is considered. These conditions conflict with reality. Therefore, recruitment levels of eight muscles of *Caiman crocodilus* are determined by means of quantitative EMG. For 12 crushing bites and one holding bite, these levels were normalized (per muscle) to the maximal activity level ever observed in a total of 72 bites. These activity levels were used as input for a static bite model. A large numbers of simulations are run in which the orientation of the food reaction forces varies over a large range. Several bite points are considered. The model calculates the magnitude of the bite forces and the orientation and magnitude of the joint forces. The results for the individual bites are compared to a model simulation where all muscles are fully active (100 %) and to an averaged bite representing a generalized crushing bite of *Caiman*. This allows to assess the biological meaning of such simulations. It turns out that (apart from the absolute size of the forces) both the 100 % model and the generalized bite simulation result in an equilibrium condition which closely approximates the actual *in vivo* equilibria. Some functional and morphological implications are discussed.

Keywords : bite force, joint force, electromyography, biomechanics, reptiles, *Caiman*.

INTRODUCTION

For basic mechanical analysis, the lower jaw in tetrapods can safely be described as a simple lever (one rotational degree of freedom). Nevertheless, several pairs of jaw closers are always present, which makes that bite forces can theoretically be generated by a large number of combinations of muscle forces. When food particles are held or crushed, muscle forces must be in balance with all other external forces acting on the jaw. These are predominantly forces situated at the level of the jaw suspension and forces exerted by the food particle in reaction upon the biting forces themselves (*i.e.*, food reaction forces). A static force analysis allows to assess this

balance in a relatively simple way (*e.g.*, ALEXANDER, 1983 ; SINCLAIR and ALEXANDER, 1987 ; for a more elaborate 3D-example see KOOLSTRA *et al.*, 1988).

Among tetrapods, the reptilian jaw musculature must be considered as the most complex one. SINCLAIR and ALEXANDER (1987) deal in their analyses with three types of reptiles : a turtle (*Chrysemys*), a lizard (*Varanus*) and a crocodilian (*Caiman*). The simulations are based on two assumptions. (1) Muscle forces were proportional to the physiological cross-section (*i.e.*, muscle volume/mean fibre length), and all muscles are fully active simultaneously. (2) Food reaction forces or joint reaction forces have a premised fixed orientation. In case of *Caiman* and *Chrysemys* food reaction forces are oriented perpendicular to the axis interconnecting their point of application and the centre of the jaw joint. For *Varanus*, the orientation of the joint reaction force was predefined (in line with the quadrate), resulting in one fixed orientation for the food reaction force too.

Such assumptions, however, conflict with reality. The orientation of food reaction forces may well diverge from one fixed (perpendicular) condition. The actual orientation is highly unpredictable, as it depends upon shape and texture of the food particle, its position with respect to the jaws, the shape and position of the teeth, the gape angle, etc. (see also KOOLSTRA *et al.*, 1988). In addition, various papers show that patterns of co-contraction of jaw closing muscles differ between bite types (*e.g.*, prey capture, positioning, crushing, reduction, killing, *etc.* : THROCKMORTON, 1980 ; GORNIAK *et al.*, 1982 ; SMITH, 1982 ; VAN DRONGELEN and DULLEMEIJER, 1982 ; BUSBEY, 1989).

Moreover, recent quantitative analyses of electromyographic data recorded from a large number of jaw muscles during feeding in *Caiman crocodilus* (CLEUREN and DE VREE, in prep.) and in agamid lizards (A. HERREL, pers.comm.) demonstrate that even within groups of similar bites, clustered by a factor analysis (PCA ; groups coincide with bite types), the recruitment level of individual muscles changes drastically from one bite to the other. Within the group of truly static crushing bites, not only the general level of recruitment of the involved muscles varies (reflecting changing biting efforts), but also the pattern of the individual recruitment level of all muscles changes (see further).

For all these reasons, the biological significance of static bite simulations with prescribed orientation of reaction forces and full scale activity of the jaw closing muscles must be questioned. Therefore, the present study applies anew a static bite force analysis to 12 crushing bites of a young caiman, taking into account the individual recruitment level of each muscle. Calculations are performed for a wide range of orientations of food reaction forces at several biting points. Magnitude and orientation of joint forces, and the magnitude of the bite forces are computed. The results are compared to those of simulations in which all muscles are fully active (*i.e.* force level accords directly to the physiological cross section), and with simulations in which the activity level of the muscles equals the average of the actual crushing bites. This allows to assess the biological relevance and meaning of such simulation conditions. Further, one recording of another static bite type, namely holding of the prey, is included in the comparison. Morphological and functional implications will be discussed.

MATERIAL AND METHODS

Juvenile *Caiman crocodilus* were commercially purchased. The specimen used for quantitative EMG-experiments had a length of 0.74 m and a mass of 1.5 kg. Muscle masses were determined on a specimen which died a natural death (0.65 m ; 1.0 kg). EMG-signals of 8 muscles (see Table 2) were recorded by means of teflon isolated stainless steel bipolar electrodes (diameter 0.045 mm ; Leico Industries Inc.). The EMG signals were passed through Tektronix 26A2 differential amplifiers and Honeywell Accudata 117 DC amplifiers and stored on a Honeywell medium-bandpass 96 FM 14-channel tape recorder. The analogue signals were digitized afterwards at 10 kHz (Keithley DAS, series 500). The digital signal was integrated using the procedure of BEACH *et al.* (1982). In this way one value per interval of 10 milliseconds was obtained for each muscle, being a measure for the intensity of its recruitment, and thus for the force development too (BASMAJIAN and DE LUCA, 1985). Throughout two complete feeding bouts (encompassing several cycles ($n=72$) of all bite types : acquisition/holding ($n=1$), killing/crushing ($n=12$), repositioning ($n=31$), transport ($n=12$) and swallowing ($n=16$) (see CLEUREN and DE VREE, 1992) ; the maximal integrated value ever observed for each muscle was determined. The integrated EMG-data of all crushing and the holding bites were then normalized per muscle according to these maxima (expressed as a percentage). An extensive description of the EMG-quantification and factor analysis will be published elsewhere.

An estimate of maximal force development by each muscle was made based on the physiological cross sections (volume/mean fibre length). Muscle volume was approximated from its mass, assuming a density of 1000 kg m^{-3}. The musculus adductor mandibulae externus (MAME) consists of three parts (MAMES = superficialis, MAMEM = medialis and MAMEP = profundus ; see SCHUMACHER, 1973 ; VAN DRONGELEN and DULLEMEIJER, 1982). In the present setup, no EMG-data were available for the MAMEM, but previous experiments revealed that the activity of the pars medialis resembles this of both other parts. Therefore, half the mass of the MAMEM was added to the MAMES, the other half to the MAMEP (Table 1) in order to represent the entire MAME in the model as two separate bundles only. The total jaw muscle mass from one side was 24.95 g. Mean fibre lengths were obtained from SINCLAIR and ALEXANDER (1987 ; from a specimen of identical length and mass). The fibre lengths of the MAMES and MAMEP were taken identical, based on the evidence provided by the ranges of fibre lengths published by BUSBEY (1989). The same holds for both components of the pterygoideus. A muscle stress of 0.25 MPa was used to convert physiological cross-section to force (see for instance HERZOG, 1994). The 3D orientation of the force vectors (see Fig. 1) was determined for one specific state of jaw depression by measuring the coordinates of the centres of the origins and insertions of the considered bundles on orthogonal X-rays (Siemens Tridoros Optimatic 880, at 35 kV, 400 Mas ; positioning based on dissections). The centre of the frame of reference was situated in the mid-sagittal plane at the level of the jaw rotation centres. The X-axis ran parallel to the neurocranial base (see Fig. 2A).

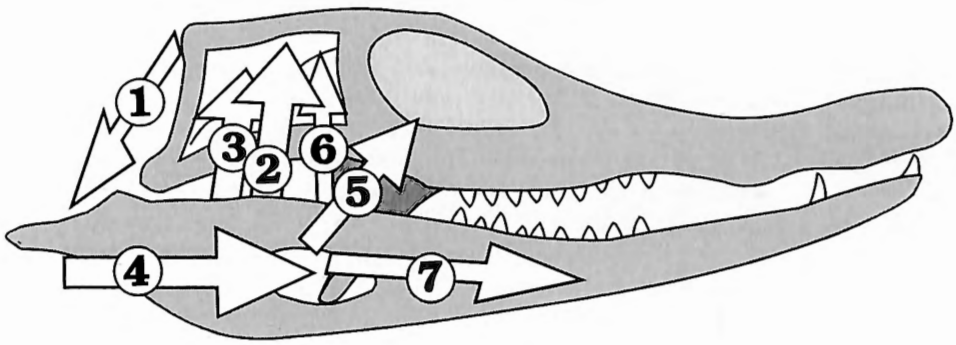

Fig. 1. — *Caiman crocodilus* : Lines of action of the jaw muscles, the width of the arrows refers to the physiological cross section. 1= M. depressor mandibulae ; 2= M. adductor mandibulae externus (sup., med., prof.) ; 3= M. adductor mandibulae posterior ; 4= M. pterygoideus posterior ; 5= M. pterygoideus anterior ; 6= M. pseudotemporalis ; 7= M. intramandibularis.

The model used to calculate the static force equilibrium was planar : calculations were carried out in a sagittal plane. Nevertheless, the 3D configuration of the muscular system (which is coupled to the state of jaw depression) was taken into account. For muscles diverging from the sagittal plane, only the sagittal component of the force vector was considered. Therefore, the model can be regarded 3D in cases where biting activity is symmetrical, as any laterally directed forces cancel each other at both body sides. [Notice that in this case left + right lower jaw constitute the free body, which is reasonable because of the very strong immobile mandibular symphysis]. This holds true for crushing and holding bites of caiman (and other reptiles too), as it is evidenced by former EMG-experiments where bilateral records of jaw muscles were made. The magnitude of the sagittal component and its orientation were automatically adjusted according to the introduced gape angle. Gape angles and biting points (= point of application of the food-reaction forces) were selected on the basis of observations of feeding in unrestrained animals.

Bites were simulated by multiplying the normalized integrated EMG-level (see above and Table 2) with the maximal contraction forces (see also De Gueldre and De Vree, 1990). These values were averaged per muscle to run the « averaged » (AVG) simulation. In a final simulation, all muscles were set to 100 % (« Model »). As mentioned in the introduction, « crushing » and « holding » were selected, as these are genuine static bites which furthermore show the necessary symmetrical muscle activity pattern. The model calculated the magnitude of the bite force, and the magnitude and orientation of the joint forces for every given orientation of the food reaction forces (see Fig. 2A). These were set to vary between -50 and -130 degrees with respect to the lower jaw (Fig. 2A). The direction of the joint forces is expressed relative to the cranium (Fig. 2A).

RESULTS

Maximal muscle forces and recruitment patterns

Masses, fibre lengths and physiological cross-sections of all muscles involved in the simulations are given in Table 1. Table 2 represents the normalized recruitment for each muscle during actual holding and crushing phases. The « model » bite (with all jaw adductors fully active) and the averaged crushing bite are added.

TABLE 1

Caiman crocodilus *: Jaw muscle masses in terms of percentage, fibre lengths (from* SINCLAIR *and* ALEXANDER*, 1987 ; also for a 1.00 kg specimen of* Caiman crocodilus*) and physiological cross sections. MAMP= M. adductor mandibulae posterior, MAMES= M. adductor mandibulae externus superficialis and part of pars medialis, MAMEP= M. adductor mandibulae externus profundus and part of pars medialis, PTP= M. pterygoideus posterior, PTA= M. pterygoideus anterior, IM= M. intramandibularis, DM= M. depressor mandibulae*

MUSCLE	*MASS (%)*	*FIBRE LENGTH (cm)*	*PHYS. CROSS (g/cm)*
MAMP	15	1.70	2.21
MAMES	7	0.96	1.77
MAMEP	8	0.96	2.08
PTP	34	2.00	4.25
PTA	18	2.00	2.25
PST	5	1.35	0.93
IM	5	0.47	2.65
DM	8	1.58	1.27

Bite simulations

Bite forces are given for one body side only. They have to be multiplied by two to obtain the overall bite force on the prey. They must be regarded as only a rough estimate of the forces exerted by a young caiman with a skull length of about 10 cm. Data are only presented for the extreme ($-50°$ and $-130°$) and the perpendicular ($-90°$) orientations of the food reaction force. All variables change gradually from the one extreme to the perpendicular, and then to the other extreme condition again.

TABLE 2

Caiman crocodilus *: Normalized recruitment level of the jaw muscles during holding and crushing phases. Model : hypothetical model with all jaw adductors fully (100 %) active simultaneously ; Hold :* in vivo *level during holding ; AVG : mathematical average of all crushing bites ; 1-12 :* in vivo *recruitment level of all crushing bites from two complete feeding sequences. For abbreviations of the muscles see Table 1*

	Model	*Hold*	*AVG*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
MAMP	100	97	69	43	58	59	69	76	100	60	61	62	88	78	72
MAMES	100	80	67	27	63	55	65	63	100	57	87	56	74	72	82
MAMEP	100	75	78	70	41	56	84	97	89	92	100	85	67	77	80
PTP	100	75	69	49	66	60	69	69	100	68	62	78	79	65	67
PTA	100	48	70	56	73	61	69	62	100	76	64	93	71	52	63
PST	100	100	67	31	61	55	61	77	83	80	70	66	84	65	75
IM	100	71	79	64	49	53	95	72	100	86	87	89	99	93	66
DM	0	4	5	0	3	0	0	5	3	19	6	12	2	10	1

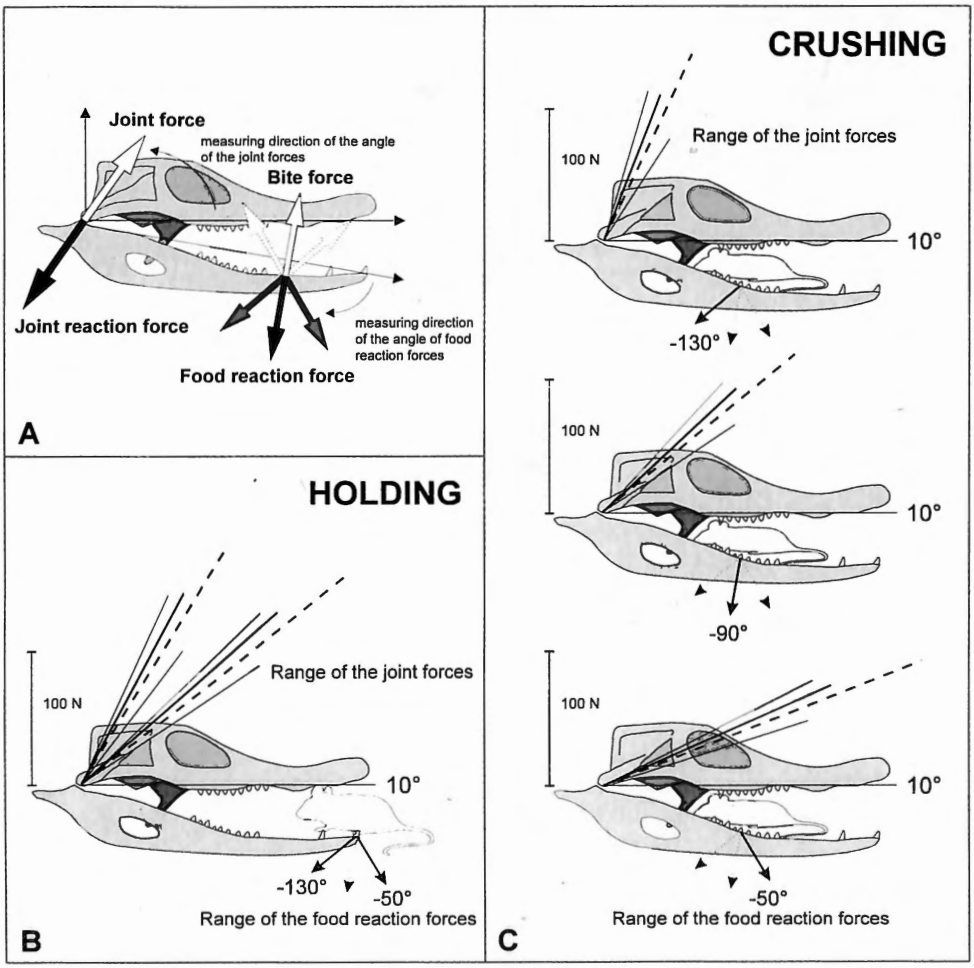

Fig. 2. — *Caiman crocodilus* — **A**. Action (white arrows) and reaction forces (black arrows) at the jaw joint and at the bite point. The direction of the joint force is measured relative to the line interconnecting the jaw joint and the anterior tip of the upper jaw, bite forces are measured relative to the lower jaw. — **B**. Holding at bite point 4 with a gape of 10° and with food reaction force angles of −50° and −130°. The magnitude of the FRF is not scaled, see Fig. 3A for absolute values. Presented are the concordant joint forces of the actual holding phase (bold solid line) and the 100 % model (dashed line). Although joint forces resulting from the recruitment levels as observed during the actual crushing phases do not reflect natural conditions, caused by the artificial forward shift of the bite point, this range is also presented as thin solid lines. — **C**. Crushing in the middle of the crushing range with a gape angle of 10° and for a food reaction force angle of −130°, −90° and −50°. The magnitude of the FRF is not scaled, see Fig. 3A for absolute values. Presented are the scaled joint forces when introducing the recruitment levels of the model conditions (100 %, dashed line), the averaged crushing values (bold solid line), and the *in vivo* recruitment levels (range presented between the thin solid lines).

Holding

Catching and holding occurs in the region of the anterior canine (bite point 4 in Fig. 3) and with a gape angle of 10° to 20° (Fig. 2B). The recruitment levels in terms of percentage presented in the second column of Table 2 are fed into the model. The latter predicts bite forces (BF) of 42 N for a 10° gape (Figs 2B, 3A) when food reaction forces (FRF) are perpendicular to the occlusal plane. A shift from the FRF in either direction results in an increase of the bite force : 56 N for FRF at −50° and 54 N for FRF at −130° (Figs 2B, 3A). Joint forces (JF), however, increase from 173 N (FRF at −90°) to 193 N (Figs 2B, 3B) when food reaction forces (FRF) point forward (−50°), but decrease to 160 N when the prey pushes backward on the lower jaw (−130°). This is accompanied by a change in slope from 41° to 62° relative to the cranium (Fig. 3C).

In Fig. 2B, the orientation and magnitude of the joint force of the actual holding phase (bold solid line) are compared for both extreme FRF orientations to those of the model bite (fully active jaw adductors ; dashed line) and with the range of joint forces generated by the observed recruitment levels of crushing phases when biting at the most anterior canine (thin solid lines). Notwithstanding the relatively large difference in jaw muscle recruitment level (see Table 2), both the joint force angle of the holding bite and the model bite (with all jaw muscles maximally active) fit into the envelope formed by the minimal and maximal values of the crushing bites.

Crushing

For these simulations the normalized recruitment values of the third (AVG ; averaged values of all actual crushing bites), and the fourth to last column (observed recruitment levels) of Table 2 are used. Crushing occurs in the posterior molar region *cf.* KIESER *et al.*, 1993 (biting points 1, 2 and 3 in Fig. 3), with a gape angle of 0° to 10°. The crushing region is situated near the insertions of the jaw adductors to minimize the load arm. The following numerical values refer to observed crushing phases at biting point 2 (Fig. 3) for a gape angle of 10° (Fig. 2C). During crushing, bite forces of 49 to 101 N (AVG = 78 N) are generated when the bite force is perpendicular (90°) to the tooth row. Analogous to holding, a shift from the FRF away from the perpendicular axis causes an increase in bite forces. The bite forces increase to 70 N − 144 N (AVG = 112 N) for FRF at −50°, and 59 N − 121 N (AVG = 94 N) for FRF at −130° (Figs 2C, 3A). Joint forces range from 90 N to 181 N (AVG = 138 N) with FRF at −90° at gape 10° (Fig. 3B). The range of joint angles is 33 to 47°. When food reaction forces are no longer perpendicular to the occlusal plane, but pointing forward (−50°), joint forces increase, ranging from 121 N to 248 N (AVG = 186 N) (Figs 2C, 3B), joint angles range from 17 to 26° (Fig. 3C). If the FRF slopes backward from −90° toward −130°, JF decrease to 74 N − 150 N (AVG = 118 N) (Figs 2C, 3), joint angles range from 58 to 74° (Fig. 3C). Apart from the relation between the direction of the food reaction forces and the magnitude of the bite forces and joint forces, also a clear relation with the direction of the joint forces exists : larger joint forces, resulting from a forward shift

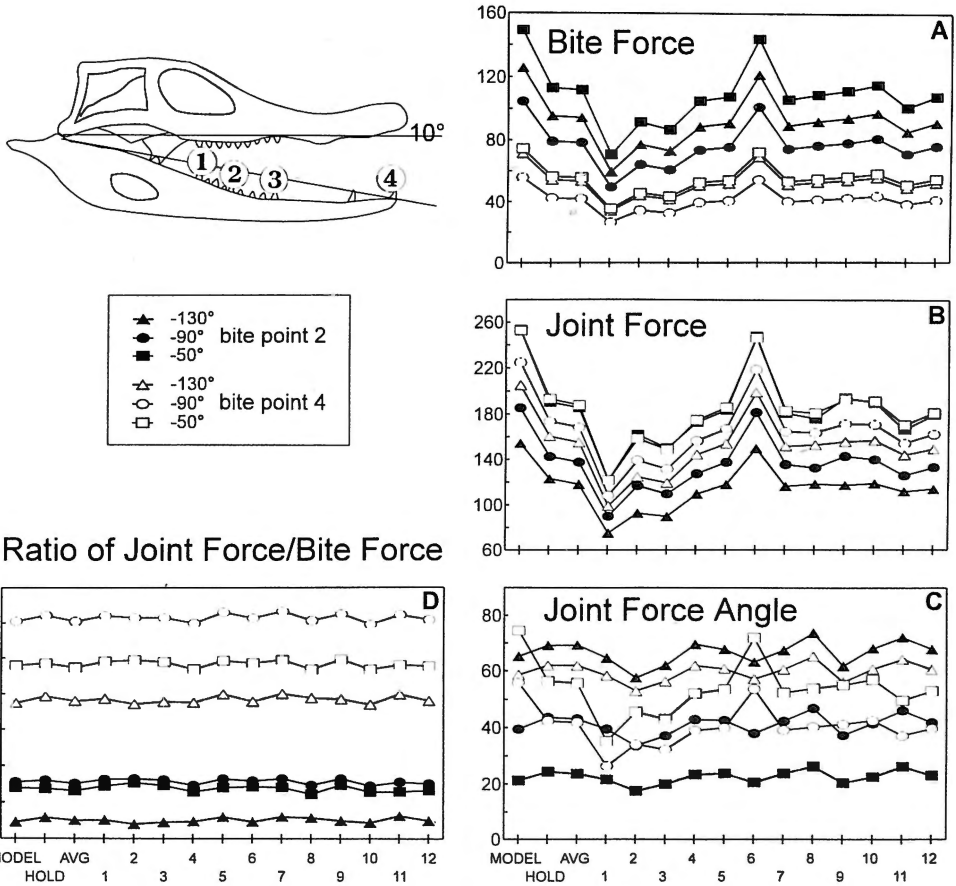

Fig. 3. — *Caiman crocodilus* — **A** : Magnitude of the bite force (values refer to one side only) — **B, C** : magnitude and orientation of the joint force at gape 10° and for food reaction forces (FRF) ranging from −130° to −50° (see legend) in function of the recruitment level (see X-axis and Table 2) and of the position of the bite point (open symbols : Holding at bite point 4 ; solid symbols : Crushing at bite point 2). — **D** : Ratio of joint force and bite force in function of the position of the bite point.

of the food reaction forces, show smaller angles. Thus, an increase of the magnitude of the joint forces coincides with a decrease of the angle this force makes with the cranium (Fig 2C, 3).

Fig. 2C demonstrates that, for the entire range of the FRF, the angle of the joint force of the averaged crushing phase (bold solid line) and the model bite (dashed line) always fits in the actual observed range of joint force angles during crushing (thin solid lines).

General

When the bite point moves from the posterior molars to the anterior incisors the magnitude of the bite forces logically decreases (see bite point 2 versus bite point 4 in Fig. 3A). This forward shift also results in a narrower range of the angle of the joint forces (compare range open symbols with closed symbols in figure 3C), and in an increase of their magnitude the more the FRF points backwards (Fig. 3B). Biting in the region of the anterior canine (during holding, Figs 3A, 3B) tends to load the joint to a larger extent than an equally sized bite force in the molar region (during crushing, Fig. 3), as also illustrated by the ratio of joint force and bite force for bite point 2 and bite point 4 (Fig. 3). This statement remains valid within the crushing region (shift from bite point 3 to 1 ; see Fig. 3).

The ratio of the joint force to the bite force is remarkably constant for a given bite point and FRF-orientation, notwithstanding the large variation in the recruitment pattern of the muscles (Fig. 3D). This factor is maximal when FRF equals 90°. The smallest factor results from biting in the molar region with an FRF pointing backwards (joint and bite forces have about the same magnitude).

DISCUSSION

De Vree and Gans (1994) argued that modelling of muscle functions starting from its architecture only is often based on several questionable assumptions. Two of these assumptions are that all muscles act simultaneously, and that muscles are fully active. To date, it is still impossible to determine the relative recruitment level of a muscle *in vivo*. Only quantitative electromyography can give an indication of the activity level and it has been shown that the link with force output exists (Basmajian and De Luca, 1985 ; Loeb and Gans, 1986 ; Gans, 1992). On the other hand, it is unpredictable what portion of the contracting muscle is sampled by the electrodes. Moreover, physiological cross section as a measure for *in vivo* maximal force is ambivalent too, because in most EMG studies it is unknown whether the motor units are ever active all together. For all these reasons, it remains uncertain whether the maximal activities used in this study to normalize the recruitment levels of the jaw closers (Table 2) indeed correspond to fully active muscles.

Figs 2B and 2C reveal no obvious difference between the model simulation (all adductor activities set to 100 % ; *i.e.* conditions as used by Sinclair and Alexander, 1987) and simulations with the *in vivo* and averaged distributions for holding and crushing presented in Table 2. In case of maximally active adductors, bite for-

ces are logically larger, ranging from 104 N to 149 N at the middle of the crushing region and from 56 N to 74 N at the level of the most anterior canine for an orientation of the food reaction forces ranging from −50 to −130 degrees. Concordant joint forces for these conditions range from 154 N to 253 N (*i.e.*, dashed lines in Fig. 2C) and from 204 N to 252 N (*i.e.*, dashed lines in Fig. 2B). These force levels have to be compared to the simulations with the adjusted recruitment levels during crushing : 50 N to 144 N, and 74 N to 248 N for the bite and joint forces respectively (Figs 2C, 3A,B), and during holding : 42 N − 56 N bite force and 160 N − 193 N joint force. For crushing bites, *Caiman crocodilus* uses between 47 and 96 % (AVG = 70 %) of its theoretical maximal bite force, but also retains between 48 and 98 % (AVG = 70 %) of the theoretical maximal joint force. This variation, resulting from the shifts in recruitment level of the individual muscles (see Table 2), is likely dictated by instantaneous feedback from the jaw system. As mentioned, magnitude and orientation of food reaction forces are unpredictable from one bite to the next, especially when the food item is inhomogeneous in texture and shape. Moreover, the position of the food particle with respect to the jaws changes during the course of a feeding sequence too. Even for the bites six to 11, which represent six consecutive crushing bites of the same sequence, table 2 shows considerable variation in the recruitment pattern, resulting in the fluctuations of forces and angles exemplified in Fig. 3.

In the middle of the crushing region, the ratio of joint force and bite force ranges from 1.2 to 1.83 for gape 10° for FRF ranging from −130° to −50°. During the holding of prey items, 75 % of the theoretical maximal bite force is retained, which equals to the retained portion (75 %) of the theoretical maximal joint force (Figs 2B, 3A, 3B). However, absolute values differ significantly, as the ratio of joint force and bite force ranges from 2.87 to 4.04 (gape 10°) at the level of the most anterior canine (Fig. 3). In other words, biting more forcefully (in absolute terms) involves a disproportional increase in joint force with a factor one to four. Therefore, it seems to make sense to adjust muscular effort to the actually required level, as in this way joint forces are kept minimal. Figures 2B, 2C and 3 further illustrate that the angular ranges of the joint forces are only slightly affected by raising all recruitment levels to 100 % and fit into the envelope of the *in vivo* crushing bites. The same findings apply to the averaged crushing bite. Apparently, the magnitude and orientation of the bite and joint forces generated by the model and average simulations perfectly fit into the observed range of *in vivo* forces (see Figs 2 and 3). Therefore, it must be concluded that both the maximal model and averaged model simulations give biologically meaningful results, especially when only relative force levels are considered. This means that static biting models are also applicable when no detailed information on the muscular recruitment level is available.

Thus, the present results for *Caiman* can be compared to those of SINCLAIR and ALEXANDER (1987). In order to do so several differences must be taken into account : the intramandibularis muscle is not included in their analyses, the musculus pterygoideus and the MAME are each represented by one force vector only, and all forces apply on the axis interconnecting the bite points with the jaw joint. A perpendicular orientation of the food reaction forces and a 100 % activity

level of all muscles is premised. First, as a test, input data gathered from Fig. 3C in SINCLAIR and ALEXANDER (1987) are fed in our model. All results were identical, except for the orientation of the joint force in case of biting in the molar region. Fig. 3C in SINCLAIR and ALEXANDER (1987) shows a joint force angle of 65° whereas our model predicts 18° [notice that SINCLAIR and ALEXANDER (1987) present joint reaction force; *cf.* Fig. 2A]. This new angle, however, makes the results consistent with our findings : a drop of the angle with a backwards shift of the bite point. Most likely, this error slipped in during the preparing of their Fig. 3C.

In an effort to mimic the model input by SINCLAIR and ALEXANDER (1987) using our data, a simulation was run in which the intramandibularis (*i.e.*, force 7 in figure 1) was excluded, and the pterygoideus was represented by one single vector (force of bundle 4 in Fig. 1 is added to bundle 5). The bite points 1 and 4 (see Fig. 3) give the best approximation of the points of application used by SINCLAIR and ALEXANDER (1987). The bite forces and according joint forces thus found largely exceed those found by SINCLAIR and ALEXANDER (1987). Bite forces are about 3.5 times larger. For the joint forces a factor equal to 1.6 (bite point 1) or 2 (bite point 4) must be applied. This is surprising as these authors used a scaling factor of 0.33 MPa to convert physiological cross-section to force. Apparently, the specimens they used to determine the physiological cross-section had a more slender jaw musculature, notwithstanding the comparable overall length and mass. This is confirmed by making the sum of all muscle masses presented by SINCLAIR and ALEXANDER (1987) : this equals only half the jaw muscle mass of the specimen used for the present study (Table 1). Also the ratio of bite to joint forces differs considerably between both studies (0.13 versus 0.23 for bite point 4 and 0.32 versus 0.66 for bite point 1 (Fig. 3); first value of each couple from SINCLAIR and ALEXANDER, 1987). The same holds for the angles of the joint forces : 25° versus 49° (see above) and 18° versus 33° for bite point 4 and 1 respectively (the first of each couple from SINCLAIR and ALEXANDER, 1987).

These divergences must relate to the large differences in the relative masses of the individual muscles in both studies (compare Table 1 with Table 1 in SINCLAIR and ALEXANDER, 1987), and the differences (although small) in the orientation and point of application of the muscle force vectors. If muscle forces taken from SINCLAIR and ALEXANDER (1987) are combined with the orientation and application of the vectors in this study, joint forces are quite identical (125 N versus 127 N and 114 N versus 112 N for bite point 1 and 4 respectively), and the orientations of these forces approximate each other closely (25° versus 38° and 18° versus 21° for bite point 1 and 4 respectively). Bite forces, however, are still about 1.8 times higher than those found by SINCLAIR and ALEXANDER (1987), notwithstanding the identical muscle force input.

These findings, together with the results of the comparison between the simulations with adjusted recruitment level and fully active muscles or an averaged crushing bite (see first paragraph of the discussion), suggest that joint forces are most sensitive to changes in the magnitude of the muscle forces, but rather insensitive to changes in their orientation. However, bite forces are also largely determined by (even small) changes in the orientation of the muscle forces. The biological implica-

tions are multiple : using different compartments of complex muscles allows an extensive modulation of bite force and slight morphometric differences may determine shifts in feeding ecology of closely related species, consequently a rigorous determination of the orientation of the muscle force vectors appears to be of crucial importance.

BUSBEY (1989) also estimated the reaction forces at the jaw joint during biting with a nearly closed gape in a 87 cm long *Alligator mississippiensis*. This phase is identical to the crushing phase in *Caiman crocodilus*. For crushing, BUSBEY (1989) found a joint force of 317 N at 47° (data transformed to the present reference system ; Fig. 2A). For holding a force of 225 N at 94° is found. These data are largely different from the *Caiman* results. The description of the followed procedure does not allow to judge whether food reaction forces were taken into account or not. If they are omitted from the equilibrium calculations (as we are inclined to believe), the above mentioned results are erroneous and cannot be used for comparison.

KOOLSTRA *et al.* (1988) showed that in humans, the direction of the largest possible bite force does not coincide with the direction perpendicular to the occlusal plane. This is of course determined by the static equilibrium conditions. For a given muscular force input, forces exerted on the food *must* be minimal in a direction perpendicular to the axis interconnecting the biting point and the jaw rotation centre (Figs 2, 3). However, in *Caiman*, joint forces increase with a shift in the orientation of the food reaction force from pointing backwards to forward. In other words : the more a bite force points forwards relative to the lower jaw, the smaller the joint forces will be (Figs 2, 3). SMITH and SAVAGE (1959) argued that in mammals different directions of pull may reduce forces in the jaw articulation. IORDANSKY (1964) showed that this may also be valid for crocodilians. This author stated that the M. adductor mandibulae posterior and the M. pterygoideus posterior prevent luxation of the mandibular joint. Our results show that not only the different direction of pull of all muscles, but also a modification of the force level of each individual muscle, determines (although to a larger extend than initially expected ; see Fig. 3) the magnitude and angle of the joint forces.

Nevertheless, joint forces are still relatively high during holding and crushing of prey items. However, irrespective the orientation of the food reaction forces and the pattern of the jaw muscle forces, the orientation of the joint forces always fits within the heavily ossified triangle at the level of the jaw suspension (see Figs 2B, 2C). The anterodorsally pointing leg of this triangle is formed by the massive quadrate which inclines medially. The quadratojugal and jugal form the other leg : the lower temporal bar, a strong bony strut pointing rostrally in a sagittal plane. This means that joint forces in *Caiman* always result in compressive loading of both bony legs of the triangle (vector resolution). The more the food reaction forces point forwards, the higher the lower temporal bar will be loaded. This is not only because higher joint forces are involved (see above, Figs 2, 3), but also because forward pointing food reaction forces coincide with decreasing joint force angles which tend to come in line with the lower temporal bar. As crushing and holding show symmetrical muscle activity (see Material and Methods), joint forces are most

likely confined to a sagittal plane, premised that also biting occurs symmetrical. The sagittal position of the lower temporal bar thus ensures pure axial loading. In case of the quadrate, the joint forces participate in a bending moment too. This might explain why, in spite of the much smaller axial loading, the quadrate appears to be stronger built than the lower temporal bar.

As the orientation in which the caiman can expect and thus also must absorb joint forces is highly determined by its jaw muscle morphology, reinforcements of the skull can be focused to the essential structures and therefore also kept minimal. This fits into the hypothesis of BRAMBLE and WAKE (1985) that terrestrial species specializing in cranio-inertial feeding are expected to show modification for the cranio-cervical mass in order to minimize inertial forces on the body (see also CLEUREN and DE VREE, 1992).

SINCLAIR and ALEXANDER (1987) premised a relation between the direction of the joint forces and the structure and position of the quadrate. The present results adjust this view in that the role of the temporal bar in stabilizing the joint is as important as this of the quadrate. For *Chrysemys* (turtle), the joint forces slope dorsally and backwards, also more or less in line with the slope of the quadrate. Based on results of SMITH (1982) on *Varanus,* SINCLAIR and ALEXANDER (1987) predicted joint forces in line with the quadrate. The according food reaction force pointed backwards relative to the lower jaw. However, in the present paper it is argued that the slope of the joint forces might not coincide with the orientation of the quadrate. Then the quadrate tends to swing anteriorly or posteriorly depending upon the specific orientation of the joint forces. In crocodilians and chelonians, this is prevented by an immobile quadrate which is wedged between the other cranial bones. However, in many lizard species the quadrate is freed for movement by the loss of the lower temporal bar by reduction of the jugal and the disappearance of the quadratojugal. If the quadrato-squamosal joint allows rotations, the quadrate is streptostylic. It is remarkable that in many lizard species a ligament (the quadrato-maxillary ligament) is found precisely at the position where crocodilians have the lower temporal bar (refs, own observations). This morphological fact strongly suggests that in such lizards joint forces predominantly point backwards, sloping behind the quadrate. As the ligament is loaded in tension, it can fulfil a role in stabilizing the quadrate, as does the lower temporal bar in crocodilians.

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REFERENCES

ALEXANDER, R.McN. (1983) — *Animal Mechanics*, 2nd edition. Oxford, Blackwell Scientific : 346 pp.

BASMAJIAN, J.V. and C.J. DE LUCA (1985) — *Muscles alive : Their functions revealed by electromyography*. Baltimore, Williams & Wilkins : 561 pp.

BEACH, J., G.C. GORNIAK and C. GANS (1982) — A method for quantifying electromyograms. *J. Biomech.*, **15** : 611-617.

BRAMBLE, D.M. and D.B. WAKE (1985) — Feeding mechanisms of lower tetrapods. *In* M. Hildebrand, D.M. Bramble, K.F. Liem, and D.B. Wake (eds) : *Functional Vertebrate Morphology*. Cambridge, Harvard University Press : 230-261.

BUSBEY, A.B. (1989) — Form and function of the feeding apparatus of *Alligator mississippiensis*. *J. Morphol.*, **202** : 99-127.

CLEUREN, J. and F. DE VREE (1992) — Kinematics of the jaw and hyolingual apparatus during feeding in *Caiman crocodilus*. *J. Morphol.*, **212** : 141-154.

DE GUELDRE, G. and F. DE VREE (1990) — Biomechanics of the masticatory apparatus of *Pteropus giganteus* (Megachiroptera). *J. Zool.*, London, **220** : 311-332.

DE VREE, F. and C. GANS (1994) — Feeding in tetrapods. *In* Bels, V.L., M. Clıardon and P. Vandewalle (eds.) : *Biomechanics of Feeding in Vertebrates, Advances in Comparative and Environmental Physiology*, Vol. **18**. Berlin, Springer Verlag : 93-118.

GANS, C. (1992) — Electromyography. *In* Biewener, A.A. (ed) : *Biomechanics — Structures and systems*. Oxford, Oxford University Press : 175-204.

GORNIAK, G.C., H.I. ROSENBERG and C. GANS (1982) — Mastication in the tuatara, *Sphenodon punctatus* (Reptilia, Rhynchocephalia), structure and activity of the motor system. *J. Morphol.*, **171** : 321-353.

HERZOG, W. (1994) — Muscle. *In* Nigg, B.M. and W. Herzog (eds) : *Biomechanics of the musculoskeletal system*. Chichester, John Wiley & Sons : 154-187.

IORDANSKY, N.N. (1964) — The jaw muscles of the crocodiles and some relating structures of the crocodilian skull. *Anat. Anz.*, **115** : 256-280.

KIESER, J.A., C. KLAPSIDIS, L. LAW and M. MARION (1993) — Heterodonty and patterns of tooth replacement in *Crocodylus niloticus*. *J. Morphol.*, **218** : 195-201.

KOOLSTRA, J.H., T.M.G.J. VAN EIJDEN, W.A. WEIJS and M. NAEIJE (1988) — A three-dimensional mathematical model of the human masticatory system predicting maximum possible bite forces. *J. Biomech.*, Vol. **21** : 563-567.

LOEB, G.E. and C. GANS (1986) — *Electromyography for Experimentalists*. Chicago, The University of Chicago Press : 373 pp.

SINCLAIR, A.G. and R. McN. ALEXANDER (1987) — Estimates of forces exerted by the jaw muscles of some reptiles. *J. Zool.*, London, **213** : 107-115.

SCHUMACHER, G.H. (1973) — The head muscles and hyolaryngeal skeleton of turtles and crocodilians. *In* C. Gans and T.S. Parsons (eds) : *Biology of the Reptilia*. Vol. **4**. London, Academic Press : 101-199.

SMITH, K.K. (1982) — An electromyographic study of the function of the jaw adducting muscles in *Varanus exanthematicus* (Varanidae). *J. Morphol.*, **173** : 137-158.

SMITH, J.M. and R.J.G. SAVAGE (1959) — The mechanics of the mammalian jaws. *Sch. Sci. Rev.*, **40** : 289-301.

THROCKMORTON, G.S. (1980) — The chewing cycle in the herbivorous lizard *Uromastix aegyptiticus* (Agamidae). *Arch. Oral Biol.*, **25** : 225-233.

VAN DRONGELEN, W. and P. DULLEMEIJER (1982) — The feeding apparatus of *Caiman crocodilus* : a functional-morphological study. *Anat. Anz.*, **151** : 337-366.

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CYTOCHEMICAL DEMONSTRATION OF ACID MUCOPOLYSACCHARIDES IN THE EPICUTICULAR SURFACE COAT OF THE CRAB *CARCINUS MAENAS* (L.) (CRUSTACEA, DECAPODA)

by

PHILIPPE COMPÈRE and GERHARD GOFFINET
Laboratoire de Biologie générale et de Morphologie ultrastructurale,
Institut de Zoologie, Université de Liège,
quai Ed. Van Beneden 22, B-4020 Liège (Belgium)

SUMMARY

The epicuticle of marine decapod crustaceans is composed of three sublayers : the inner epicuticle, the cuticulin layer, and the surface coat which was previously described as an external layer rich in tannophilic proteins. Positive staining by ruthenium red and alcian blue suggests the presence in this layer of polyanionic sites and acid mucopolysaccharides. The surface coat might be viewed as a hydrophilic glycoproteinaceous layer reducing the surface tension between the hydrophobic cuticulin layer and the aqueous environment.

Keywords : Acid mucopolysaccharides, cuticle, *Carcinus maenas*, cytochemistry.

INTRODUCTION

The epicuticle is defined as a thin, multilayered, outer covering of the arthropod cuticle. Its complex structure and chemical composition vary considerably with the ecological conditions and the physiology of the integument. The presence of surface waxes reducing water loss is one of the most important adaptative features of terrestrial arthropods (insects, arachnids). In marine decapod crustaceans, the epicuticle is composed of three sublayers : the inner epicuticle, the cuticulin layer, and the outermost surface coat (KÜMMEL *et al.*, 1970 ; COMPÈRE, 1988, 1995). The inner epicuticle and the cuticulin layer are common structures of the arthropod cuticle while the surface coat was only described in crustaceans (COMPÈRE, 1995). In *Carcinus maenas (op. cit.)*, the surface coat is deposited during late premoult after the cuticulin layer, the inner epicuticle, and a great part of the pigmented layer. After the moult, it appears as a relatively thick (0.1 μm), electron-dense, fuzzy coat overlying the cuticulin layer. The recent demonstration that this coat is mainly proteinaceous (showing a strong tannophilic reaction, COMPÈRE and GOFFINET,

1992) and hydrophilic raises the question : might this surface coat contain acid mucopolysaccharides and electrostatic surface charges ? The hypothesis that it is negatively charged is supported by the fact that, in the gill cuticle of fresh water acclimatised crabs *Eriocheir sinensis* (MILNE-EDWARDS, 1854) intoxicated with $HgCl_2$, it accumulates Hg^{2+} ions (BARRADAS and PÉQUEUX, 1995).

The purpose of this study is to detect polyanionic sites and acid mucopolysaccharides at the ultrastructural level in the epicuticular surface coat of the Atlantic shore crab *Carcinus maenas,* using cytochemical methods based on staining with ruthenium red and alcian blue.

MATERIAL AND METHODS

Cuticular samples excised from branchiostegites and gills of the crab *Carcinus maenas* were fixed by immersion for 2 h at 20° C in 2.5 % glutaraldehyde, 50 mM $MgCl_2$, 0.05 M Na-acetate buffer (pH 7.4 or 5.8) containing either 0.1 % ruthenium red (LUFT, 1971) or 1 % alcian blue 8GX (SCOTT and DORLING, 1965 ; SCOTT, 1970 ; LEWIS and KNIGHT, 1977). Control experiments were carried out in the presence of 1 M MgCl2 according to Scott's (1970) critical electrolyte method. Branchiostegite samples were decalcified under the same conditions (pH, buffers, $MgCl_2$ and stain concentrations) for 24 h at 4° C in a solution containing 0.1 M EGTA and 1.25 % glutaraldehyde, then fixed again for 90 min in 2.5 % glutaraldehyde solutions as detailed above. To enhance staining, we post-fixed all the samples for 1 h at 4° C in 1 % OsO_4, using the same buffer, pH and $MgCl_2$ concentration. Ruthenium-red- and Alcian-blue-treated samples were washed in buffer and OsO_4-fixed, respectively, with or without the stain. All samples were then processed classically without further staining and examined in a transmission electron microscope (JEOL 100-SX) at 80 kV accelerating voltage.

RESULTS

The ruthenium red and alcian blue staining methods give quite similar qualitative results, staining being only slightly more intense with ruthenium red. In the samples fixed at pH 7.4 (Figs 1, 2, 5 and 6), the surface coat appears very electron-dense, but less so than the outer leaflets of the cuticulin layer (COMPÈRE, 1988, 1995). The chitin-protein fibres of the pigmented layer are also contrasted while the inner epicuticle remains electron-lucent. In all layers but the cuticulin layer, positive

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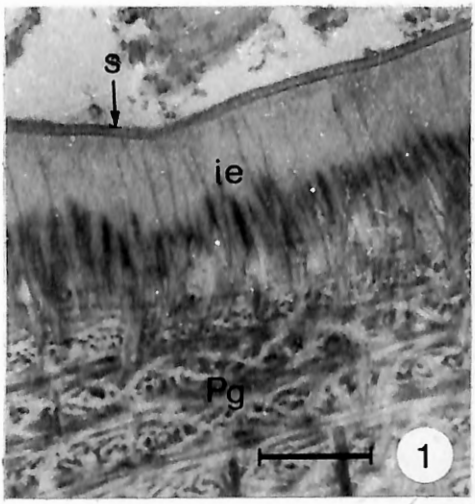
Figs 1-8. Branchiostegite epicuticle of the crab *C. maenas* — 1-4. Treated with alcian blue — 5-8. Treated with ruthenium red — 1, 2, 6. at pH 7.4 and 50 mM $MgCl_2$ — 3, 5, 7 at pH 5.8 and 50 mM $MgCl_2$ — 4, 8. at pH 7.4 and 1 M $MgCl_2$. c, cuticulin layer ; ie, inner epicuticle ; Pg, pigmented layer ; s, surface coat ; ie, inner epicuticle ; Pg, pigmented layer ; s, surface coat. Scale bars in 1, 5 : 1 μm ; in 2-4, 6-8 : 100 nm.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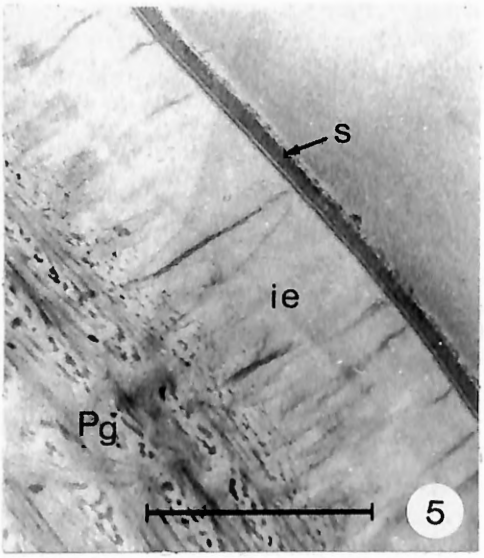
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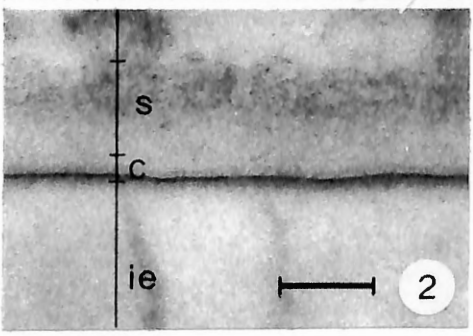
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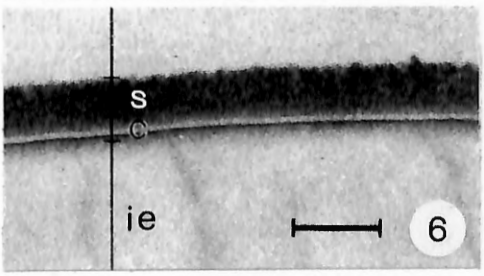
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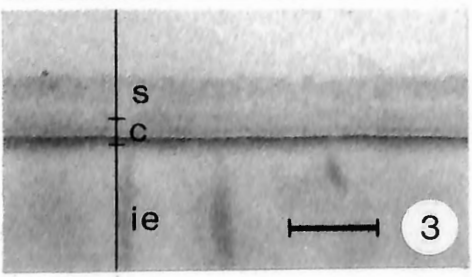
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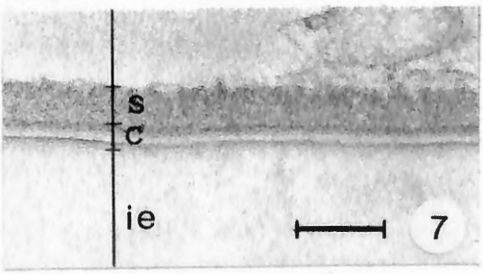
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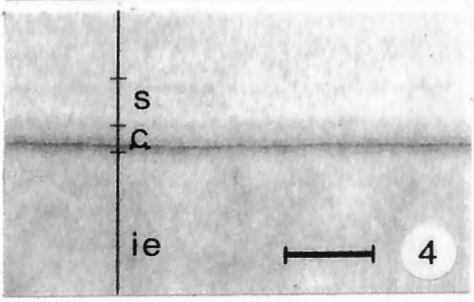
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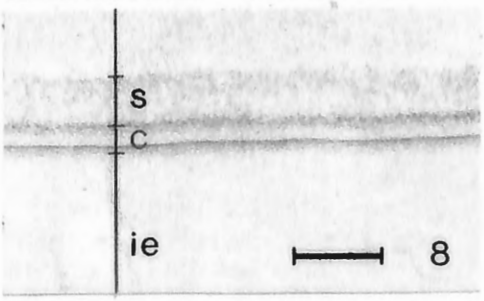
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staining appears slightly less intense in the samples fixed at pH 5.8 (Figs 3 and 7) and completely absent in control samples treated in the presence of 1 M $MgCl_2$ (Figs 4 and 8).

DISCUSSION

Our results point to the presence of polyanionic sites and acid mucopolysaccharides in the epicuticular surface coat of the branchiostegite and gill cuticle of the shore crab *C. maenas*. The slightly higher contrast in samples treated at pH 7.4 than in ones treated at pH 5.8 is presumably attributable to weakly negative sites, *i.e.* the carboxyl groups of proteins, which are not stained at pH 5.8 in the presence of a low concentration (50 mM) of $MgCl_2$ (SCOTT and DORLING, 1965 ; LEWIS and KNIGHT, 1977). At pH 5.8, the appearance of contrast strongly suggests the presence of negatively charged acid mucopolysaccharides. Furthermore, the absence of contrast in control samples treated in the presence of a high concentration of electrolyte (1 M $MgCl_2$) confirms the electrostatic nature and the specificity of the reaction (*op. cit.*). The persistence of the high electron-density of the cuticulin layer leaflets in these controls is probably due to a nonspecific reaction.

In combination with the previous demonstration of tannophilic proteins (COMPÈRE, 1990a ; COMPÈRE and GOFFINET, 1992), the presence of polyanionic sites and acid mucopolysaccharides suggests that the epicuticular surface coat of the crab *C. maenas* forms a hydrophilic, glycoproteinaceous, external coat. This composition explains the affinity of this sublayer for Hg^{2+} ions in *E. sinensis* gills after intoxication by $HgCl_2$ (BARRADAS and PÉQUEUX, 1995). Accumulation of Hg^{2+}, moreover, is greater in fresh-water-acclimatised animals than in seawater-acclimatised ones, being inversely proportional to the electrolyte concentration.

The presence of this hydrophilic external sublayer is probably a general feature of the epicuticle of aquatic arthropods, since a surface coat or corresponding layers have been reported by several authors or can be structurally identified on published micrographies in many crustaceans (decapods : KÜMMEL *et al.*, 1970 ; FOSTER and HOWSE, 1978 ; GREEN and NEFF, 1972, COMPÈRE, 1990a, 1995 ; COMPÈRE and GOFFINET, 1992 ; copepods GHARAGOZLOU-VAN GINNEKEN and BOULIGAND, 1973, 1975 ; cladoceran branchiopods : HALCROW, 1976) and different pycnogonid species (COMPÈRE *et al.*, 1993, FAHRENBACH, 1994). In the terrestrial oniscoid isopods, the surface coat is also present but appears much thinner than in marine decapods (COMPÈRE, 1990b). On the other hand, POQUET *et al.* (1994) have shown that in a parasitic copepod in mussel gills, the external coat of the epicuticle (« el », according to the terminology of GHARAGOZLOU-VAN GINNEKEN and BOULIGAND, 1973, 1975) gives a positive reaction with phosphotungstic acid (PTA) at pH 0.3, indicating the presence of glycoproteins (RAMBOURG, 1971). No homology can be proposed, however, between this external coat and the epicuticular surface coat of decapods. Apart from the observation of the surface coat deposition in the sclerites of *C. maenas* (COMPÈRE, 1988, 1995) and *Oniscus asellus* (L., 1758) (COMPÈRE, 1990b)

during late premoult, there is no information in the litterature on the morphogenesis of these external epicuticular layers.

Concerning the role of these outermost layers, many different highly specialised functions have been proposed but all are hypothetical. In some pycnogonids, the filamentous coat is believed to act as a protective layer against nematocysts (FAHRENBACH, 1994). POQUET *et al.* (1994) suggest that the glycoproteinaceous coat of parasitic copepods plays a role in the attachment of bacteria or protects the copepod against host reactions. According to ARNAUD *et al.* (1988), sublayer « e1 » plays a key role in the agglutination of food particles in the labral glands of calanoid copepods, but the presence of similar or at least analogous external epicuticle sublayers in a variety of aquatic arthropods suggests, rather, that these layers have a common basic function. In all cases, the surface coat directly overlies the cuticulin layer, which is known to be hydrophobic and membrane-like structure (NEVILLE, 1975 ; FILSHIE, 1982 ; WIGGLESWORTH, 1985). Similarly, *C. maenas* (COMPÈRE and GOFFINET, 1992), the cuticulin layer appears to form the main permeability barrier of the cuticle. The surface coat might thus be viewed as a hydrophilic layer protecting the cuticulin layer and/or reducing the surface tension between the hydrophobic cuticulin layer and the aqueous environment. In this respect, the surface coat would resemble the cell coat or the polar heads of plasma membrane phospholipids.

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REFERENCES

ARNAUD, J., M. BRUNET and J. MAZZA (1988) — The labral glands in *Centropages typicus* (Copepoda, Calanoïda) : I. Site of synthesis. *J. Morphol.*, **197** : 21-32.

BARRADAS, C. and A. PÉQUEUX (1995) — Influx and accumulation of mercury in the gills of euryhaline chinese crab *Eriocheir sinensis* (Milne-Edwards). In *Proc. 1st Iberian Cong. of Contamination and Environmental Toxicology. Proscientia,* Coimbra, Portugal : 67.

COMPÈRE, PH. (1988) — Mise en place de l'épicuticule chez le crabe *Carcinus maenas*. In P. LE GAL and A. VAN WORMHOUDT : *Aspects récents de la Biologie des Crustacés*. IFREMER, Brest, A.C. **8** : 4754.

COMPÈRE, PH. (1990a) — Structure, mise en place et cytochimie de l'épicuticule et des systèmes de canaux intracuticulaires du crabe *Carcinus maenas* au cours d'un cycle de mue et en relation avec la specialisation physiologique de diverses regions tégumentaires. Doctorat thesis, Université de Liège : 167 pp.

COMPÈRE, PH. (1990b) — Fine structure and elaboration of the epicuticule and pore canal system in tergite cuticle of the land isopod *Oniscus asellus* during a molting cycle. In

P. JUCHAULT and J.P. MOCQUARD : *Biology of terrestrial Isopods III.* Université de Poitiers, Poitiers : 169-175.

COMPÈRE, PH. (1995) — Fine structure and morphogenesis of the sclerite epicuticle in the Atlantic shore crab *Carcinus maenas. Tissue Cell* (in press).

COMPÈRE, PH. and G. GOFFINET (1992) — Organisation tridimensionnelle et cytochimie de l'épicuticule et des systèmes canaliculaires des sclérites du crabe *Carcinus maenas* (Crustacé décapode). *Mém. Soc. r. belge Ent.*, **35** : 715-720.

COMPÈRE, PH., THIRY PH., J.-C. BUSSERS and G. GOFFINET (1993) — Ultrastructure of the integument of the sea spider *Pycnogonum littorale* (Ström) (Pycnogonida). *Belgian J. Zool.*, **123** (suppl. 1) : 11-12.

FAHRENBACH, W.H. (1994) — Microscopic anatomy of Pycnogonida : 1. cuticle, epidermis and muscle. *J. Morphol.*, **222** : 33-48.

FILSHIE, B.K. (1982) — Fine structure of the cuticle of insects and others Arthropods. In R.C. KING and H. AKAI : *Insect Ultrastructure*, vol. 1. Plenum Press : 281-312.

FOSTER, C.A. and H.D. HOWSE (1978) — A morphological study on gills of the brown shrimp, *Penaeus aztecus. Tissue Cell.*, **10** : 77-92.

GHARAGOZLOU VAN GINNEKEN, I.O. and Y. BOULIGAND (1973) — Ultrastructures tégumentaires chez un Crustacé Copépode, *Cleptocampus retrogressus. Tissue Cell.*, **5** : 413-439.

GHARAGOZLOU VAN GINNEKEN, I.O. and Y. BOULIGAND (1975) — Studies on the fine structure of the cuticle of *Porcellidium*, Crustacea Copepoda. *Cell Tissue Res.*, **159** : 399-412.

GREEN, J.P. and M.R. NEFF (1972) — A survey of the fine structure of the integument of the fiddler crab *Uca pugilator. Tissue Cell*, **4** :137-171.

HALCROW, K. (1976) — The fine structure of the carapace integument of *Daphnia magna* Strauss (Crustacea, Branchiopoda). *Cell Tissue Res.*, **169** : 267-276.

KÜMMEL, F., H. CLAASSEN and R. KELLER (1970) — Zur Feinstruktur von Cuticula und Epidermis beim Flusskrebs *Orconectes limosus* während eines Haütungszyklus. *Z. Zellforsch. mikrosk. Anat.*, **109** : 517-551.

Lewis, P.R. and D.P. KNIGHT (1977) — Staining methods for sectioned material. A.M. GLAUERT : *Practical Methods in electron Microscopy, vol. 6.* North Holland Publ. Company, Amsterdam, N.-Y., Oxford : 311 pp.

LUFT, J.H. (1971) — Ruthenium red and violet. II. Fine structural localization in animal tissues. *Anat. Rec.*, **171** : 369-392.

NEVILLE, A.C. (1975) — Biology of the arthropod cuticle. W.S. HOAR, J. JACOBS, H. LANGER and M. LINDAUER : *Zoophysiology and Ecology vol. 4/5.* Springer Verlag, Berlin Heidelberg, New-York : 448 pp.

POQUET, M., E. RIBES, G.M. BOZZO and M. DRUFORT (1994) — Ultrastructure and cytochemistry of the integument of *Modiolicola gracilis*, parasitic copepod in mussel gills (*Mytilus galloprovincialis* and *Mytilus edulis*). *J. Morphol.*, **221 : 87-99.**

RAMBOURG, A. (1971) — Morphological and histochemical aspects of glycoproteins at the surface of animals. *Int Rev. Cytol.*, **31,** 57-114.

Scott, J.E. (1970) — Histochemistry of alcian blue. I. Metachromasia of alcian blue, astrablau and other cationic phthalocyanin dyes. *Histochemie*, **21** : 277-285.

SCOTT, J.E. and J. DORLING (1965) — Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. *Histochemie*, **5** : 221-233.

WIGGLESWORTH, V.B. (1985) — Sclerotin and lipid in the waterproofing of the insect cuticle. *Tissue Cell*, **17** : 227-248.

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A COMPARISON OF GENE FLOW ESTIMATES BASED ON PRIVATE ALLELE FREQUENCIES

by

HANS DE WOLF (1), THIERRY BACKELJAU (2),
KURT JORDAENS (1), ROBERTO MEDEIROS (3), PETER VERDYCK (2)
and RON VERHAGEN (1)

(1) Department of Biology, Evolutionary Biology, University of Antwerp (RUCA)
Groenenborgerlaan 171, B-2020 Antwerpen (Belgium)
(2) Royal Belgian Institute of Natural Sciences,
Vautierstraat 29, B-1040 Brussel (Belgium)
(3) Departamento de Biologia, Universidade dos Açores
Rua da Mae de Deus 58, P-9502 Ponta Delgada, Açores (Portugal)

SUMMARY

The frequency of private alleles is often used to assess the amount of gene flow (Nm) between populations, with the equations proposed by SLATKIN (1985b) and by SLATKIN and BARTON (1989). Although these equations express the same relationship, they may yield different estimates of gene flow for the same data. These differences increase with decreasing frequencies of private alleles. Comparisons of Nm estimates, based on different equations can therefore be misleading. It is advisable to use these equations method only to distinguish between $Nm > 1$ and $Nm < 1$.

Key words : Population genetics, gene flow, private alleles.

INTRODUCTION

The amount of gene flow (Nm = Number of migrants) between natural populations is usually estimated by indirect methods based on allele frequency data. One of these methods uses the mean frequency $\overline{P}$ (1) of so called private alleles, *i.e.* alleles that are found in one population only (SLATKIN, 1985a). The likelihood that such alleles are exchanged between populations is indeed related to the degree of migration, for the probability of exchange of private alleles between populations will be very low unless migration is frequent. Gene flow estimates based on $\overline{P}$ (1) rely on a simulation model suggesting that in the case of the stepping stone and island models the logarithm of Nm is approximately linearly related to the logarithm of $\overline{P}$ (1). This relationship was first formulated by a nepperian logarithm

(SLATKIN, 1985b) and subsequently by a $\log_{10}$ ébased equation (SLATKIN and BARTON, 1989). Despite expressing the same relationship, different estimates of gene flow can be obtained when both equations are applied to the same data.

In this paper, both equations are compared and some literature on the calculation of gene flow using private alleles is reviewed.

MATERIAL AND METHODS

Using SLATKIN's (1985b) formula, gene flow (Nm) is calculated as follows

$$Nm = Nm_{ref} \cdot \frac{25}{N_{sam}}$$

Where Nm_{ref} is a reference gene flow estimate for an arbitrary theoretical sample size (*i.e.* average number of individuals per population) of $N_{ref}=25$ and where N_{sam} is the actual number of individuals sampled per population. Nm_{ref} (for $N_{ref}=25$) can be calculated using following equation

$$Nm_{ref} = e^{(\ln(\overline{P}(1)) - b)(\frac{1}{a})}$$

or

$$\ln(\overline{P}(1)) = a\ln(Nm_{ref}) + b$$

with $a=-0.505$ and $b=-2.440$ and where $\overline{P}(1)$ is the average frequency of private alleles over all populations and loci sampled.

Equivalent to these formulae is SLATKIN and BARTON's (1989) equation where

$$Nm_{ref} = 10^{(\log_{10}(\overline{P}(1)) - b)(\frac{1}{a})}$$

or

$$\log_{10}(\overline{P}(1)) = a\log_{10}(Nm_{ref}) + b$$

With values of $a=-0.49$ and $b=-0.95$ for $N_{ref}=10$; $a=-0.58$ and $b=-1.1$ for $N_{ref}=25$ and $a=-0.61$ and $b=-1.2$ for $N_{ref}=50$

Correction for sample sizes different from 10, 25 or 50 is made as follows :

$$Nm = Nm_{ref} \cdot \frac{N_{ref}}{N_{sam}}$$

The formulae of SLATKIN (1985b) and SLATKIN and BARTON (1989) are graphically compared (Fig. 1). $\overline{P}(1)$ values, ln and $\log_{10}$ transformed, ranging from 0.01 to 0.10 are plotted against corresponding ln and $\log_{10}$ transformed Nm estimates obtained using both equations under the assumption that $N_{ref}=25$. Using $\overline{P}(1)$ and average sample sizes adopted from literature, Nm values are recalculated and com-

pared (Table 1). If F_{ST} values (fixation index measuring the degree of genetic differentiation between subpopulations) are known, Nm values are also estimated according to WRIGHT's (1951) method, which is based on the following equation

$$Nm = \frac{1}{4}\left(\frac{1}{F_{ST}} - 1\right)$$

Selected F_{ST} and derived Nm estimates are also given in Table 1.

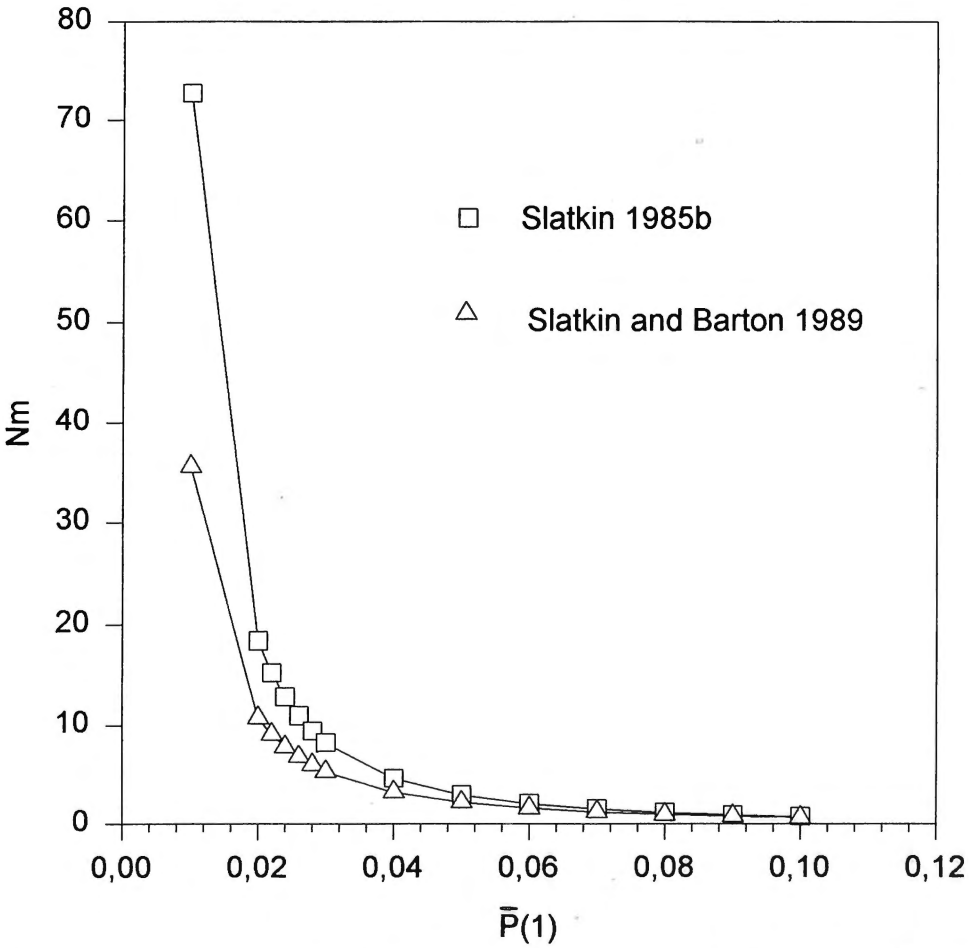

Fig.1. — Graphical comparison of gene flow calculation according to SLATKIN (1985b) and SLATKIN and BARTON (1989) for $N_{sam}=25$, which are respectively.

$$Nm_{ref} = e^{(\ln(\bar{P}(1)) - b)\left(\frac{1}{a}\right)}$$

$$Nm_{ref} = 10^{(\log_{10}(\bar{P}(1)) - b)\left(\frac{1}{a}\right)}$$

with

$$Nm = Nm_{ref} \cdot \frac{25}{N_{sam}}$$

RESULTS

Fig. 1 shows Nm estimates as function of theoretical $\overline{P}(1)$ values with an average sample size of 25, based on SLATKIN's (1985b) and SLATKIN and BARTON's

TABLE 1

Nm estimates according to SLATKIN (1985b), SLATKIN and BARTON (1989) and WRIGHT (1951). N_{sam} = sample ; $\overline{P}(1)$ = mean private alleles frequency ; Nm = gene flow estimate

Species	N_{sam}	$\overline{P}(1)$	*Nm Slatkin 1985b*	*Nm Slatkin, Barton 1989*	F_{st} *Wright 1951*	*Nm Wright 1951*
Pteridium aquilinum	47.0	.0103	36.51[1]	19.99	0.110	2.022
Pinus ponderosa Deme 157.1	39	.026	7.03	5.39[2]		
Pinus ponderosa Deme 160.5	13	.039	9.45	6.82[2]		
Stephanomeria exigua	45.8	.054	1.4[3]	1.3	0.152	1.394
Mytilus edulis	67.4	.008	42.0[3]	21.1	0.006	41.41
Strombus gigas		.026	10.97[4]	6.85[4]		8.7
Haliotis rubra	90.27	.0071	39.17	19.67[5]		
Haliotis laevigata	72.37	.0106	22.27	14.01[5]		
Gammarus fossarum	33.31	.190	0.16[6]	0.16	0.68	0.117
Gammarus pulex	21.06	.110	0.76[6]	0.67	0.36	0.444
Speonomus hydrophhilus	70	.042	12.53[7]	1.39	0.112	1.982
			1.51			
Sitobion avenae 9 sites	± 165	.027	1.6[8]	1.22		
Sitobion avenae 13 sites	± 165	.019	3.0[8]	2.16		
Drosophila willistoni	94	.014	9.9[3]	6.0		
Drosophila pseudoobscura	33.2	.075	1.0[3]	0.8	0.200	8.70
Chanos chanos	48.9	.030	4.2[3]	3.3	0.056	4.21
Salmo salar	56	.030	7.7[9]	3.02		
			3.68			
Batrachoseps campi	10.6	.338	0.16[3]	0.09		
Batrachoseps pacifica spp. 1	21.7	.117	0.64[3]	0.59	0.281	0.64
Batrachoseps pacifica spp. 2	22.2	.207	0.20[3]	0.21	0.556	0.20
Hyla regilla	20.1	.081	1.4[3]	1.2		
Plethodon ouachitae	31.4	.054	2.1[3]	1.5	0.106	2.108
Plethodon cinereus	22.1	.200	0.22[3]	0.23		
Plethodon dorsalis	22.3	.294	0.10[3]	0.11		
Lacerta melisellensis	22.4	.066	1.9[3]	1.5		
Peromyscus polionotus	25.2	.158	0.31[3]	0.30	0.446	0.31
Peromyscus californicus	20.0	.066	2.2[3]	1.6		
Thomomys bottae	29.1	.087	0.86[3]	0.73		

[1]WOLF *et al.*, 1991 ; [2]ALSTAD *et al.*, 1991 ; [3]SLATKIN, 1985b ; [4]MITTON *et al.*, 1989 ; [5]BROWN and MURRAY, 1992 ; [6]SCHEEPMAKER, 1990 ; [7]CROUAU-ROY, 1989 ; [8]LOXDALE, 1990 ; [9]ELO, 1993.

(1989) equations. When $\overline{P}$ (1) values are high, both curves overlap, however as soon as $\overline{P}$ (1) decreases, both curves begin to diverge with the ln based curve (SLATKIN, 1985b) rapidly exceeding the $\log_{10}$ based one (SLATKIN and BARTON, 1989). When $\overline{P}$ (1) reaches for instance 0.01, gene flow estimates drop from 70 to 35 respectively when SLATKIN's (1985b) or SLATKIN and BARTON's (1989) equation is used.

The difference between both equations observed in our simulation model is also observed in the literature (Table 1). When Nm values are high (small $\overline{P}$ (1) values), both equations yield substantially different Nm estimates. Nm estimates calculated with SLATKIN (1985b) will then exceed Nm values obtained with SLATKIN and BARTON's (1989) equation.

This is for example the case in the blue mussel *Mytilus edulis* where the original Nm value drops from 42.0 (see SLATKIN, 1985b) to 21.1 when SLATKIN and BARTON's (1989) equation is used instead. Similar differences are observed in the bracken *Pteridium aquilinum*. Gene flow between seven British populations was estimated using SLATKIN's (1985b) equation (WOLF *et al.*, 1991) and yielded $Nm = 36.51$. This value drops to 19.9 when estimated with SLATKIN and BARTON's (1989) equation. In the blacklip abalones *Haliotis rubra* and *H. laevigata*, the original Nm values, which are respectively 19.67 and 14.01 (BROWN and MURRAY, 1992) increase to 39.17 and 22.27 when recalculated using SLATKIN's (1985b) equation.

Although under realistic conditions the method of WRIGHT (1951) is likely to be more accurate than the private alleles method (SLATKIN and BARTON, 1989), one would expect both methods to yield comparable Nm estimates. This is true except for *Pteridium aquilinum* (WOLF *et al.*, 1991). Since the formulae of SLATKIN (1985b) and/or SLATKIN and BARTON (1989) do not show a consistent pattern of difference compared to WRIGHT's (1951), there is no obvious way to determine which of both formulas matches best with WRIGHT's (1951) method.

Besides the differences due to the equation used, differences in Nm estimates can also be the result of sample size correction. If the correction is not made according to SLATKIN (1985b) and SLATKIN and BARTON (1989), Nm values could be over- or underestimated. This is the case when sample size correction is ignored as was done in studies of the queen conch *Strombus gigas* (MITTON *et al.*, 1989) and the fruit fly *Ceratitis capitata* (GASPERI *et al.*, 1991). Depending on the values of N_{sam} and N_{ref}, gene flow estimates will then under- ($N_{ref} > N_{sam}$) or overestimate ($N_{ref} < N_{sam}$) the actual Nm value. The same is true when Nm_{ref} is multiplied by an inverted correction term, which was done in a study of the troglobitic beetle *Speonomus hydrophilus* (CROUAU-ROY, 1989). The original Nm value (obtained by multiplying Nm_{ref} with N_{sam} and dividing it by N_{ref}) drops from 12.53 to 1.51 when appropriately corrected.

DISCUSSION

As demonstrated above, the formulae of SLATKIN (1985b) and SLATKIN and BARTON (1989) can give different results when applied to the same data. Since this

is particularly the case when $\overline{P}(1)$ values are small it is obvious that this discrepancy will mostly affect gene flow estimates derived from species with a high dispersal potential and hence a high degree of gene flow and low expected frequency of private alleles.

According to WRIGHT (1931) one immigrant per generation ($Nm = 1$) is sufficient to prevent population differentiation due to random genetic drift. The transition from large to small amounts of population differentiation will not occur abruptly with an Nm value of one, yet $Nm = 1$ is very often used as a decisive limit. If gene flow is expressed in terms of its ability ($Nm > 1$) or disability ($Nm < 1$) to prevent population differentiation due to random drift, both equations will yield comparable results. If on the other hand Nm estimates are used to compare gene flow estimates, both equations can not be used interchangeably and the equation used together with $\overline{P}(1)$ and N_{sam} should be specified. Even when specified, confusion may still persist. In ALSTAD *et al.* (1991) SLATKIN's (1985b) equation, together with values of a and b belonging to SLATKIN and BARTON's (1989) equation were described. However, Nm was not estimated with SLATKIN's (1985b) but with SLATKIN and BARTON's (1989) equation (Table 1). Furthermore it seems that, even when equations are specified, Nm estimates are sometimes compared regardless of the equation used. This was the case in the blacklip abalone *H. rubra* (BROWN, 1991). The $\log_{10}$ based Nm estimate of 19.67 is compared to the ln based Nm estimate of *Mytilus edulis* (see SLATKIN, 1985b), whereas in fact it should be compared with the recalculated Nm value of 21.1, as shown in Table 1.

Given the fact that there seems to be confusion regarding the use of private alleles to estimate gene flow, it is advisable to use SLATKIN's method only to distinguish between $Nm > 1$ or $Nm < 1$, with $Nm = 1$ as limit. If Nm estimates are to be compared quantitatively, WRIGHT's (1951) method, based on the mean F_{ST} value, is more appropriate.

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REFERENCES

ALSTAD, N.D., C.S. HOTCHKISS, and W.K. CORBIN (1991) — Gene flow estimates implicate selection as a cause of scale insect population structure. *Evol. Ecol.*, **5** : 88-92.

BROWN, L.D. (1991) — Genetic variation and population structure in the blacklip abalone *Haliotis rubra*. *Aust. J. Mar. Freshw. Res.*, **42** : 77-90.

BROWN, L.D. and N.D. MURRAY (1992) — Population genetics, gene flow and stock structure in *Haliotis rubra* and *Haliotis laevigata*. In SHEPERD, S.A., TEGNER, M.J. and GUZMANN DEL PROO, S.A. (eds.) *Abalone of the world, Biology, Fisheries and culture*. Proceedings of the 1st International Symposium on Abalone : 24-33.

CROUAU-ROY, B. (1989) — Population studies on an endemic troglobitic beetle : geographical patterns of genetic variation, gene flow and genetic structure compared with morphometric data. *Genetics*, **121** : 571-582.

ELO, K. (1993) — Gene flow and conservation of genetic variation in anadromous Atlantic salmon (*Salmo salar*). *Hereditas*, **119** : 149-159.

GASPERI, G., C.R. GUGLIELMINO, A.R. MALACRIDA and R. MILANI (1991) — Genetic variability and gene flow in geographical populations of *Ceratitis capitata* (Wied.) (medfly). *Heredity*, **67** : 347-356.

LOXDALE, D.H. (1990) — Estimating levels of gene flow between natural populations of cereal aphids (Homoptera : Aphididae). *Bull. Entomol. Res.*, **80** : 331-338.

MITTON, B.J., J.C. BERG Jr, and S.K. ORR (1989) — Population structure, larval dispersal, and gene flow in the Queen Conch, *Strombus gigas*, of the Caribbean. *Biol. Bull.*, **177** : 356-362.

SCHEEPMAKER, M. (1990) — Genetic differentiation and estimated levels of gene flow in members of the *Gammarus pulex*-group (Crustacea, Amphipoda) in western Europe. *Bijdr. Dierk.*, **60** : 3-30.

SLATKIN, M. (1985a) — Gene flow in natural populations. *Annu. Rev. Ecol. Syst.*, **16** : 393-430.

SLATKIN, M. (1985b) — Rare alleles as indicators of gene flow. *Evolution*, **39** : 53-65.

SLATKIN, M. and N.H. BARTON (1989) — A comparison of three indirect methods for estimating average levels of gene flow. *Evolution*, **43** : 1349-1368.

WOLF, G.P., F.L.S.E. SHEFFIELD and H.C. HAUFLER (1991) — Estimates of gene flow, genetic substructure and population heterogeneity in bracken (*Pteridium aquilinum*). *Biol. J. Linn. Soc.*, **42** : 407-423.

WRIGHT, S. (1931) — Evolution in Mendelian populations. *Genetics*, **16** : 97-159.

WRIGHT, S. (1951) — The genetical structure of populations. *Ann. Eugen.*, **15** : 323-354.

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SEASONAL SUCCESSION AND SPATIAL DISTRIBUTION OF THE ZOOPLANKTON COMMUNITY IN THE RESERVOIR OF ESCH-SUR-SÛRE (LUXEMBOURG)

by

ALAIN DOHET (1) and LUCIEN HOFFMANN (1, 2)
(1) C.R.P. Centre Universitaire,
Cellule d'Hydrobiologie,
162a, avenue de la Faïencerie, L-1511 Luxembourg, Luxembourg
(2) Université de Liège,
Département de Botanique B 22,
Sart Tilman, B-4000 Liège, Belgium

ABSTRACT

During three years (1990, 1992 and 1993), the zooplankton community of the Esch-sur-Sûre reservoir was investigated. A total of 75 taxa, dominated by rotifers, was observed. Zooplankton densities increased from the damsite towards the headwaters of the reservoir ; they peaked in spring and at the end of summer. Despite quantitative differences, the same species succession was observed during the three years and at all the lake sampling stations. Thus the zooplankton community shifted from rotifers in early spring to small-bodied cladocerans (*Bosmina* spp.), and, finally, to larger cladocerans (*Daphnia* spp.) in early summer. Shifts in zooplankton composition have also frequently been observed in midsummer, when the cladoceran assemblage of *Daphnia-Bosmina* was generally replaced by a combination of *Ceriodaphnia-Diaphanosoma*. These species successions are discussed in relation to size-selective predation, resource limitation and interspecific competition.

Key words : competition, community structure, predation, reservoir, seasonal succession, zooplankton.

INTRODUCTION

In early studies, zooplankton species succession was usually considered to be the result of differences in ecological tolerance to various abiotic environmental factors,

such as light intensity and water density or viscosity (*e.g.* EDMONDSON, 1965 ; MIRACLE, 1977). More recently, descriptive field studies and laboratory experiments have implicated competition (*e.g.* SMITH and COOPER, 1982 ; EDMONDSON, 1985 ; KERFOOT *et al.*, 1985 ; LAMPERT and MUCK, 1985 ; TAYLOR, 1985 ; THRELKELD, 1985 ; VANNI, 1986 ; GILBERT, 1988 ; LAMPERT and ROTHHAUPT, 1991 ; WICKHAM and GILBERT, 1991 ; CONDE-PORCUNA *et al.*, 1994) or predation (*e.g.* ZARET, 1978 ; LANE, 1979 ; STENSON, 1982 ; RIESSEN, 1985 ; GULATI, 1990 ; ARCIFA *et al.*, 1992 ; RONNEBERGER *et al.*, 1993 ; KASPRZAK *et al.*, 1993) as factors which may alternately favour one species over another in successional events. In their publication of the « size-efficiency hypothesis », BROOKS and DODSON (1965) popularised the general belief that the coexistence of large — and small-bodied zooplankton species could be explained by a balance between predatory forces and competition. These authors postulated that all planktonic herbivores compete for the « fine particulate matter (1-15 μm) of the open water », but that large-bodied species are superior competitors, because of their assumed increased filtering efficiency and ability to ingest larger particles. It is presumed that competition forces communities towards larger-bodied species (*e.g. Daphnia*), while fish predation forces them towards smaller-bodied species (*e.g. Bosmina*, rotifers) by selectively removing the larger-bodied forms. In this way, both factors, competition and predation, are believed to interact to cause species replacements in seasonal successions (NEILL, 1975 ; LYNCH, 1979 ; GLIWICZ *et al.*, 1981 ; DEMOTT and KERFOOT, 1982 ; DEMOTT, 1983 ; BENDORF and HORN, 1985 ; GLIWICZ, 1985 ; GOPHEN and POLLINGER, 1985). At the same time, the role of abiotic factors cannot be ignored because they are well synchronised with changes in the magnitude of food limitation and predation intensity (GLIWICZ and PIJANOWSKA, 1989 ; SCHMID-ARAYA and ZUÑIGA, 1992).

Until now, there was virtually no work published on the zooplankton of the reservoir of Esch-sur-Sûre. This first paper briefly presents the field data of a three-year study (1990, 1992, 1993) on the zooplankton structure, i.e. vertical and longitudinal distribution, composition and abundance, in the reservoir. The seasonal successions of rotifers and cladocerans during these three years of investigations are described and discussed in relation to predation and competition as explaining factors.

MATERIAL AND METHODS

Study area

The Esch-sur-Sûre reservoir (Fig. 1) lies in the Northern part of the G.-D. of Luxembourg and is the result of a dam built on the Sûre river in 1960. The reservoir, located at an altitude of 320 m, has an area of 3.2 km^2 and a length of 18 km

from the major influent river to the dam. The reservoir has a volume of 55.10^6 m^3, a mean depth of 17 m and a maximum depth of 46 m. The watershed has a surface of about 430 km^2. The maximum water flow occurs in the winter period (December-February) ; the annual mean retention time amounts to about 3 months.

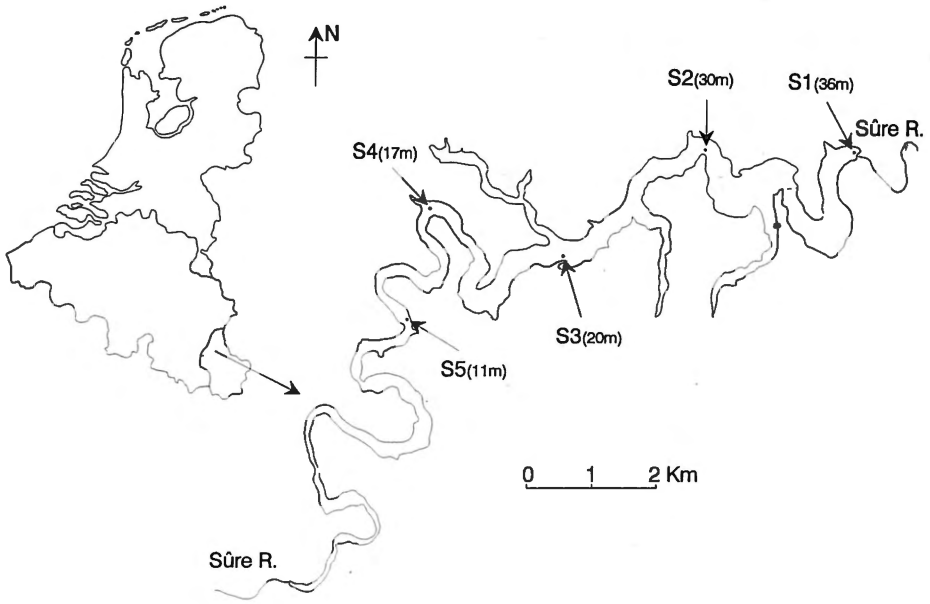

Fig. 1. — Map of the Esch-sur-Sûre reservoir showing the locations of sampling stations. Numbers in parentheses show the mean depth of the station.

According to the OECD classification (1982), the reservoir is considered as a meso-eutrophic waterbody (annual mean chlorophyll a concentration in the euphotic zone : 5.0-8.8 µg/l ; annual maximum total chlorophyll concentration : 16.3-34.9 µg/l ; annual mean Secchi transparency 4.2-4.7 m ; annual minimum transparency : 1.8-2.5 m ; data for the sampling station near the dam from 1990 to 1993). In winter the reservoir may be partly ice-covered, but this was only the case in 1992. During the study period, the water temperature remained below 10° C until the beginning of April when it rapidly increased in the epilimnion. From mid-June until the end of August, the surface water temperature was higher than 17° C. From the beginning of September, water temperature decreased reaching 8° C by November. During the whole period the temperature of the hypolimnion always remained below 10° C at station 1. The stratification of the reservoir generally starts at the end of April-beginning of May and the fall destratification begins in September. The metalimnion occurs in summer between 10 and 15 m. The hypolimnion remains oxic throughout the year. Most dominant fish species in the reservoir are planktivorous (roach : *Rutilus rutilus* ; bleak : *Alburnus alburnus* ; perch : *Perca*

fluviatilis ; coregone : *Coregonus nasus*...). The spring algal bloom is dominated by small centric diatoms and Cryptophyceae ; it is followed by a clear water phase in June and then by a summer phytoplankton bloom dominated by large species that are inedible for most zooplankton species (*e.g. Aphanizomenon flos-aquae, Anabaena* spp., *Ceratium hirundinella, Peridinium* gr. *cinctum*).

Zooplankton analysis

Samples were collected weekly from January to November in 1990 and twice a month from April to November in 1992 and 1993. In 1991, the reservoir was emptied in order to repair the main dam wall. Zooplankton was sampled every 2.5 metres in the epilimnion and every 5 metres in the meta- and hypolimnion at 5 stations (S 1-S 5) between the dam and the influent river. The samples were taken between 9 and 11 o'clock with a 10-litre sampling bottle.

The zooplankton was concentrated by gently filtering 2 litres of lake water through a 55 µm sieve ; the sieve was thoroughly rinsed and its content preserved with a 4 % formalin solution. In the laboratory, the organisms contained in a 100 ml sample were allowed to settle down for at least one day. The water was then removed with a vacuum pump until only 10 ml remained.

Species in the zooplankton were counted at a magnification of 63 with an inverted microscope (Leitz Labovert) ; depending on the zooplankton densities, either an entire or half a settling chamber was examined.

In addition, samples were taken with a plankton net towed vertically from the bottom to the surface. This sampling technique was used to obtain a large number of living organisms for the estimation of the biomass and for the identification of some species difficult to recognize after preservation with formalin.

In order to compare seasonal successions of zooplankton species between years and between different sampling stations, the densities recorded at each depth (expressed in individuals per litre) were converted to values per m^2 of surface area. Since the volume of water at different depths is not the same, conversion to m^2 was weighted by a depth factor.

RESULTS AND DISCUSSION

Zooplankton composition

Zooplankton species and indication of their frequency of occurrence throughout the study period are shown in Table 1. A total of 75 taxa, comprising 49 rotifers,

TABLE 1

Zooplankton species and indication of their frequency of occurrence among the samples taken from 1990 to 1993 at 5 stations in the reservoir of Esch-sur-Sûre :••• ... dominant species, •• ... common species, • ... sporadic species, ¤ ... one or two observations

Rotifers

Anuraeopsis fissa (Gosse, 1851)•
Ascomorpha ecaudis (Perty, 1850)••
Ascomorpha saltans Bartsch, 1870•
Ascomorpha sp.•
Asplanchna priodonta Gosse, 1850••
Brachionus angularis Gosse, 1851••
Brachionus calyciflorus (Pallas, 1766)••
Brachionus quadridentatus (Hermann, 1783)••
Brachionus sp.•
Cephalodella gibba (Ehrenberg, 1838)•
Cephalodella sp.•
Colurella adriatica (Ehrenberg, 1831)•
Colurella sp.•
Conochilus unicornis (Rousselet, 1892)•••
Epiphanes senta (O.F. Müller, 1773) ¤
Euchlanis dilatata (Ehrenberg, 1832)••
Filinia longiseta (Ehrenberg, 1834)••
Filinia sp.•
Gastropus hyptopus (Ehrenberg, 1838) ¤
Kellicottia longispina (Kellicott, 1879)••
Keratella cochlearis (Gosse, 1851)•••
Keratella cochlearis tecta (Lauterborn, 1900)••
Keratella hiemalis (Carlin, 1943)••
Keratella quadrata (O.F. Müller, 1786)••
Keratella ticinensis (Calleris, 1920) ¤
Keratella valga (Ehrenberg, 1834) ¤
Lecane (Monostyla) sp.••
Lecane (Lecane) sp.••
Lepadella ovalis (O.F. Müller, 1786)•
Mytilina mucronata (O.F. Müller, 1773) ¤
Notholca acuminata (Ehrenberg, 1832)••
Notholca squamula (O.F. Müller, 1786)••
Notholca sp.•
Ploesoma hudsoni (Imhof, 1891)••
Polyarthra dolichoptera (Idelson, 1985)••
Polyarthra major (Burckhart, 1900)••
Polyarthra vulgaris (Carlin, 1943)••
Polyarthra « vulgaris x dolichoptera »•••
Pompholyx sulcata (Hudson, 1845)••
Proales sp.•
Scaridium sp. ¤
Synchaeta pectinata Ehrenberg, 1832••
Synchaeta tremula O.F. Müller, 1786••
Trichocerca bicristata (Gosse, 1887) ¤
Trichocerca pusilla (Lauterborn, 1898)••
Trichocerca rousseleti (Voigt, 1902)•
Trichocerca similis (Wierzejski, 1893)••
Trichocerca sp.•
v*Trichotria* sp.•

Cladocerans

Alona quadrangularis (O.F. Müller, 1785)••
Alona rectangula Sars, 1862•
Alona sp.•
Biapertura affinis (Leydig, 1860)•
Bosmina (Eubosmina) coregoni Baird, 1857•••
Bosmina (Bosmina) longirostris (O.F. Müller, 1785)••
Ceriodaphnia pulchella Sars, 1862••
Ceriodaphnia sp.•
Chydorus brevilabris (Frey, 1980)•
Chydorus sphaericus (O.F. Müller, 1785)••
Chydorus sp.•
Daphnia cucullata Sars, 1862••
Daphnia galeata Sars, 1864•••
Daphnia longispina O.F. Müller, 1785•
Daphnia sp.•
Diaphanosoma brachyurum (Liévin, 1848)•••
Eurycercus lamellatus (O.F. Müller, 1785) ¤
Holopedium gibberum Zaddach, 1855 ¤
Leptodora kindtii (Focke, 1844)••
Polyphemus pediculus Linnaeus, 1761•
Simocephalus sp. ¤

Copepods

Cyclops venustus (Norman and Scott, 1906)•
Cyclops vernalis americanus (Marsh, 1893)•
Cyclops vicinus (Ulianine, 1875)•••
Eudiaptomus gracilis (Sars, 1862)•••
Halicyclops christianensis (Boeck, 1872)•••

21 cladocerans and 5 copepods, were identified. However, about half of them were only sporadically found and their density was very low. This species number is similar to that found in some other lakes of the temperate zone (*e.g.* GULATI, 1990 ; ARNDT *et al.*, 1993), but considerably higher than the diversity observed in other reservoirs (*e.g.* ADALSTEINSSON, 1979 ; SELIN and HAKKARI, 1982 ; URABE and MURANO, 1986 ; ARCIFA *et al.*, 1992 ; SCHMID-ARAYA and ZUÑIGA, 1992). Among the rotifers, *Keratella cochlearis, Polyarthra vulgaris-dolichoptera* and *Conochilus unicornis* were the dominant species throughout the year. *Synchaeta tremula* was very abundant only in early spring. Among the cladocerans, the most abundant species were *Daphnia galeata, D. cucullata* and hybrids (from the end of April to the beginning of October) and *Diaphanosoma brachyurum* (during summer). *Bosmina (Eubosmina) coregoni* and *Bosmina longirostris* were common in spring. Among the copepods, *Eudiaptomus gracilis, Halicyclops christianensis* and *Cyclops vicinus* were abundantly and frequently found throughout the year. The species diversity assessed by the Shannon index (RICHARD *et al.*, 1985) did not significantly differ between the downlake and the uplake stations (Table 2).

TABLE 2

Annual mean Shannon diversity index at 5 stations in the reservoir of Esch-sur-Sûre

	Station 1	*Station 2*	*Station 3*	*Station 4*	*Station 5*
1992	1.84	1.76	2.09	2.33	2.35
1993	1.70	2.19	2.16	2.25	2.44

Vertical and longitudinal distribution

Except for quantitative differences, the zooplankton showed the same vertical and longitudinal distribution patterns during the three investigated years. Fig. 2 shows, as an example, the vertical and temporal distribution of the zooplankton at the 5 sampling stations in the reservoir in 1993. The zooplankton was concentrated in the top 15 m at the times of sampling ; in the parts with the greatest depth (stations 1, 2 and 3), a distinct vertical gradient in zooplankton density was thus observed whereas in the shallower parts of the reservoir (stations 4 and 5), the zooplankton was as a result distributed more uniformly over the whole water column. With the exception of *Ceriodaphnia pulchella* which appeared preferentially in the hypo or metalimnion of the reservoir, this vertical distribution was quite similar for all the species and was also observed by URABE (1990) in the Ogochi reservoir (Tokyo).

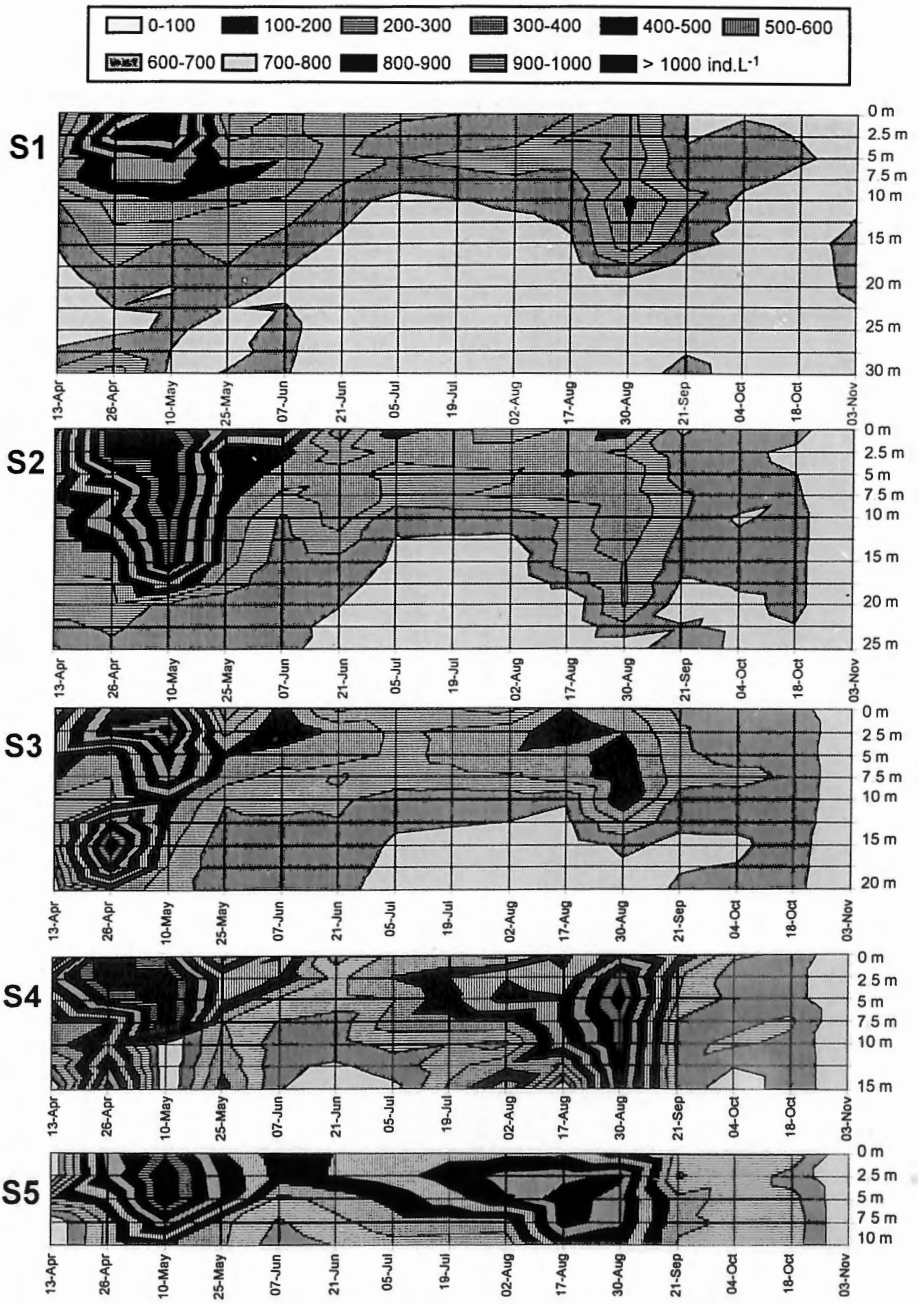

Fig. 2. — Temporal and vertical distribution of the zooplankton at the five sampling stations in the reservoir of Esch-sur-Sûre during 1993.

The highest zooplankton densities (> 800 ind/l) were observed in all the stations during spring ; a second peak occurred at the end of the summer. During spring, total zooplankton density was quite similar in all the stations whereas, from July to September, zooplankton density increased from the damsite (station 1) to the headwaters with a maximum at station 4. This gradual uplake increase was commonly found for most of the dominant species (Table 3). This was the case for

TABLE 3

Longitudinal variation in the mean density (10^3 ind.m^{-2}) of dominant species in 1993. Dgr : Eudiaptomus gracilis ; *C :* Cyclops *spp.* ; *Dg :* Daphnia galeata ; *Db :* Diaphanosoma brachyurum ; *Kc :* Keratella cochlearis ; *P :* Polyarthra *spp.*

	Dgr	*C*	*Dg*	*Db*	*Kc*	*P*
S1	285	475	179	157	1.887	1.390
S2	235	636	159	328	2.027	1.133
S3	239	720	224	385	2.004	1.744
S4	233	1.025	312	466	2.445	1.934
S5	276	632	183	465	1.672	1.430

Keratella cochlearis and *Polyarthra* spp. among the rotifers and for *Cyclops* spp., *Daphnia galeata* and *Diaphanosoma brachyurum* among the crustaceans. On the contrary, mean densities of *Eudiaptomus gracilis* did not appreciably differ. Some rotifers, like *Brachionus* spp., *Filinia longiseta* and *Pompholyx sulcata*, species which are commonly used as indicators of eutrophic conditions (GANNON and STEMBERGER, 1978 ; PEJLER, 1983), also increased from the damsite to the uplake region.

Gradual uplake increase in the abundance of the zooplankton has been observed in some other lakes or reservoirs (*e.g.* GANNON and STEMBERGER, 1978 ; URABE and MURANO, 1986). This longitudinal zooplankton density gradient is probably the result of high nutrient conditions near the river mouth which stimulate an increase in primary production resulting in a higher food availability. The algal biomass showed indeed a similar horizontal gradient to the zooplankton densities (for example, the annual mean chlorophyll a concentrations in the epilimnion increased from 5.3 µg/l at station 1 to 7.0 µg/l at station 5 in 1993). This higher algal biomass can support an increase in secondary production, as was shown by GANNON and STEMBERGER (1978) for lake Michigan.

On the other hand, it is known that the hydrological regime, such as the flushing and dilution effects of inflowing water, sometimes affects the horizontal planktonic populations, decreasing their abundances, especially for stations nearest to the river (SOTO *et al.*, 1984 ; HAYWARD and VAN DEN AVYLE, 1986). The facts that the highest values of zooplankton density were observed at station 4 in the reservoir of Esch-sur-Sûre, would indicate that this station represents the optimal conditions for the zooplankton development, influenced by the dilution impact

of the inflowing water on one hand and high nutrient conditions on the other hand.

Seasonal succession

Seasonal variations in the density of rotifers and dominant species of cladocerans at station 1, in 1990, 1992 and 1993 are shown in Fig. 3. Large interannual differences were observed in the dominance of the species. Thus for example, *Bosmina* reached by far the highest densities among the crustaceans in 1992, whereas in 1990 and 1993, its densities were comparable to or lower than those of *Daphnia*. Furthermore, there were considerable annual differences in total zooplankton biomass (52 $kg.ha^{-1}$ in 1990, 67 $kg.ha^{-1}$ in 1992 and 45 $kg.ha^{-1}$ in 1993). Despite these quantitative differences, the seasonal succession of zooplankton species remained the same during the three years of investigations, at all the sampling stations (example of 1993, Fig. 4). Thus, after the spring phytoplankton bloom formed by fast growing algae such as Cryptophyceae and small centric diatoms, a peak in rotifer species (in order of appearance : *Synchaeta tremula, Polyarthra vulgaris-dolichoptera, Keratella cochlearis* and *Conochilus unicornis*) occurred which was followed by a peak of *Bosmina* spp., and then by a peak of *Daphnia* spp. This early spring sequence of the peaks in densities from small to large zooplankton species has been observed in many lakes of the temperate zone (ELORANTA, 1982 ; DEMOTT, 1983 ; LEHTOVAARA and SARVALA, 1984 ; SOMMER *et al.*, 1986 ; ARNDT *et al.*, 1993).

The smallest species, which also have the shortest generation time, thus appear first and are subsequently replaced by larger species with longer generation time. The species sequence can thus be understood as a typical successional replacement of populations caused by resource limitation or interspecific competition alone. Indeed, each subsequent species can be assumed to be a more effective competitor for resources than the one before it since competitive advantage is believed to increase with increasing body size (BROOKS and DODSON, 1965). According to GLIWICZ and PIJANOWSKA (1989), the possibility cannot be excluded, however, that other forces are involved. First, the sequence may be produced by temperature-cued successive hatching of resting eggs. Second it may be caused by predation ; during this period (end of April to beginning of June), indeed, when temperature is increasing, planktivorous fish hatch (GLIWICZ and PIJANOWSKA, 1989). Juvenile fish thus appear as a synchronised cohort of gape-limited predators that grow and switch their feeding mode from smaller to larger prey (WONG and WARD, 1972 ; ZARET, 1978), and may cause the observed sequence from small to large zooplankton species (*e.g.* case of juvenile roach, CRYER *et al.* 1986).

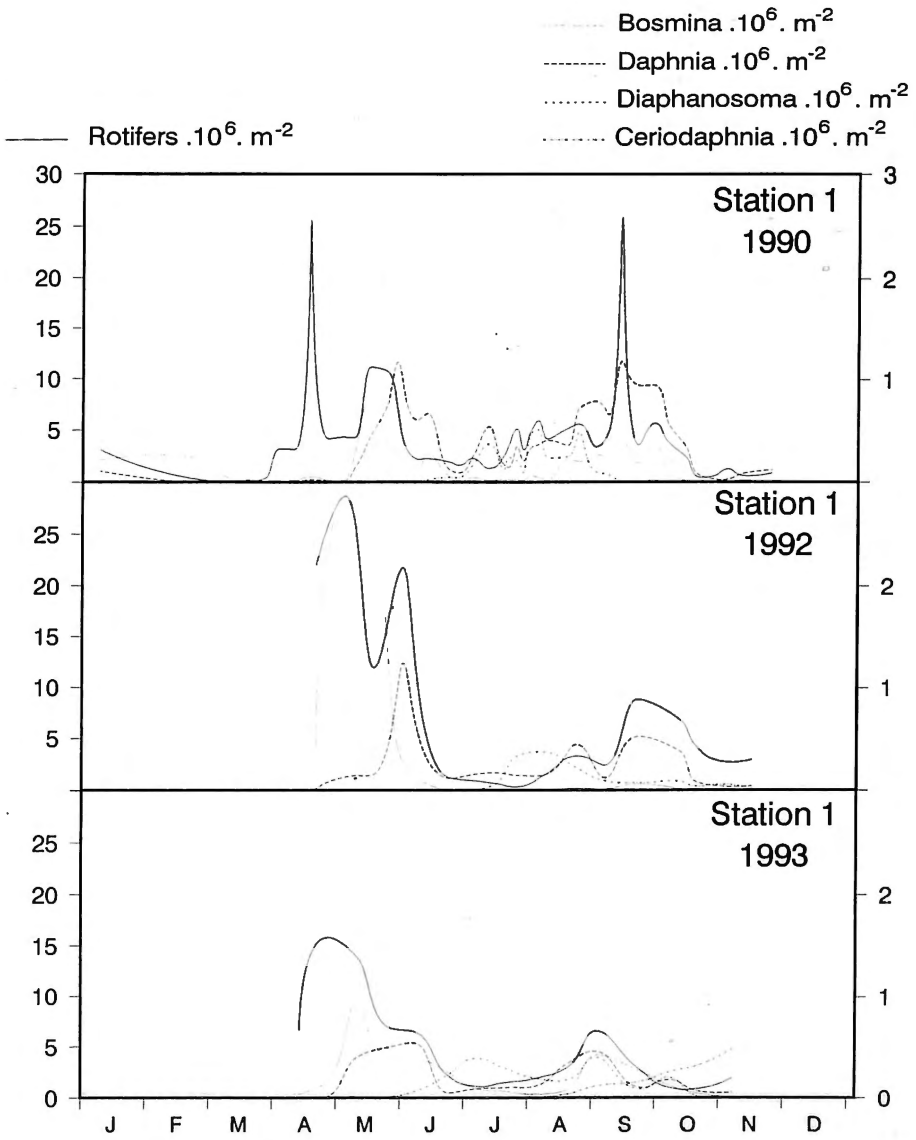

Fig. 3. — Seasonal changes in the abundance of rotifers, *Bosmina* spp., *Daphnia* spp., *Diaphanosoma brachyurum* and *Ceriodaphnia pulchella* in the reservoir of Esch-sur-Sûre from 1990 to 1993 (station 1).

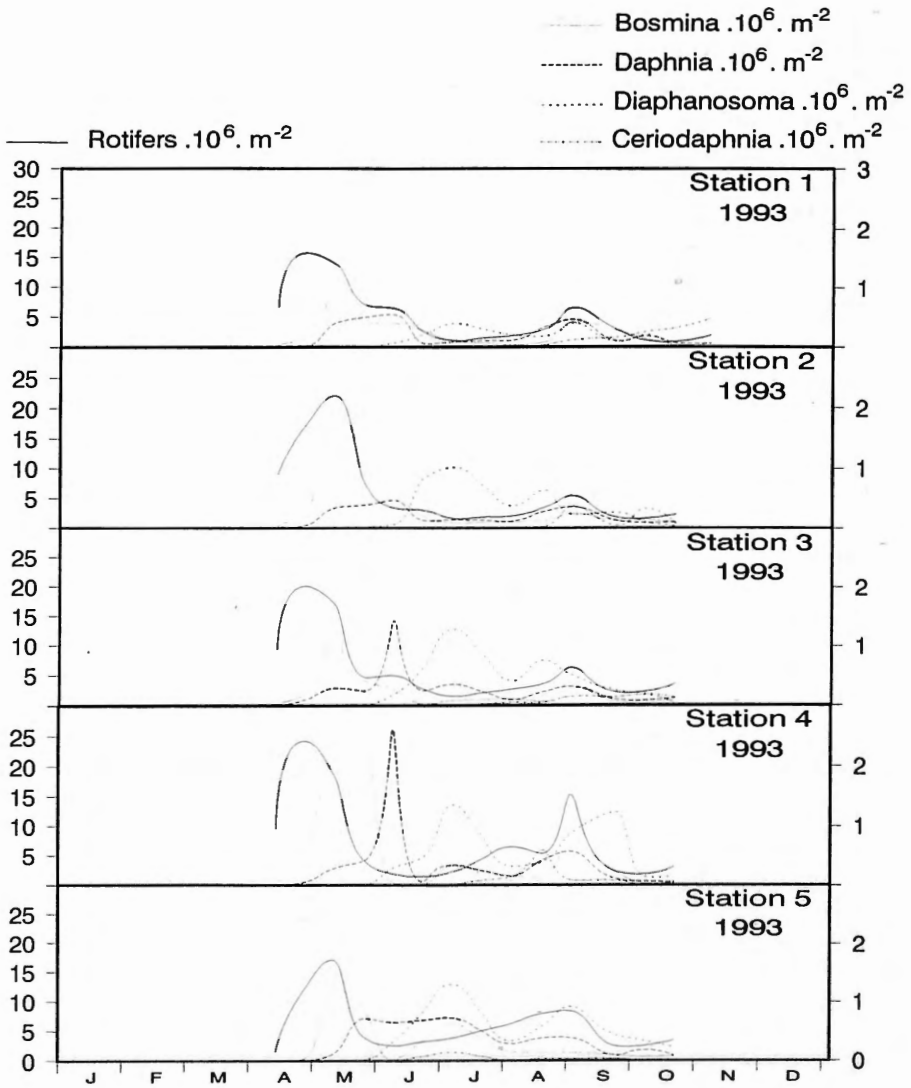

Fig. 4. — Seasonal changes in the abundance of rotifers, *Bosmina* spp., *Daphnia* spp., *Diaphanosoma brachyurum* and *Ceriodaphnia pulchella* in the reservoir of Esch-sur-Sûre from station 1 to station 5 in 1993.

Another common phenomenon observed in the Esch-sur-Sûre reservoir, is the midsummer replacement of the cladoceran assemblage of *Bosmina* spp. and *Daphnia galeata* by *Diaphanosoma brachyurum* or by a combination of *Diaphanosoma brachyurum* and *Ceriodaphnia pulchella*. The latter species showed indeed a significant peak in the hypolimnion during the end of summer 1993. These shifts in zooplankton composition were also frequently observed in other temperate

lakes (BEATTIE *et al.*, 1978 ; SOMMER *et al.*, 1986 ; GLIWICZ and PIJANOWSKA, 1989) and are often attributed to fish predation, especially when a large or less evasive species (like *Daphnia galeata*) is replaced by a smaller, less conspicuous one or by a species that has developed antipredator defences such as the escape ability of *Diaphanosoma brachyurum* (DRENNER and MCCOMAS, 1980 ; GLIWICZ and PIJANOWSKA, 1989). However, midsummer declines in *Daphnia* in eutrophic lakes and subsequent increases in *Diaphanosoma* have also been attributed to the greater tolerance of *Diaphanosoma* to interference from filamentous cyanobacteria (GLIWICZ, 1977). Radiotracer studies have indeed confirmed that *Diaphanosoma*'s feeding rate on small edible algae is less affected than *Daphnia*'s by the presence of cyanobacteria (FULTON and PAERL, 1987 ; FULTON, 1988). Because *Diaphanosoma brachyurum* exhibits midsummer peaks even in years without significant densities of cyanobacteria, greater tolerance to interfering particles cannot be the sole reason for *Diaphanosoma*'s success during midsummer in the Esch-sur-Sûre reservoir. Furthermore, DEMOTT and KERFOOT (1982) and DEMOTT (1985) have demonstrated the ability of *Diaphanosoma* to feed very effectively on bacteria ; this could explain the success of *Diaphanosoma* during midsummer minima in edible algae. However, despite correlations, it is often difficult to be sure which factor leads to the midsummer decline in *Daphnia* (DEMOTT, 1989).

CONCLUSIONS

The early spring sequence of the peaks in densities from small to large zooplankton species and the midsummer declines in *Daphnia* were the only phenomena observed in all years. Predation and food limitation are two possible factors to explain the zooplankton species replacements in the Esch-sur-Sûre reservoir. An experimental approach and demographic analyses (birth and death rates, average clutch size) are needed to determine the respective impacts of predation, competition, various environmental factors and their interactions.

REFERENCES

ADALSTEINSSON, H. (1979) — Zooplankton and its relation to available food in Lake Mývatn. *Oikos*, **32** : 162-194.

ARCIFA, M.S., E.A.T. GOMES and A.J. MESCHIATTI (1992) — Composition and fluctuations of the zooplankton of a tropical Brazilian reservoir. *Arch. Hydrobiol.*, **123** : 479-495.

ARNDT, H., M. KROCKER, B. NIXDORF and A. KÖHLER (1993) — Long-term annual and seasonal changes of meta- and protozooplankton in Lake Müggelsee (Berlin) : Effects of eutrophication, grazing activities and impact of predation. *Int. Revue ges. Hydrobiol.*, **78** : 379-402.

BEATTIE, D.M., H.L. GOLTERMAN and J. VIJVERBERG (1978) — Introduction to the limnology of Friesian lakes. *Hydrobiologia*, **58** : 49-64.

BENDORF, J. and W. HORN (1985) — Theoretical considerations on the relative importance of food limitation and predation in structuring zooplankton communities. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 383-396.

BROOKS, J.L., and S.I. DODSON (1965) — Predation, body size and composition of plankton. *Science,* **150** : 28-35.

CONDE-PORCUNA, J.M., R. MORALES-BAQUERO and L. CRUZ-PIZARRO (1994) — Effects of *Daphnia longispina* on rotifer populations in a natural environment : relative importance of food limitation and interference competition. *J. Plankton Res.*, **16** : 691-706.

CRYER, M., G. PEIRSON and C.R. TOWNSEND (1986) — Reciprocal interactions between roach, *Rutilus rutilus*, and zooplankton in a small lake : Prey dynamics and fish growth and recruitment. *Limnol. and Oceanogr.*, **31** : 1022-1038.

DEMOTT, W.R. (1983) — Seasonal succession in a natural *Daphnia* assemblage. *Ecol. Monogr.*, **53** : 321-340.

DEMOTT, W.R. (1985) — Relations between filter mesh-size, feeding mode, and capture efficiency for cladocerans feeding on ultrafine particules. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 125-134.

DEMOTT, W.R. (1989) — The role of competition in zooplankton succession. *In :* SOMMER U. (ed.), *Plankton Ecology : Succession in Plankton Communities.* Brock/Springer series in contemporary bioscience. Springer-Verlag Berlin : 195-252.

DEMOTT, W.R. and W.C. KERFOOT (1982) — Competition among cladocerans : nature of the interaction between *Bosmina* and *Daphnia. Ecology*, **63** : 1949-1966.

DRENNER, R.W. and S.R. MCCOMAS (1980) — The roles of zooplankter escape ability and fish size selectivity, the selective feeding and impact of planktivorous fish. *In :* KERFOOT W.C. (ed.), *Evolution and ecology of zooplankton communities.* University Press of New England, Hanover, New Hampshire : 587-593.

EDMONDSON, W.T. (1965) — Reproductive rate of planktonic rotifers as related to food and temperature in nature. *Ecol. Monogr.*, **35** : 61-112.

EDMONDSON, W.T. (1985) — Reciprocal changes in abundance of *Diaptomus* and *Daphnia* in Lake Washington. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 475-481.

ELORANTA, P.V. (1982) — Zooplankton in the Vassikalampi pond, a warm water effluent recipient in Central Finland. *J. Plankton Res.*, **4** : 813-837.

FULTON, R.S. (1988) — Grazing on filamentous algae by herbivorous zooplankton. *Freshw. Biol.*, **20** : 263-271.

FULTON, R.S. and H.W. PAERL (1987) — Effects of colonial morphology on zooplankton utilization of algal resources during blue-green algal (*Microcystis aeruginosa*) blooms. *Limnol. and Oceanogr.*, **32** : 634-644.

GANNON, J.E. and R.S. STEMBERGER (1978) — Zooplankton (especially Crustaceans and Rotifers) as indicators of water quality. *Trans. Amer. Micros. Soc.*, **97** : 16-35.

GILBERT, J.J. (1988) — Suppression of rotifer populations by *Daphnia* : A review of the evidence, the mechanisms, and the effects on zooplankton community structure. *Limnol. and Oceanogr.*, **33** : 1286-1303.

GLIWICZ, Z.M. (1977) — Food size selection and seasonal succession of filter feeding zooplankton in a eutrophic lake. *Ekol. Polska*, **25** : 179-225.

GLIWICZ, Z.M. (1985) — Predation or food limitation : an ultimate reason for extinction of planktonic cladoceran species. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 419-430.

GLIWICZ, Z.M., A.M. GHILAROV and J. PIJANOWSKA (1981) — Food and predation as major factors limiting two natural populations of *Daphnia cucullata* Sars. *Hydrobiologia,* **80** : 205-218.

GLIWICZ, Z.M. and J. PIJANOWSKA (1989) — The role of predation in zooplankton succession. *In :* SOMMER U. (ed.), *Plankton ecology : Succession in Plankton Communities.* Brock/ Springer series in contemporary bioscience. Springer-Verlag Berlin : 253-296.

GOPHEN, M. and U. POLLINGHER (1985) — Relationships between food availability, fish predation and the abundance of the herbivorous zooplankton community in Lake Kinneret. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 397-405.

GULATI, R.D. (1990) — Zooplankton structure in the Loosdrecht lakes in relation to trophic status and recent restoration measures. *Hydrobiologia*, **191** : 173-188.

HAYWARD, R.S. and M.J. VAN DEN AVYLE (1986) — The nature of zooplankton spatial heterogeneity in a nonriverine impoundment. *Hydrobiologia*, **131** : 261-271.

KASPRZAK, P., L. KRIENITZ and R. KOSCHEL (1993) — Changes in the plankton of Lake Feldberger Haussee (Germany, Mecklenburg-Vorpommern) in response to biomanipulation. *Arch. Hydrobiol.*, **128** : 149-168.

KERFOOT, W.C., W.R. DEMOTT and D.L. DEANGELIS (1985) — Interactions among cladocerans : Food limitation and exploitive competition. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 431-451.

LAMPERT, W. and P. MUCK (1985) — Multiple aspects of food limitation in zooplankton communities : the *Daphnia-Eudiaptomus* example. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 311-322.

LAMPERT, W. and K.O. ROTHHAUPT (1991) — Alternating dynamics of rotifers and *Daphnia magna* in a shallow lake. *Arch. Hydrobiol.*, **120** : 447-456.

LANE, A.L. (1979) — Vertebrate and invertebrate predation intensity on freshwater zooplankton communities. *Nature*, **280** : 391-392.

LEHTOVAARA, A. and J. SARVALA (1984) — Seasonal dynamics of total biomass and species composition of zooplankton in the litoral of an oligotrophic lake. *Verh. Internat. Verein. Limnol.*, **22** : 805-810.

LYNCH, M. (1979) — Predation, competition, and zooplankton community structure : an experimental study. *Limnol. and Oceanogr.*, **24** : 253-272.

MIRACLE, M.R. (1977) — Migration, patchiness and distribution in time and space of planktonic rotifers. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **8** : 19-37.

NEILL, W.E. (1975) — Experimental studies of microcrustacean competition, community composition and efficiency of resource utilization. *Ecology*, **56** : 809-826.

OECD (1982) — Eutrophisation des eaux. OECD, Paris.

PEJLER, B. (1983) — Zooplanktic indicators of trophy and their food. *Hydrobiologia*, **101** : 111-114.

RICHARD, D.I., J.W. SMALL and J.A. OSBORNE (1985) — Response of zooplankton to the reduction and elimination of submerged vegetation by grass carp and herbicide in four Florida lakes. *Hydrobiologia*, **123** : 97-108.

RIESSEN, H.P. (1985) — Exploitation of prey seasonality by a planktonic predator. *Can. J. Zool.*, **63** : 1729-1732.

RONNEBERGER, D., P. KASPRZAK and L. KRIENITZ (1993) — Long-term changes in the rotifer fauna after biomanipulation in Haussee (Feldberg, Germany, Mecklenburg-Vorpommern) and its relationship to crustacean and phytoplankton communities. *Hydrobiologia*, **255/256** : 297-304.

SCHMID-ARAYA, J.M. and L.R. ZUÑIGA (1992) — Zooplankton community structure in two Chilean reservoirs. *Arch. Hydrobiol.*, **123** : 305-335.

SELIN, P. and L. HAKKARI (1982) — The diversity, biomass and production of zooplankton in Lake Inarijärvi. *Hydrobiologia*, **86** : 55-59.

SMITH, D.W. and S.D. COOPER (1982) — Competition among Cladocera. *Ecology*, **63** : 1004-1015.

SOMMER, U., Z.M. GLIWICZ, W. LAMPERT and A. DUNCAN (1986) — The PEG-model of seasonal succession of planktonic events in fresh waters. *Arch. Hydrobiol.*, **106** : 433-471.

SOTO, D., I. VILA and B. VILLALOBOS (1984) — Temporal and spatial distribution in a Chilean reservoir : A possible effect of impoundment hydrodynamics. *Hydrobiologia*, **114** : 67-74.

STENSON, J.A. (1982) — Fish impact on rotifer community structure. *Hydrobiologia*, **87** : 57-64.

TAYLOR, B.E. (1985) — Effects of food limitation on growth and reproduction of *Daphnia*. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 285-296.

THRELKELD, S.T. (1985) — Resource variation and the initiation of midsummer declines of cladoceran populations. *Arch. Hydrobiol. Beih. Ergebn. Limnol.*, **21** : 333-340.

URABE, J. (1990). Stable horizontal variation in the zooplankton community structure of a reservoir maintained by predation and competition. *Limnol. and Oceanogr.*, **35** : 1703-1717.

URABE, J. and M. MURANO (1986) — Seasonal and horizontal variations in the zooplankton community of Ogochi Reservoir, Tokyo. *Bull. Plankt. Soc. Jap.*, **33** : 101-112.

VANNI, M.J. (1986) — Competition in zooplankton communities : Suppression of small species by *Daphnia pulex*. *Limnol. and Oceanogr.*, **31** : 1039-1056.

WICKHAM, S.A. and J.J. GILBERT (1991) — Relative vulnerabilities of natural rotifer and ciliate communities to cladocerans : laboratory and field experiments. *Freshw. Biol.*, **26** : 77-86.

WONG, B. and F.J. WARD (1972) — Size selection of *Daphnia pulicaria* by yellow perch (*Perca flavescens*) fry in West Blue Lake, Manitoba. *J. Fish. Res. Board Can.*, **29** : 1761-1764.

ZARET, T.M. (1978) — A predation model of zooplankton community structure. *Verh. Internat. Verein. Limnol.*, **20** : 2496-2500.

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FINE STRUCTURE OF THE PODIA IN THREE SPECIES OF PAXILLOSID ASTEROIDS OF THE GENUS *LUIDIA* (ECHINODERMATA)

by

PATRICK FLAMMANG *
Laboratoire de Biologie Marine, Université de Mons-Hainaut,
19 av. Maistriau, B-7000 Mons, Belgium

SUMMARY

Individuals of the paxillosid asteroid genus *Luidia* use their podia in locomotion and burrowing. Each podium in the three considered species consists of a stem with a pointed knob at its tip. The knob consists of four tissue layers that are, from the inside to the outside, a mesothelium, a connective tissue layer, a nerve plexus, and an epidermis. The latter is made up of four cell categories : secretory cells, neurosecretory cells, non-secretory ciliated cells, and support cells. The epidermal cells of the podial knob are presumably functioning as a duo-gland adhesive system in which adhesive secretions would be produced by secretory cells while de-adhesion, on the other hand, would be due to neurosecretory cell secretions. Although the podia of the three considered species of *Luidia* share numerous similarities, there are nevertheless several important morphological differences between, on the one hand, the podia of *L. ciliaris* and *L. maculata*, and, on the other hand, the podia of *L. penangensis*. These differences stress that carefulness is required when generalizations, drawn from the morphology of a single species, are applied to related species having the same life style ; but also that the genus *Luidia* needs to be re-examined from a taxonomic point of view.

Keywords : ultrastructure, podia, adhesive organs, Asteroidea, Echinodermata.

INTRODUCTION

The podia, *i.e.* the external appendages of the ambulacral system of echinoderms, are multifunctional organs. Indeed, according to the considered class, they can take part in locomotion, feeding, burrowing, respiration or sensory perception. In asteroids, the main function of the podia is locomotion (LAWRENCE, 1987). Most species possess podia which end with a flat disk allowing the podium to adhere to the substratum. Yet species of the order Paxillosida, which are soft sub-

* Senior Research Assistant, National Fund for Scientific Research, Belgium.

strate dwellers, possess podia ending with a pointed knob. In these asteroids, the podia take part in both locomotion and burrowing.

Whereas asteroid disk-ending podia have been the subject of a number of ultrastructural investigations (CHAET, 1965 ; HARRISON and PHILPOTT, 1966 ; SOUZA SANTOS and SILVA SASSO, 1968 ; FLAMMANG *et al.*, 1994), the available data about knob-ending podia are scarce. In fact, there is only a single paper on these podia concerning one species of the genus *Astropecten* (ENGSTER and BROWN, 1972).

The aim of the present work is to describe the ultrastructure of the podia of three asteroid species of the paxillosid genus *Luidia* and to consider whether their burrowing behaviour and the shape of their podia correspond with a different epidermal organization compared to podia of other asteroid species.

MATERIAL AND METHODS

Individuals of three species of the paxillosid asteroid genus *Luidia* were investigated. They were all collected by dredging either in the bay of Morlaix, Brittany, France (*Luidia ciliaris* (PHILIPPI, 1837)) in August 1994, or off the east coast of Singapore mainland (*Luidia maculata* MÜLLER and TROSCHEL, 1842 and *Luidia penangensis* DE LORIOL, 1891) in March and September 1994.

For scanning electron microscopy (SEM), podia were fixed in Bouin's fluid for 24 h. They were dehydrated in graded ethanol, dried by the critical point method (with CO_2 as transition fluid), mounted on aluminum stubs, coated with gold in a sputter coater and observed with a JEOL JSM-6100 scanning electron microscope.

For transmission electron microscopy (TEM), podia were fixed in 3 % glutaraldehyde in cacodylate buffer (0.1M, pH 7.8) for 3 h at 4° C, rinsed in cacodylate buffer, and post-fixed for 1 h in 1 % osmium tetroxide in the same buffer. After a final wash in buffer, they were dehydrated in graded ethanol and embedded in Spurr. Semithin sections (1µm in thickness) were cut on a Reichert Om U2 ultramicrotome equipped with a glass knife. They were stained with a 1:1 mixture of 1 % methylene blue in 1 % natrium tetraborate and 1 % azur II. Ultrathin sections (40-70 nm) were cut with an LKB III ultramicrotome equipped with a diamond knife, stained with uranyl acetate and lead citrate, and observed with a Zeiss EM 10 transmission electron microscope.

RESULTS

Outer aspect of the podia

In the three species, the podia are arranged in two rows on the oral surface of the arms. Mean size podia measure about 1,25 mm in diameter and 15 mm in length. They consist of an extensible cylindrical stem topped by a knob (Figs 1-3). In both *L. ciliaris* and *L. maculata*, the knob is ovoid, somewhat pointed, measuring about 800 µm in diameter and 1.2 mm in height (Figs 1, 2). In *L. penangensis*,

the knob has the shape of an hemispherical cap about 300 µm high and 800 µm in diameter (Fig. 3).

Whatever the species, the knob surface is covered with cilia and pores (Figs 4, 7). The cilia are about 1 µm long (Figs 5, 8) ; the pores measure from 450 nm to 600 nm in diameter (Figs 6, 9). In *L. ciliaris* and *L. maculata*, the cilia and the pores are mixed and uniformly distributed over the knob (Fig. 4). In *L. penangensis*, they are always separated, the cilia being clustered in islets (Fig. 7). These islets range from 5 to 20 µm in diameter and enclose from 5 to 50 cilia which arise from cuticular bulges about 0.5 µm high and 0.5 µm in diameter (Figs 7, 8).

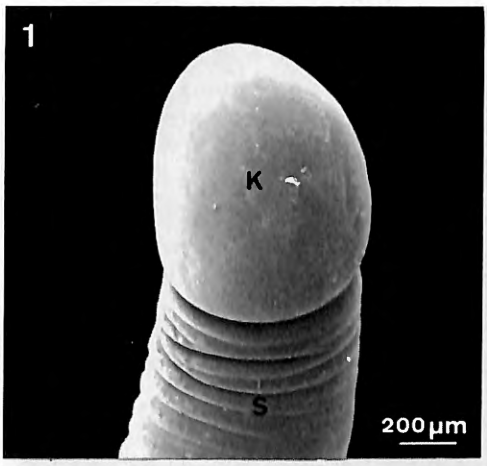
Histology and cytology of the podial knob

The knob always consists of four tissue layers that are, from the inside to the outside, a mesothelium, a connective tissue layer, a nerve plexus, and an epidermis covered by a cuticle.

The inner tissues (Fig. 10). The mesothelium, the most internal layer, surrounds the ambulacral lumen. It is a pseudostratified epithelium comprising adluminal cells and myoepithelial cells. The latter together form the retractor muscle of the podium which anchors distally to the connective tissue layer of the knob. In both *L. ciliaris* and *L. maculata*, this layer is thick (about 300 µm and 150µm in thickness, respectively) and is made up of loose connective tissue. In *L. penagensis*, the layer is thinner (about 25 µm) and consists of dense connective tissue. At the base of the knob, the nerve plexus of the stem is thickened to form the nerve ring. In both *L. ciliaris* and *L. maculata*, the nerve ring gives off radial branches, the nerve strands, that extend over the surface of the connective tissue layer, and join at the pointed apex of the knob. On the other hand, in *L. penangensis*, the nerve ring gives off a nerve plexus, thicker than the one of the stem, that extends over the whole knob surface (compare Fig. 10 A and B).

The epidermis. The epidermis of the knob is thickened compared to the stem epidermis (Fig. 10). It is about 150 µm thick in *L. ciliaris* and *L. maculata*, 100 µm in *L. penangensis*. In the three species, it is made up of four cell categories : secretory cells, neurosecretory cells, non-secretory ciliated cells, and support cells (Figs 11-14). In both *L. ciliaris* and *L. maculata*, the secretory cells are of two types (S1 and S2 cells ; Figs 11, 13, 15) whereas in *L. penangensis*, there is only one type of secretory cells (S1 cells ; Figs 12, 14). In *L. ciliaris* and *L. maculata*, cells of the four categories are mixed in the epidermis. In *L. penangensis*, they are segregated : non-secretory ciliated cells are gathered in islets surrounded by areas enclosing secretory and neurosecretory cells. Support cells occur in both the islets and the secretory areas.

Secretory cells (S1 and S2 cells) are flask-shaped. Their enlarged cell bodies are located basally, and each one sends out an apical process that reaches the surface of the epidermis (Figs 11, 12). The cytoplasm of both the cell body and the apical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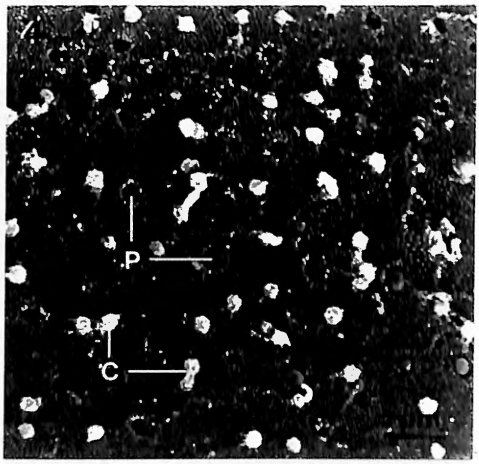
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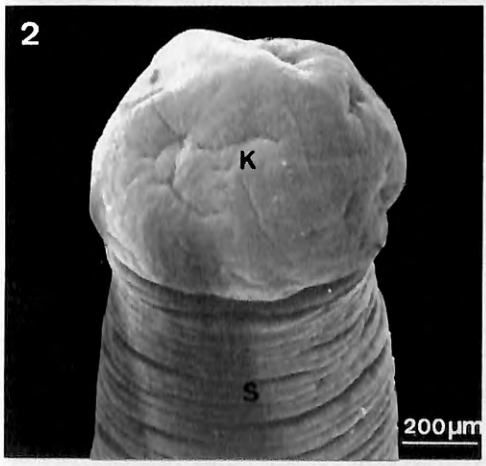
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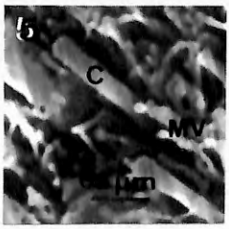
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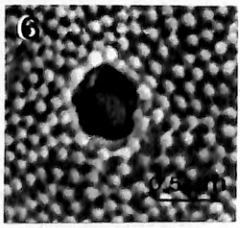
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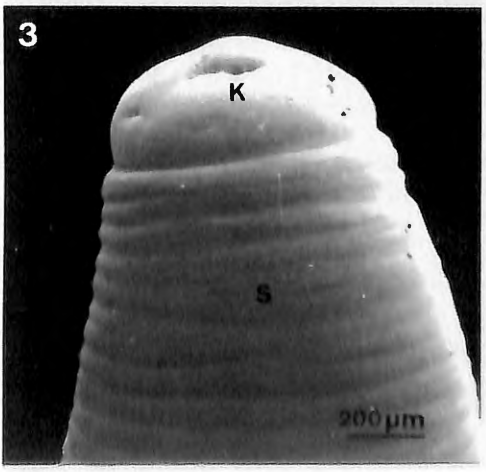
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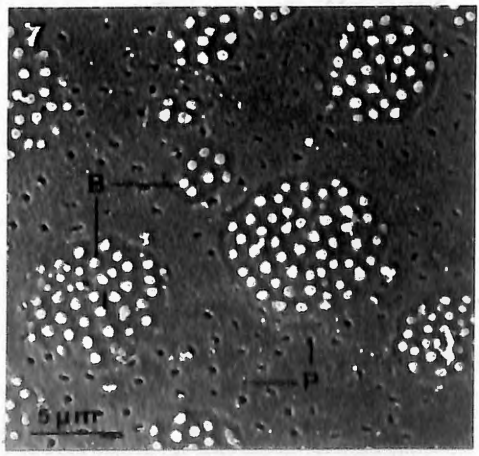
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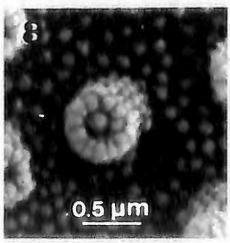
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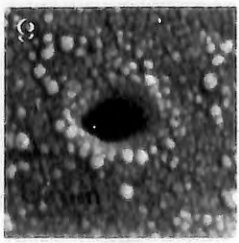

9

process is filled with densely packed membrane-bound secretory granules. The cytoplasm of the cell body also contains a well-developped Golgi apparatus and an extensive rough endoplasmic reticulum (RER) (Fig. 13). At the end of the apical processes of the secretory cells, the granules are extruded through a duct delimited by a ring of microvilli and opening onto the disk surface as a cuticular pore (Figs 11, 12). These pores correspond to those observed on SEM pictures of the knob surface (Figs 6, 9).

In the three species, S1 cell secretory granules are ellipsoids about 1,5 × 1 µm (Figs 11, 12, 14, 15). They consist of a large central core made of parallelly oriented fibrils, surrounded by a thin clear ring. In *L. ciliaris* and *L. maculata*, S2 cell secretory granules are spherical, about 1 µm in diameter (Figs 11, 13, 15). They enclose an electron-dense granular material limited by very electron-dense dots and surrounded by a belt of electron luscent granular material.

Neurosecretory cells are narrow and have a centrally located nucleus (Fig. 16). Apically, they end with a bulge just beneath the cuticle (Figs 12, 14, 15). The entire cytoplasm of the cell is filled with spherical membrane-bound secretory granules, ranging from 200 to 350 nm in diameter (Figs 15, 16). They contain an electron-dense homogeneous material surrounded by a thin clear belt. The cytoplasm also contains a Golgi apparatus, numerous mitochondria and RER cisternae (Fig. 16).

Non-secretory ciliated cells have the same shape and size as neurosecretory cells. Their characteristic feature is a single short cilium (about 3 µm long) whose apex protrudes into the outer medium (Fig. 17). These cilia are visible on SEM pictures of the knob surface (Figs 5, 8).

Support cells have a centrally to apically located nucleus. One longitudinal bundle of intermediate filaments traverses the cell and joins its apical and basal membranes (Figs. 11, 17).

DISCUSSION

The three species of *Luidia* considered in this work live on soft substrata, in which they can burrow, and feed on mollusks and other echinoderms (JANGOUX, 1982 ; LAWRENCE, 1987). The main functions of their podia are locomotion and burrowing ; the knob-shaped apical part of the podia is indeed well adapted to dig in the sediment.

Figs 1-9. — Outer aspect of the podia – 1. Protracted podium of *L. maculata* – 2. Retracted podium of *L. ciliaris* – 3. Protracted podium of *L. penangensis* – 4. Knob surface (*L. maculata*) – 5. Detailed view of cilia (the cuticle has been removed) (*L. ciliaris*) – 6. Detailed view of a pore (*L. maculata*) – 7. Knob surface (*L. penangensis*) – 8. Detailed view of a cilium arising from a cuticular bulge (*L. penangensis*) – 9. Detailed view of a pore (*L. penangensis*).
B cuticular bulge ; C cilium ; I islet of cilia ; K knob ; MV microvilli ; P pore ; S stem.

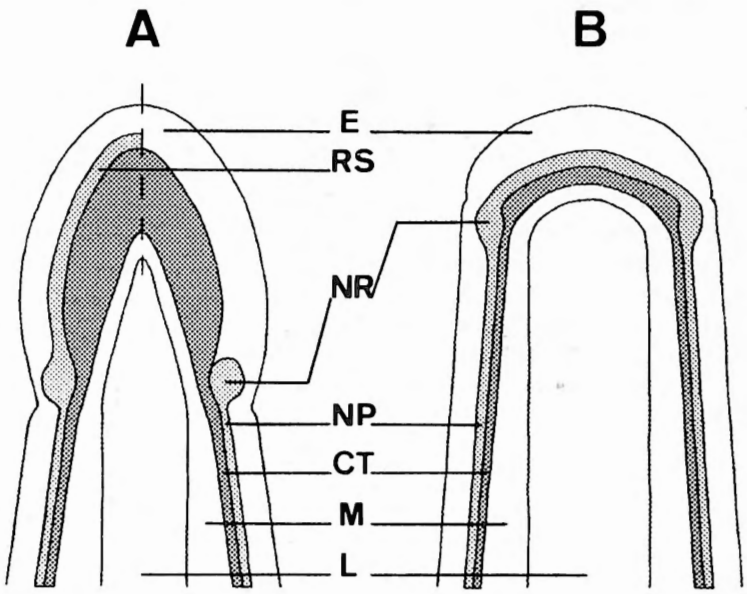


Fig. 10. — Schematic representation of two longitudinal sections through the podial knob of *Luidia ciliaris* and *Luidia maculata* (A), and *Luidia penangensis* (B).
CT connective tissue layer ; E epidermis ; L ambulacral lumen ; M mesothelium ; NP nerve plexus ; NR nerve ring ; RS radial nerve strand.

The podial knob has the classical tissular stratification of echinoderm podia, that is, from the inside to the outside : a mesothelium, a connective tissue layer, a nerve plexus, and an epidermis (KAWAGUTI, 1964 ; FLOREY and CAHILL, 1977 ; CAVEY and WOOD, 1991). In *L. ciliaris* and *L. maculata*, the connective tissue layer is thick and the nerve tissue is arranged in radial strands. This structure is similar to the one found in the disk of forcipulatid asteroid podia (FLAMMANG *et al.*, 1994). In *L. penagensis*, on the other hand, the connective tissue layer is thinner and the nerve tissue is arranged in a thick plexus. This structure is more reminiscent of the one observed in the podial knob of the paxillosid genus *Astropecten* (ENGSTER and BROWN, 1972).

In the three species, the epidermis of the knob encloses the same cell categories : secretory cells, neurosecretory cells, non-secretory ciliated cells, and support cells. These four cell categories occur in the podial adhesive areas of all other asteroid species so far examined.

In *L. ciliaris* and *L. maculata*, the secretory cells are of two types (S1 and S2 cells) whereas in *L. penangensis*, all secretory cells are S1 cells. The podial disk epidermis of forcipulatid asteroids also encloses both S1 and S2 cells (CHAET, 1965 ; FLAMMANG *et al.*, 1994). On the contrary, the podial knob epidermis of *Astropecten* only contains one type of secretory cell which, according to the size of its secretory granules and the aspect of their content, could be S2 cells. In all species so far

studied, secretory cells (both S1 and S2 cells) have always been considered to be adhesive in function.

S1 secretory granules are much less complex in *Luidia* spp than in valvatid and forcipulatid asteroids in which they are made up of highly ordered parallel rods (CHAET, 1965 ; HARRISON and PHILPOTT, 1966 ; SOUZA SANTOS and SILVA SASSO, 1968 ; FLAMMANG *et al.*, 1994). ENGSTER and BROWN (1972) pointed out a possible relationship between the internal organization of the secretory granules of adhesive cells and the possible adhesive strength of the podia : asteroids confined to hard rocky substratum have complex granules enclosing a highly organized core whereas soft substratum dwelling species have granules of considerably simpler ultrastructure. Similarly, the adhesive enclosed in the granules of the secretory cells of *Luidia*'s podia is presumably adapted to the functions the podia fulfil, *e.g.* particle handling or burrow wall consolidation. As for S2 secretory granules, they are similar in all asteroid species (CHAET, 1965 ; ENGSTER and BROWN, 1972 ; FLAMMANG *et al.*, 1994).

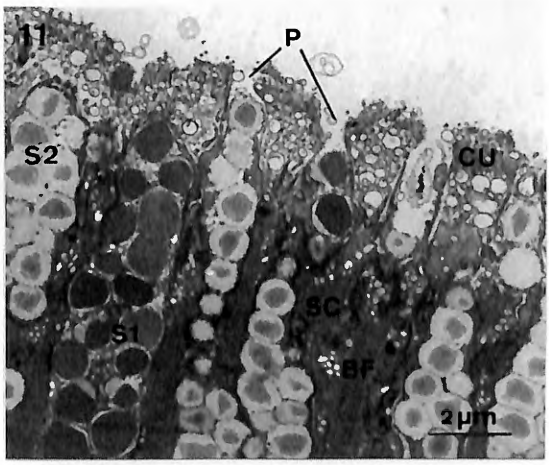
Neurosecretory cells in the podia of *Luidia* are almost identical to those in the podia of *Astropecten* and of forcipulatid asteroids (ENGSTER and BROWN, 1972 ; FLAMMANG *et al.*, 1994). However, in the latter, the neurosecretory cells possess a subcuticular cilium (FLAMMANG *et al.*, 1994). These cells are thought to be de-adhesive in function.

Non-secretory ciliated cells are sensory and are almost identical in the podia of all asteroid species studied so far (ENGSTER and BROWN, 1972 ; FLAMMANG *et al.*, 1994).

As in other asteroids, *Luidia*'s podia would thus enclose a duo-gland adhesive system in which S1 and S2 cells would be adhesive and neurosecretory cells would be de-adhesive in function (HERMANS, 1983 ; THOMAS and HERMANS, 1985 ; FLAMMANG *et al.*, 1994).

Like forcipulatid asteroid podia, the podia of *L. ciliaris* and *L. maculata* enclose two types of adhesive cells. However, the podia of *L. penangensis*, a species living in the same habitat and presumably having the same behaviour, only enclose one type of adhesive cell, as do the podia of *Astropecten*. The significance of having two types of adhesive cells in the knob epidermis of both *L. ciliaris* and *L. maculata* therefore remains obscure.

Secretory, neurosecretory and non-secretory ciliated cells are always closely associated in echinoderm podial adhesive areas but, generally, they are differently arranged according to whether the podia are handling or locomotory (FLAMMANG and JANGOUX, 1992). In the epidermis of handling podia, these cells join together to form sensory-secretory complexes (wherein the three cell types are present). Conversely, in the epidermis of locomotory podia, these three cell types form an homogeneous cellular layer together with support cells. This last arrangement is the one found in the knob epidermis of the podia of *L. ciliaris* and *L. maculata*. On the other hand, in the knob epidermis of the podia of *L. penangensis*, non-secretory ciliated cells are gathered in islets surrounded by areas enclosing secretory and neurosecretory cells, support cells occurring in both the islets and the secretory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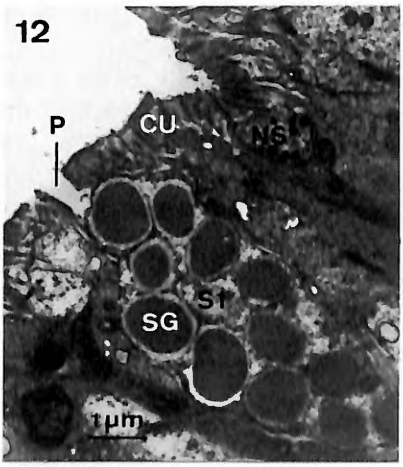
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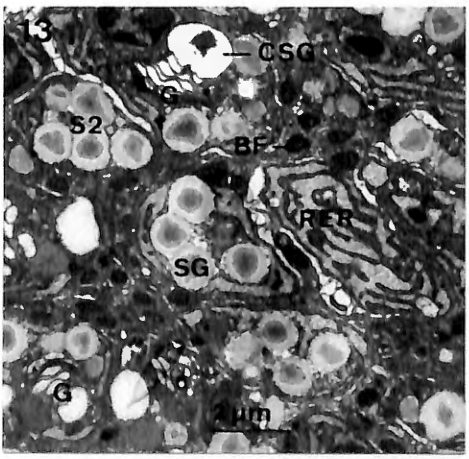
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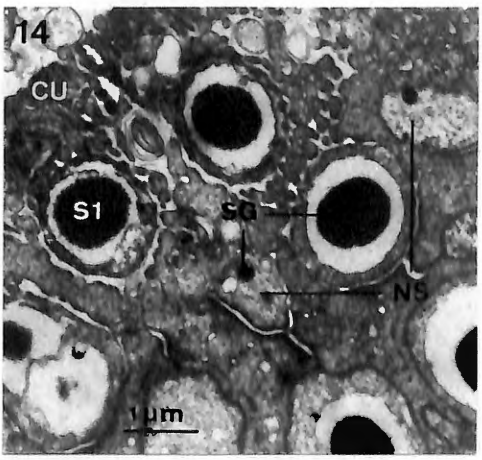
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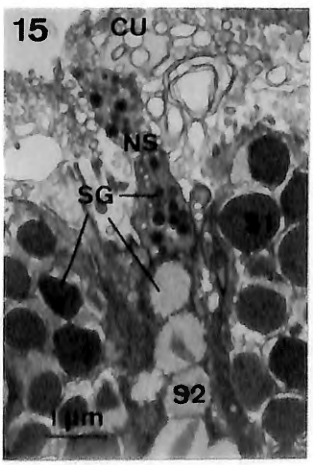
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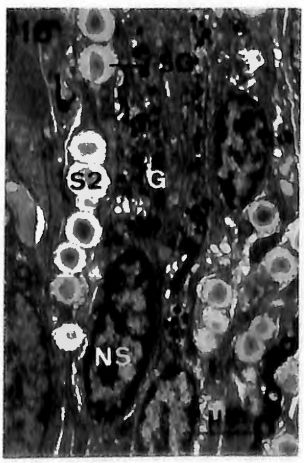
16
SG
S2
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17
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SC
1µm

areas. So far, this kind of cell organization has never been observed in any echinoderm podia.

This work has enlighted morphological differences between, on the one hand, the podia of *L. ciliaris* and *L. maculata*, and, on the other hand, the podia of *L. penangensis*. These differences concern the shape of their terminal knob, its tissular organization, the number of cell types enclosed in the knob epidermis and the way they are arranged. Yet the three species belong to the same genus and have the same life style. These marked differences stress that carefulness is required when generalizations, drawn from the morphology of a single species, are applied to related species having the same life style ; but also that the genus *Luidia*, which is generally considered as homogeneous (it is the only genus of the family Luidiidae), needs to be re-examined from a taxonomic point of view (see also DÖDERLEIN, 1920 and FELL, 1963).

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REFERENCES

CAVEY, M.J. and R.L. WOOD (1991) — Organization of the adluminal and retractor cells in the coelomic lining from the tube foot of a phanerozonian starfish, *Luidia foliolata*. *Can. J. Zool.*, **69** : 911-923.

CHAET, A.B. (1965) — Invertebrate adhering surfaces : secretions of the starfish, *Asterias forbesi*, and the coelenterate, *Hydra pirardi*. *Ann. NY Acad. Sci.*, **118** : 921-929.

DÖDERLEIN, L. (1920) — Die Asteriden der Siboga-Expedition. 2. Die Gattung *Luidia* und ihre Stammesgeschichte. *Siboga Exped.*, **46b** : 193-291.

ENGSTER, M.S. and S.C. BROWN (1972) — Histology and ultrastructure of the tube foot epithelium in the phanerozonian starfish, *Astropecten*. *Tissue & Cell*, **4** : 503-518.

Figs 11-17. — Fine structure of the epidermis of podial knob – 11, 12. Longitudinal sections through the apex of the epidermis (*L. ciliaris* and *L. penangensis*, respectively) – 13. Transverse section through the base of the epidermis (*L. ciliaris*) – 14. Oblique section through the apex of the epidermis (*L. penangensis*) – 15, 16. Neurosecretory cell (apical and basal parts, respectively) (*L. ciliaris*) – 17. Non-secretory ciliated cell (*L. penangensis*).
BB basal body ; BF bundle of filaments ; C cilium ; CSG condensing secretory granule ; CU cuticle ; G Golgi zone ; NS neurosecretory cell ; NSC non-secretory ciliated cell ; P pore ; RER rough endoplasmic reticulum ; S1 type 1 secretory cell ; S2 type 2 secretory cell ; SC support cell ; SG secretory granule.

FELL, H.B. (1963) — The phylogeny of sea-stars. *Phil. Trans. Roy. Soc. Lond.*, **246B** : 381-435.

FLAMMANG, P., S. DEMEULENEARE and M. JANGOUX (1994) — The role of podial secretions in adhesion in two species of sea stars (Echinodermata). *Biol. Bull.*, **187** : 35-47.

FLAMMANG, P. and M. JANGOUX (1992) — Functional morphology of the locomotory podia of *Holothuria forskali* (Echinodermata, Holothuroida). *Zoomorphology*, **111** : 167-178.

FLOREY, E. and M.A. CAHILL (1977) — Ultrastructure of sea urchin tube feet. Evidence for connective tissue involvement in motor control. *Cell Tissue Res.*, **177** : 195-214.

HARRISON, G. and D. PHILPOTT (1966) — Subcellular particles in echinoderm tube feet. I. Class Asteroidea. *J. Ultrastruct. Res.*, **16** : 537-547.

HERMANS, C.O. (1983) — The duo-gland adhesive system. *Oceanogr. Mar. Biol. Ann. Rev.*, **21** : 281-339.

JANGOUX, M. (1982) — Food and feeding mechanisms : Asteroidea. In : *Echinoderm Nutrition*. JANGOUX, M. and J.M. LAWRENCE (eds). Balkema, Rotterdam : 117-159.

KAWAGUTI, S. (1964) — Electron microscopic structure of the podial wall of an echinoid with special references to the nerve plexus and the muscle. *Biol. J. Okayama Univ.*, **10** : 1-12.

LAWRENCE, J.M. (1987) — *A functional Biology of Echinoderms*. Croom Helm, London and Sidney : 340 pp.

SOUZA SANTOS, H. and W. SILVA SASSO (1968) — Morphological and histochemical studies on the secretory glands of starfish tube feet. *Acta Anat.*, **69** : 41-51.

THOMAS, L.A. and C.O. HERMANS (1985) — Adhesive interactions between the tube feet of a starfish, *Leptasterias hexactis*, and substrata. *Biol. Bull.*, **169** : 675-688.

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IMPACT DE LA POLLINISATION PAR LES ABEILLES (*Apis Mellifera* LINNÉ) SUR LA PRODUCTION DE POMMES, CV JONAGOLD

par

ALEXIA FOUREZ
Unité d'Écologie et de Biogéographie,
4-5 Place Croix du Sud
Université Catholique de Louvain, B-1348
Louvain-la-Neuve, Belgium

RÉSUMÉ

L'objectif du travail était d'analyser l'impact quantitatif de la pollinisation par les abeilles sur pommiers de la variété Jonagold. Plusieurs traitements de pollinisation, manuels et sous cages, ont été comparées avec ou sans abeilles (*Apis mellifera* LINNÉ) à Visé en Belgique. L'allopollinisation semble être indispensable pour assurer une production de pommes suffisante (5 % de fructification en autopollinisation sans abeilles et 40 % de fructification en allopollinisation). Les expériences ont également permis de conclure que la variété Jonagold est partiellement autocompatible étant donné le taux de fructification qui s'élève à 20 % en autopollinisation avec abeilles.

SUMMARY

With an aim to analyse the quantitative impacts of insect pollination, experiments were carried out by comparing several types of pollination with or without honey bees (*Apis mellifera* LINNÉ) on apple (cv Jonagold) at Visé, Belgium. Allopollination seems to be indispensable (5 % of fruits set by autopollination without honey bees, and 40 % of fruits set by allopollination). However, it is also found to be partially autocompatible as 20 % fruit setting was done by autopollination with honey bees.

Keywords : pollination, apple, Jonagold, honeybees, cage.

INTRODUCTION

Le pommier est une plante qui peut être qualifiée d'autoincompatible à degrés divers suivant les variétés (GRIGGS cité par MAC GREGOR, 1976). Dans un verger,

il est donc indispensable de planter au moins deux variétés différentes pouvant s'interféconder. La production fruitière dépend directement des transferts de pollen d'une variété à l'autre, transferts qui pourront être assurés par des insectes pollinisateurs.

Des études en verger (LEMANCEAU, 1976 ; JACOB-REMACLE, 1989 ; TASEI, 1986) démontrent que l'abeille domestique (*Apis mellifera* L.) intervient pour 80 à 90 % dans la pollinisation du pommier. Pour la plupart des variétés, l'apport de ruches semble être une pratique agricole permettant l'amélioration de la récolte (BARBIER, 1986).

L'objectif de ce travail est de caractériser l'impact de la pollinisation sur la production quantitative de pommiers de la variété Jonagold.

MATÉRIEL ET MÉTHODES

L'étude a été réalisée en avril 1993 à Visé dans un verger appartenant au GAWI en collaboration avec le CARI. La parcelle expérimentale était composée de jeunes arbres de la variété Jonagold, conduits en fuseau, plantés en 1989 selon un système de plantation en un rang. Dans chacun des rangs, des arbres de la variété Elstar ont été plantés en tant que variété pollinisatrice, environ tous les 12 Jonagold. Deux types de tests de pollinisation ont été réalisés au sein de la même ligne expérimentale : des tests manuels et des tests sous cages.

Les tests manuels ont été mis en place afin d'aborder les phénomènes de pollinisation chez Jonagold (BARBIER, 1986). Un jour avant les tests, 5 corymbes dont les fleurs sont au stade ballon, ont été sélectionnés sur 15 arbres de la ligne expérimentale. La fleur centrale a été éliminée et 3 fleurs périphériques ont été conservées. Chacune de ces fleurs a fait l'objet d'un traitement. Une des fleurs a été autopollinisée à l'aide d'un pinceau avec du pollen de Jonagold. Cette fleur a été ensuite ensachée et étiquetée afin d'éviter toute pollinisation extérieure à l'expérience. La deuxième fleur a été allopollinisée à l'aide d'un autre pinceau avec du pollen de James Grieve. Cette fleur a été également ensachée et étiquetée. Enfin la troisième fleur a été uniquement étiquetée dans le but de tester la pollinisation libre dans le verger.

Les tests sous cages ont permis de tester différents types de pollinisation tout en restant le plus proche possible des conditions réelles de culture du pommier (BARBIER, 1986 ; FREE, 1964). Ces tests ont fait intervenir les abeilles. Cinq séries de trois arbres ont été sélectionnées sur la ligne expérimentale. Chaque série a été placée sous une cage et a fait l'objet d'un traitement différent. **1)** Le traitement **ab** a consisté en une autopollinisation avec abeilles. Une ruche a été installée au milieu de la cage close. **2)** En ce qui concerne le traitement **ab + JG**, une allopollinisation avec abeilles et pollen de James Grieve a été testée. Une ruche a été placée au milieu de la cage close ainsi que des bouquets pollinisateurs. La technique des bouquets pollinisateurs est inspirée des méthodes employées respectivement par BARBIER, 1986 et FREE, 1964. Les bouquets pollinisateurs étaient constitués de bouteilles remplies d'eau contenant des rameaux prélevés sur des arbres de la variété James

Grieve. Trois bouteilles étaient déposées au sol dans la cage et les rameaux étaient remplacés environ tous les trois jours. **3)** Le traitement **ab + AL** était pratiquement identique au précédent, mis à part l'origine des bouquets pollinisateurs qui provenaient d'arbres de la variété Alkmene. Il s'agissait donc d'une allopollinisation avec abeilles et pollen d'Alkmene. **4)** Le traitement **vide** a permis de tester une autopollinisation stricte. Il n'y avait ni insectes pollinisateurs ni source d'allopollen dans la cage. **5)** Enfin dans le cas du dernier traitement, le traitement **libre**, la cage était ouverte sur deux côtés afin de tester la pollinisation naturelle dans le verger, tout en tenant compte de l'effet cage.

Les ruches ont été amenées de Louvain-la Neuve, elles étaient closes jusqu'à leur installation dans les cages. Dans le cas des trois premiers traitements, la cage empêchait les abeilles de sortir et d'aller prélever du pollen extérieur. Dans le cas des 4 premiers traitements, la cage isolait les arbres testés des insectes pollinisateurs extérieurs. Les variétés utilisées pour la confection des bouquets pollinisateurs (James Grieve et Alkmene) ont été suggérées par le propriétaire du verger.

Les fruits ont été récoltés le 12 septembre 1993. Les pépins ont été dénombrés et pesés. L'analyse quantitative de la récolte a été abordée grâce à l'étude des taux de fructification. Le taux de fructification représente le nombre de fruits obtenus à la récolte par rapport au nombre de fleurs testées. Les taux de nouaison ont également été étudiés mais, uniquement pour les traitements manuels. Le taux de nouaison représente le nombre de jeunes fruits par rapport aux nombre de fleurs, environ un mois après la pollinisation. Une partie d'entre eux ont fait l'objet de tests de germination. Les tests statistiques employées sont des tests d'homogénéité de proportions et des tests de Scheffé. Les tests d'homogénéité de proportions ont permis de comparer les taux de fructification et de nouaison. Les tests de Scheffé ont permis de comparer les traitements sur base du nombre moyen de pépins par pomme (GÉRARD *et al.*, 1992-1993). Ils ont été réalisés grâce au logiciel SAS (Statistical Analysis System) (SAS INSTITUTE INC., 1989, 1990).

RÉSULTATS ET DISCUSSION

En pollinisation manuelle, l'allopollinisation et la pollinisation libre donnent de meilleurs taux de fructification que l'autopollinisation (auto : 20,3 %, allo : 39,7 % et librem. : 43,8 %) (voir Fig. 1). Cette constatation est identique en ce qui concerne les taux de nouaison (auto : 43,2 % a ; allo : 75,3 % b, librem. : 76,7 % b).

En ce qui concerne les pollinisations en cages, la pollinisation libre présente le meilleur taux de fructification (librec. : 38,2 %). Il est suivi par les pollinisations avec abeilles (ab : 19 % ; ab + JG : 18,4 % et ab + AL : 21,5 %). Le traitement vide est de loin le moins performant avec seulement 5 % de fruits (voir Fig. 2).

En pollinisation sous cage, la comparaison des taux de fructification n'a pas permis de mettre en évidence des différences entre les trois traitements avec abeilles (voir Fig. 2). Le fait de placer des bouquets d'une autre variété n'a donc pas

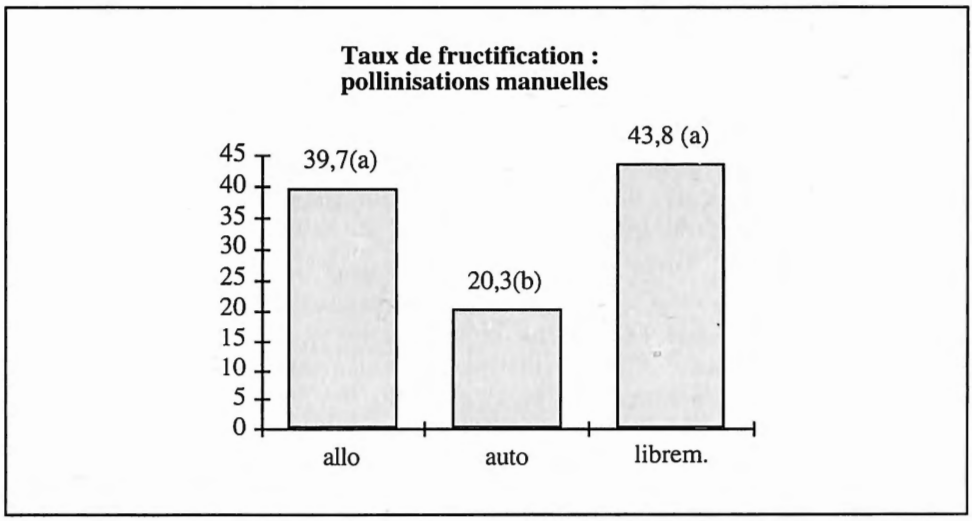

Fig. 1. — Traitements manuels : proportions de fruits à la récolte par rapport aux fleurs testées, exprimées en % : allo : pollinisation par James Grieve ; auto : pollinisation par Jonagold ; librem. : fleur non ensachée.
Les % suivis de la même lettre ne sont pas significativement différents. (a = 0.05)

influencé la fructification. Il faut signaler cependant que les variétés choisies par l'arboriculteur pour les tests (Alkmene et James Grieve) ne sont pas reconnues comme étant de bonnes variétés pollinisatrices de Jonagold. De plus, les bouquets de rameaux de la variété pollinisatrice dans des bouteilles posées au sol peuvent être remis en question quant à leur efficacité en tant que dispensateurs de pollen. En effet, lorsqu'on compare les taux de fructification en autopollinisation manuelle et en autopollinisation sous cage avec abeilles (auto : 20,3 % et ab : 19 %), les différences ne sont pas significatives (voir Fig. 1 et 2). Ce qui signifie que les abeilles pollinisent aussi bien que l'expérimentateur. Cependant, si on compare allopollinisation manuelle avec James Grieve et allopollinisation en cage toujours avec James Grieve, on obtient deux fois plus de fruits en pollinisation manuelle (allo : 39,7 % et ab + JG : 18,4 %) (voir Fig. 1 et 2). Il paraît alors évident que la technique bouquet (inspirée de BARBIER, 1986 et FREE, 1954) doit être remise en cause.

Au vu des résultats obtenus en pollinisation libre (librec. : 38,2 %), la faune pollinisatrice semble être suffisante dans ce verger pour assurer une production de pommes Jonagold rentable et il n'est pas certain que l'apport de ruchers supplémentaires puisse améliorer cette production. On peut cependant nuancer cette constatation. Premièrement, comme il a été dit précédemment, d'autres techniques expérimentales auraient sans doute révéler de meilleurs résultats pour la fructification en allopollinisation. Pour tester l'action réelle des abeilles en allopollinisation, des bouquets d'Elstar auraient dû être introduits dans l'une des cages à la place d'Alkmene ou de James Grieve. De plus, ces bouquets auraient été peut-être plus efficaces s'ils

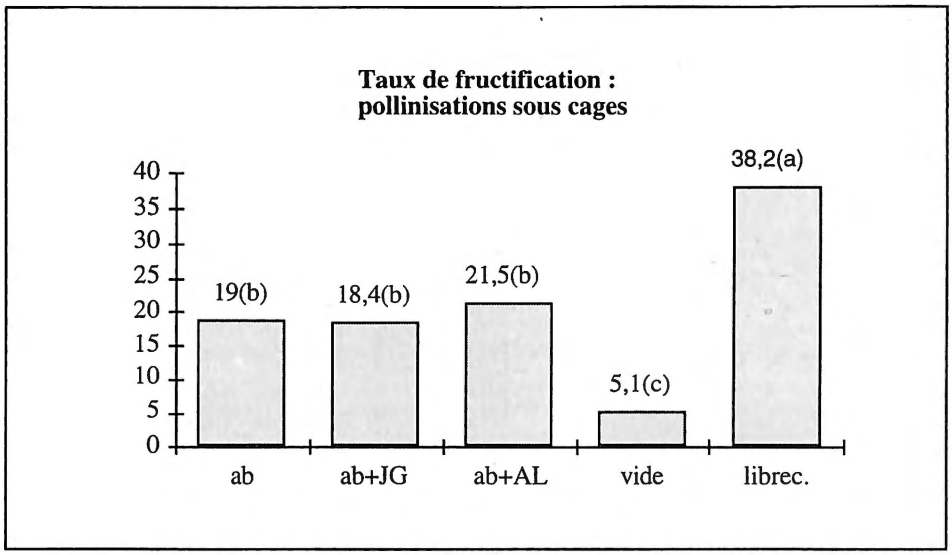

Fig. 2. — Traitements sous cages : Proportions de fruits à la récolte par rapport au nombre de fleurs testées exprimées en % : ab : cage + abeilles ; ab + JG : cage + abeilles + pollen de James Grieve ; ab + AL : cage + abeilles + pollen d'Alkmene ; vide : cage ; librec. : cage ouverte. Les % suivis de la même lettre ne sont pas significativement différents. (a = 0.05).

avaient été suspendus dans les arbres dans des sachets remplis d'eau. En effet, les abeilles seraient passées plus facilement des fleurs pollinisatrices aux fleurs à polliniser. Deuxièmement, les arbres du traitement libre se situaient à proximité immédiate d'un arbre Elstar qui est une des meilleures variétés pollinisatrices de Jonagold.

On peut cependant remarquer l'effet positif « abeilles » lorsque les traitements ab (19 %) (cage + ruche) et vide (5 %) (cage) sont comparés.

D'autre part, les taux de germination des pépins et les taux de fructification obtenus en autopollinisation manuelle et en autopollinisation sous cage ont permis de conclure que Jonagold est partiellement autocompatible. Les figure 3 et 4 représentent les nombres de pépins moyens obtenus pour chaque traitement ainsi que les taux de germination respectifs. En pollinisation manuelle, le nombre de pépins en pollinisation libre est significativement plus grand qu'en autopollinisation (librem. : 5,52, allo : 4,48, auto : 3,86) (voir Fig. 3). En pollinisation sous cage, on constate que la pollinisation libre et que les allopollinisations avec abeilles (ab + AL : 5,39 ; ab + JG : 4,87 ; librec. : 4,55) produisent plus de pépins que les autopollinisations (ab : 2,95, vide : 1,78) (voir Fig. 4). Le nombre de pépins est donc un bon indice du type de pollinisation (auto- ou allopollinisation).

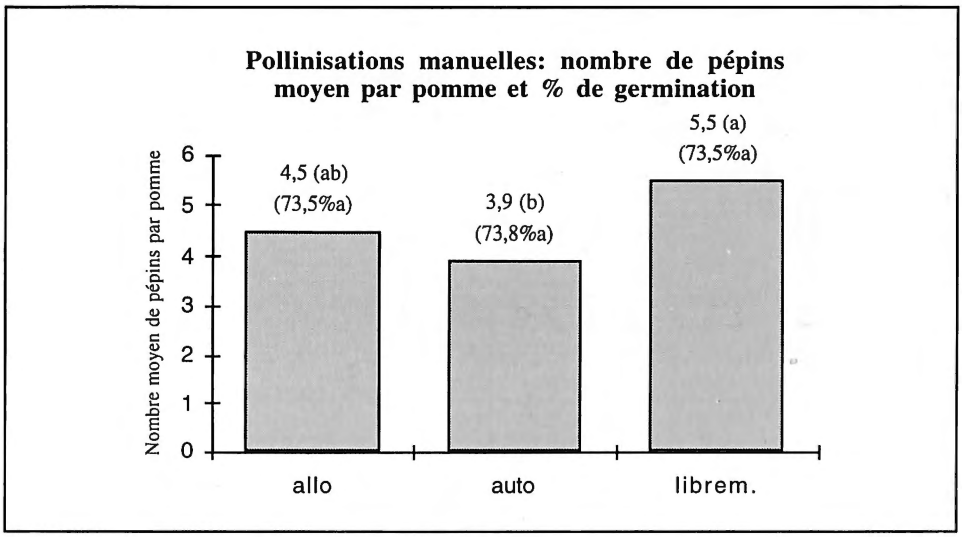

Fig. 3. — Traitements manuels : nombre moyen de pépins et pourcentages de germination (entre parenthèses). allo : pollinisation par James Grieve ; auto : pollinisation par Jonagold ; librem. : fleur non ensachée. Les nombres et les % suivis de la même lettre ne sont pas significativement différents. (a = 0.05).

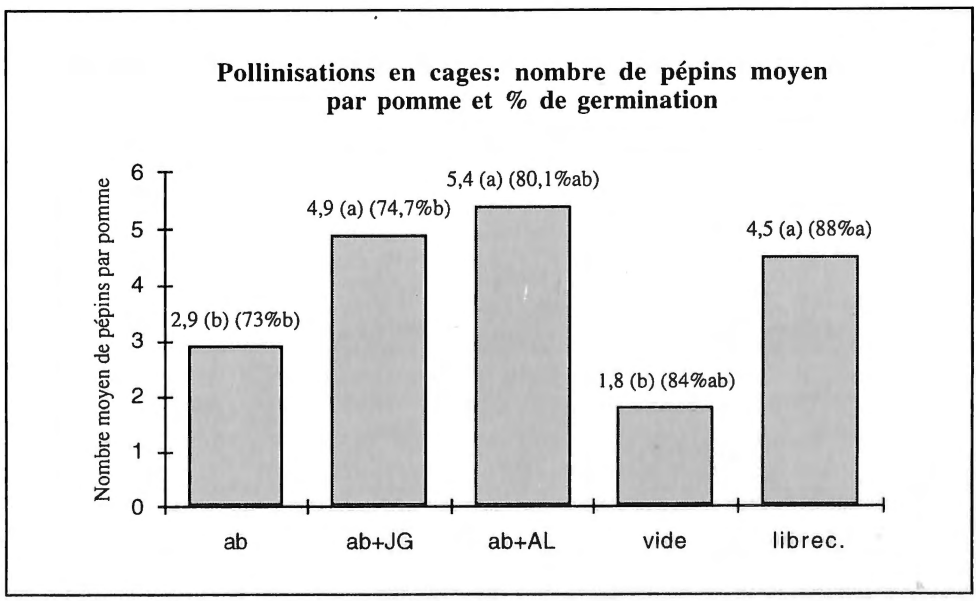

Fig. 4. — Traitements sous cages : nombre moyen de pépins et pourcentages de germination (entre parenthèses). ab : cage + abeilles ; ab + JG : cage + abeilles + pollen de James Grieve ; ab + AL : cage + abeilles + pollen d'Alkmene ; vide : cage ; librec. : cage ouverte. Les nombres et les % suivis de la même lettre ne sont pas significativement différents. (a = 0.05).

En outre, les pépins issus des autopollinisations ont germé dans des proportions identiques aux pépins issus des allopollinisations (voir Fig. 3 et 4). Ils sont donc le résultat d'une fécondation. On ne peut donc pas parler d'une parthénocarpie dans ce cas.

CONCLUSIONS

La source d'allopollen (cage ouverte versus cage fermée avec pollinisateurs) et la présence ou non d'insectes pollinisateurs (cage ouverte et cage fermée avec pollinisateurs versus cage vide) sont deux facteurs qui influencent la production de pommes, cv Jonagold. La variété Jonagold est partiellement autocompatible.

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BIBLIOGRAPHIE

BARBIER, E. (1986) — *La pollinisation des cultures. Pourquoi ? Comment ?* INRA éditions.

FREE, J.B. (1964) — Comparaison of the Importance of Insect and Wind Pollination of Apple Trees. *Nature*, **201 (4920)** : 726-727.

GÉRARD, G. et E. LEBOULENGÉ (1992-1993) — *Biométrie*. Inédit. Université Catholique de Louvain.

JACOB-REMACLE, A. (1989) — Comportement de butinage de l'abeille domestique et des abeilles sauvages dans des vergers de pommiers en Belgique. *Apidologie*, **20** : 271-285.

LEMANCEAU, P. (1979) — Contribution à l'étude du problème de la pollinisation des vergers monovariétaux de pommiers. *Bulletin Technique Apicole*, **6(2)** : 9-24.

MAC GREGOR, S.E. (1976) (c) — « Apple » In Agricultural Research Service (eds). Insect pollination of cultivated crop plant, Agricultural Handbook n° 496. U.S. Government printing office.

SAS INSTITUTE INC. SAS® — Language : Reference, Version 6, First Edition Cary, NC : SAS Institute INC., 1990, 1042 pp.

SAS INSTITUTE INC. SAS/STAT® — User's Guide, Version 6, Fourth Edition, volume 1, Cary, NC : SAS Institute INC., 1989, 943 pp.

TASEI, J.N. (1984) — Arbres fruitiers. Pages 349-372 in PESSON, P. & LOUVEAUX, J. (eds). Pollinisation et production végétale. INRA. Editions. Paris.

Belg. J. Zool. — Volume 125 (1995) — issue 1 — pages 143-156 — Brussels 1995

INFLUENCE OF CORTICOTROPIN-RELEASING FACTOR ON THE *IN VITRO* THYROXINE AND THYROTROPIN SECRETION IN NEWLY HATCHED FOWL

by

KRIS L. GERIS [1], VEERLE M. DARRAS [1], LUC R. BERGHMAN [2] and EDUARD R. KÜHN [1]
[1] Laboratory of Comparative Endocrinology,
University of Leuven, Naamsestraat 61, B-3000 Leuven (Belgium)
[2] Laboratory for Neuroendocrinology and Immunological Biotechnology,
University of Leuven, Naamsestraat 59, B-3000 Leuven (Belgium)

SUMMARY

Injections of ovine corticotropin-releasing factor (oCRF) are known to increase circulating thyroid hormone levels in the chicken embryo (MEEUWIS *et al.*, 1989). This can in part be explained by a direct effect of the hypothalamic hormone on the thyroid gland and/or the stimulation of the thyrotropin (TSH) release from the pituitary. We tested these two assumptions in two separate perifusion experiments. Our results clearly indicate that oCRF does not influence the *in vitro* thyroxine (T_4) secretion from the thyroid. The pituitaries of the newly hatched chickens, however, released significant amounts of TSH after *in vitro* treatment with oCRF. Growth hormone and luteinizing hormone secretions were also elevated after the oCRF stimulation period, but this release was less pronounced compared to the TSH release. As a conclusion we postulate that oCRF mediates its effect on the thyroidal status of the chicken through stimulation of TSH release and not through a direct effect on the thyroid.

Keywords : chicken, oCRF, *in vitro*, TSH, T_4.

INTRODUCTION

In vertebrates it is well established that the pituitary produces a thyroid-stimulating hormone (thyrotropin : TSH) which acts directly upon the thyroid gland to stimulate the synthesis and release of thyroxine (T_4) (mammals : VALE *et al.*, 1974 ; birds : WILLIAMSON and DAVISON, 1985 ; KÜHN *et al.*, 1988 ; amphibians : DARRAS and KÜHN, 1983 ; reptiles : LANCE and SAWIN, 1979 ; fish : MILNE and LEATHERLAND, 1980). The secretion of this TSH in mammals and birds is regulated by several factors, the best documented being TSH-releasing hormone (TRH) and somatostatin (SRIF), respectively a stimulating and inhibiting hypothalamic factor (VALE *et al.* 1974 ; DROUIN *et al.*, 1976 ; RADKE and CHIASSON, 1977 ; IQBAL *et al.*,

1989). Earlier reports of our research group suggested that ovine corticotropin-releasing factor (oCRF) has also a stimulatory effect on thyroid hormone secretion in the embryonic chicken. T_4 and triiodothyronine (T_3) plasma levels of dwarf and normal chicken embryos were increased after an intravenous (iv) injection of oCRF (MEEUWIS *et al.*, 1989 ; KÜHN *et al.*, 1990). It was however not clear at what level oCRF interacted with thyroid function. It is known that glucocorticoids and adrenocorticotropic hormone (ACTH), whose release in birds is controlled by CRF (ESTIVARIZ *et al.*, 1984 ; CARSIA *et al.*, 1986), depress circulating concentrations of thyroid hormones in respectively posthatch and adult chickens (DECUYPERE *et al.*, 1983 ; WILLIAMSON and DAVISON, 1987 ; MITCHELL *et al.*, 1986). During the last period of the embryonic development, on the contrary, glucocorticoids are reported to increase the T_4 and T_3 plasma concentrations (DECUYPERE *et al.*, 1983). The promptness of the response of circulating thyroid hormone levels after stimulation with oCRF, however, suggests that there may be a direct interaction of oCRF with the thyroidal axis.

The aim of the present study was to investigate if oCRF influences the thyroidal axis through a direct effect on the thyroid gland and/or through the stimulation of TSH release. We tested these two assumptions in two separate series of perifusion experiments : 1) the effect of oCRF on the *in vitro* T_4-releasing activity of the thyroid gland, 2) the response of the pituitary to oCRF. Because of the lack of specific monoclonal antibodies (mAbs) to the chickenTSH (cTSH) β-subunit we used the subtractive strategy published by BERGHMAN *et al.* (1993) to obtain an indicative cTSH value in the pituitary eluates.

MATERIAL AND METHODS

Animals

One day old male chickens (C1) from a layer strain (Hisex white) were purchased from a commercial dealer (Euribrid, Aarschot, Belgium) and the same day used for the experiments.

Perifusion experiments

In the first experiment the thyroid glands were removed from the chickens and immediately submerged in Gibco 199 medium (M199 : Life Technology, Gent, Belgium). Each pair of thyroids was placed in an individual perifusion chamber, held in a water bath at 37° C, and the perifusion was started with M199 at a constant flow rate of 12 ml per hour. After a preincubation period of 90 min to stabilise the basal secretion of T_4, collection of 1-ml samples (= period of 5 min) from each individual chamber was started. After 30 min of baseline registration, 4 chambers remained perifused with the control medium, the other 8 received M199 containing 10 or 100 nM oCRF (n = 4 per group) (UCB Bioproducts, Braine-l'Alleud, Belgium) for the next 30 min. Afterwards the perifusion continued for another 90 min with the control medium in the absence of any stimulatory agent.

The samples were stored at −20° C prior to the analysis of T_4 by radioimmunoassay (RIA).

During the second experiment 12 pituitaries from newly hatched chickens (C1) were stimulated with 10 or 100 nM oCRF (n = 6 per experimental group). The same protocol was followed (see above), but the post-stimulation period was 120 min instead of 90 min. No baseline control group was added. The samples were kept at −20° C prior to the analysis of cLH, cFSH, cα-subunit and chicken growth hormone (cGH) by RIA.

Radioimmunoassays

T_4 and cGH levels were measured by radioimmunoassay as described before (DARRAS *et al.*, 1991 ; 1992b).

The RIA of chicken α-like immunoreactivity (IR) was carried out as published by BERGHMAN *et al.* (1993). Briefly, 100 µl of the mAb (1/500,000), validated in binding studies of the mAbs with Reference Tracer Preparation (BERGHMAN *et al.*, 1993), was incubated overnight with 100 µl USDA-cLH-I-1 tracer (± 30,000 cpm), 100 µl of the sample or 50 µl of the standard (USDA-cLH-K-3 : 0.78 to 100 ng/ml). In order to obtain equal volumes in the assay, 50 µl M199 was added to the standards. Samples showing concentrations higher than 100 ng/ml were measured again with the same RIA, except in that case 25 µl of the sample and 75 µl M199 were added. The next day we used Sac-Cel anti-mouse (Innogenetics, Zwijndrecht, Belgium) to separate free and bound radioactivity. The total titer of α-containing molecules is than expressed in relative units (ng cLH-like IR/ml).

The homologous RIAs for cFSH and cLH were recently developed and validated (KRISHNAN *et al.*, 1992 ; 1993 ; PROUDMAN, KRISHNAN and BAHR, unpublished results). The radioiodination and the RIA was performed as described by BERGHMAN *et al.* (1993).

Calculation of an indicative cTSH level in the samples

Specific mAbs to the cTSH-molecule are not available yet because to date researchers have not been successful in isolating this peptide from chicken pituitary. Heterologous polyclonal Abs have been applied to localise TSH-cells in the chicken pituitary (THOMMES *et al.*, 1983). Unfortunately, such Abs tend to have too low affinity with cTSH to allow sensitive measurements by RIA. Therefore we calculated an indicative value for the cTSH levels in our samples using a subtractive strategy published by BERGHMAN *et al.* (1993). This indicative cTSH level in the samples was obtained by the subtraction of cFSH and cLH levels from the total level of α-like immunoreactive material in the same sample. This indirect method obviously assumes that free α-subunit is not being secreted under physiological conditions (BERGHMAN *et al.*, 1993). Finally, the cTSH concentration in the samples is expressed in relative units because of the lack of homologous standard TSH preparations.

Statistics

For each individual chamber (*e.g.* pair of glands) we calculated (Fig. 1) : 1) basal secretion (BS), 2) the peak value in the response to the stimulator (PV), 3) the stimulation factor (SF), 4) total secretion after stimulation (TS). Statistical analysis between the experimental groups was carried out by one-way-analysis of variance (ANOVA).

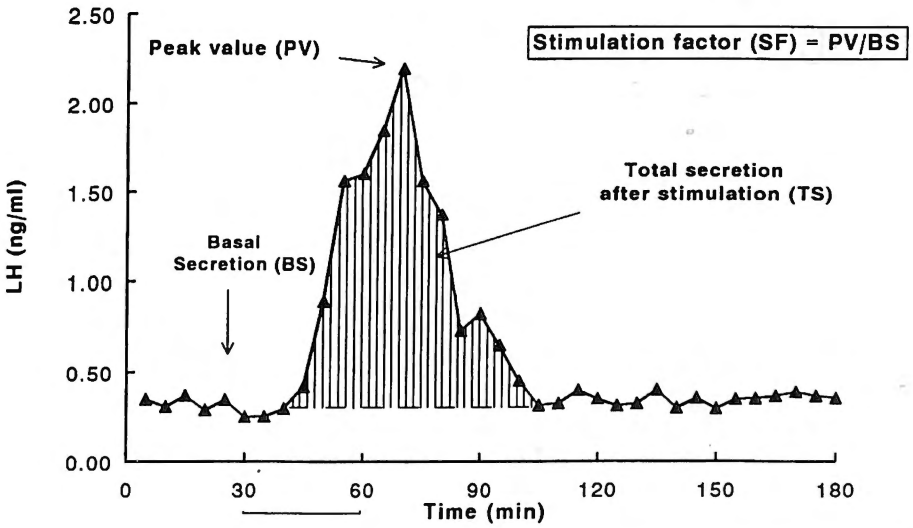

Fig. 1. — Typical individual LH response curve to oCRF in a perifusion experiment. The four different experimental parameters that are calculated, are indicated. The horizontal line beneath the X-axis indicates the stimulation period which is the same as used in the different perifusion experiments.

RESULTS

Experiment 1 : Influence of oCRF on the *in vitro* T_4 release

The *in vitro* T_4 release from thyroids dissected from C1 chickens was not affected by a treatment with 10 or 100 nM oCRF (Fig. 2). The basal T_4 secretion was higher in both oCRF conditions compared to the control group (control : BS = 0.15 ± 0.02 pmol/ml ; 10 nM : BS = 0.25 ± 0.02 pmol/ml ; 100 nM : 0.25 ± 0.02 pmol/ml : $P < 0.05$). However no stimulation of the release was seen in response to an oCRF-treatment as shown by their respective stimulation factors : control : SF = 1.00 ± 0.14 ; 10 nM : SF = 0.92 ± 0.03 ; 100 nM : SF = 1.14 ± 0.03.

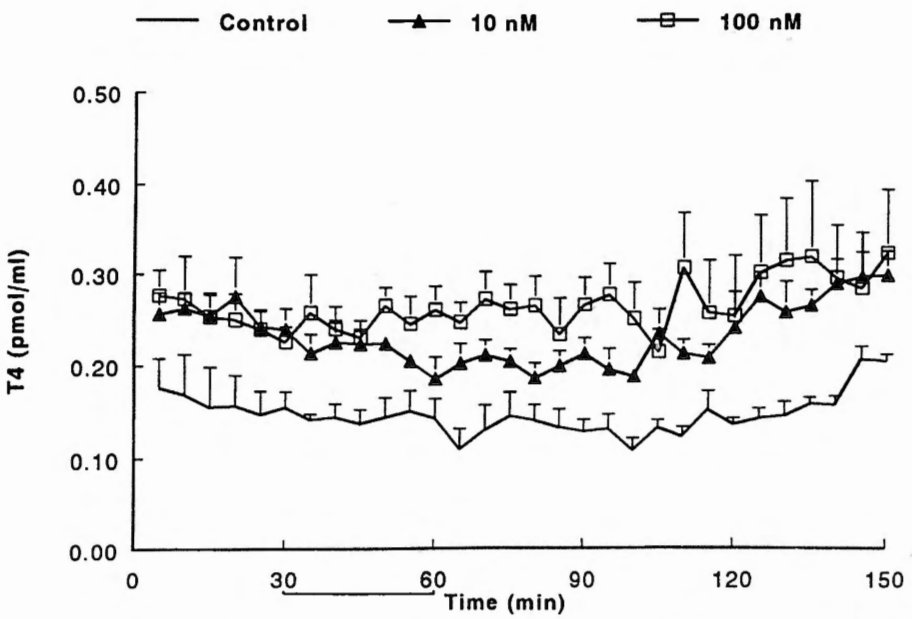

Fig. 2. — *In vitro* T_4 release by thyroid glands of one day old chicks (Cl), stimulated for 30 min with 10 or 100 nM oCRF after a 30 min baseline registration. T_4 levels are measured by RIA. Values shown are mean ± SEM of four individuals. The horizontal line beneath the X-axis indicates the stimulation period.

Experiment 2 : Influence of oCRF on the *in vitro* releasing activity of the chicken pituitary

The effect of oCRF on the α-subunit and LH release is shown in Fig. 3. The oCRF-treatment had a dose-dependent effect on the total amount of α-subunit in the samples. The highest concentration caused a significantly greater SF and TS (Table 1). Also the LH secretion was enhanced, although to a lesser extent compared to α-subunit release. Both doses induced approximately a four-fold increase in LH release. The LH response caused by 100 nM, however, lasted longer, which explains the significantly higher TS in this condition compared to the other experimental group. The amount of cFSH in the samples was either very low or below the detection limit (< 0.20 ng/ml) before, during and after the stimulation period (data not shown). Thus, no stimulatory effect of oCRF on the cFSH-release was noticed. Finally cLH values were subtracted from the α-subunit levels to yield, in each sample individually, an indicative concentration of cTSH. Because of the low or not measurable amount of cFSH we didn't take these data into account for subtraction. As shown in Figure 4 oCRF is a very strong stimulator of the *in vitro* TSH secretion in newly hatched fowl. Due to the wide variation in the individual responses to the highest oCRF concentration, stimulation factors did not differ significantly between the 2 groups, but the total TSH secretion after stimulation showed a clear significant dose-dependent effect of oCRF (Table 2).

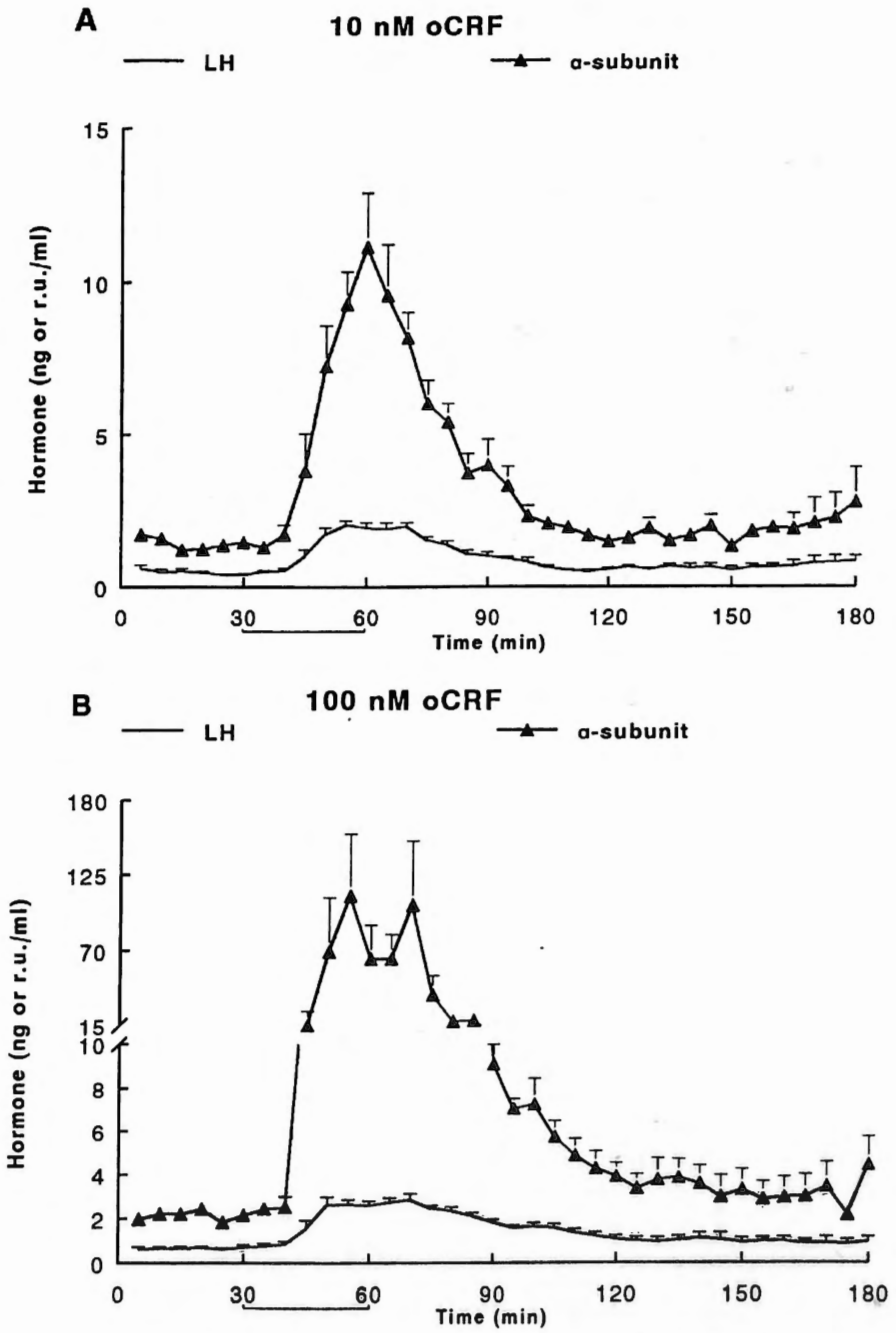

Fig. 3. — *In vitro* LH and α-subunit release by pituitaries of one day old chicks (Cl), stimulated for 30 min with 10 (A) or 100 nM oCRF (B) after a 30 min baseline registration. LH and α-subunit levels are measured by a homologous RIA. Values shown are mean ± SEM of six individuals. The horizontal line beneath the X-axis indicates the stimulation period. The α-subunit levels are expressed in relative units.

TABLE 1

Influence of 10 nM and 100 nM oCRF on the LH (ng/ml) and α-subunit (relative units/ml) secretion in one day old chicks (Cl) (n = 6 per group). Values shown are mean ± SEM. Asterisks indicate differences between the 2 doses (ANOVA : * $P < 0.05$; ** $P < 0.01$).

	basal secretion (ng or r.u./ml) (BS)	peak value (ng or r.u./ml) (PV)	stimulation factor (SF)	total secretion after stimulation (ng or r.u.) (TS)
LH (ng)				
10 nM oCRF	0.46 ± 0.05	2.01 ± 0.14	4.50 ± 0.21	11.70 ± 0.66
100 nM oCRF	0.69 ± 0.07*	2.86 ± 0.26*	4.23 ± 0.37	18.82 ± 1.57**
α-subunit (r.u.)				
10 nM oCRF	1.30 ± 0.08	11,15 ± 1.73	8.51 ± 1.09	57.08 ± 7.05
100 nM oCRF	2.22 ± 0.23**	110.08 ± 44.94	44.94 ± 16.27*	497.65 ± 131.94**

TABLE 2

Indicative TSH variations in response to 10 nM and 100 nM oCRF in one day old chicks (Cl). Subtractions (αIR-cLH) were calculated for individual animals. Values shown are mean ± SEM of 6 individual subtraction results. Asterisks indicate differences between the 2 doses (ANOVA : **$P < 0.01$).

	basal secretion (r.u./ml) (BS)	peak value (r.u./ml) (PV)	stimulation factor (SF)	total secretion after stimulation (r.u.) (TS)
10 nM oCRF	0.84 ± 0.04	9.23 ± 1.58	10.85 ± 1.59	45.88 ± 6.56
100 nM oCRF	1.53 ± 0.16**	107.23 ± 44.88	63.63 ± 23.76	478.83 ± 130.58**

The GH secretion (Fig. 5) was hardly influenced by 10 nM oCRF (SF = 1.71 ± 0.17), a concentration of 100 nM oCRF however induced a significantly higher GH release compared to the lower dose (SF = 3.35 ± 0.47 : $P < 0.01$). The total GH secretion after stimulation differed also significantly in the 100 nM condition (respectively TS = 28.33 ± 6.08 and TS = 116.33 ± 37.68 : $P < 0.05$).

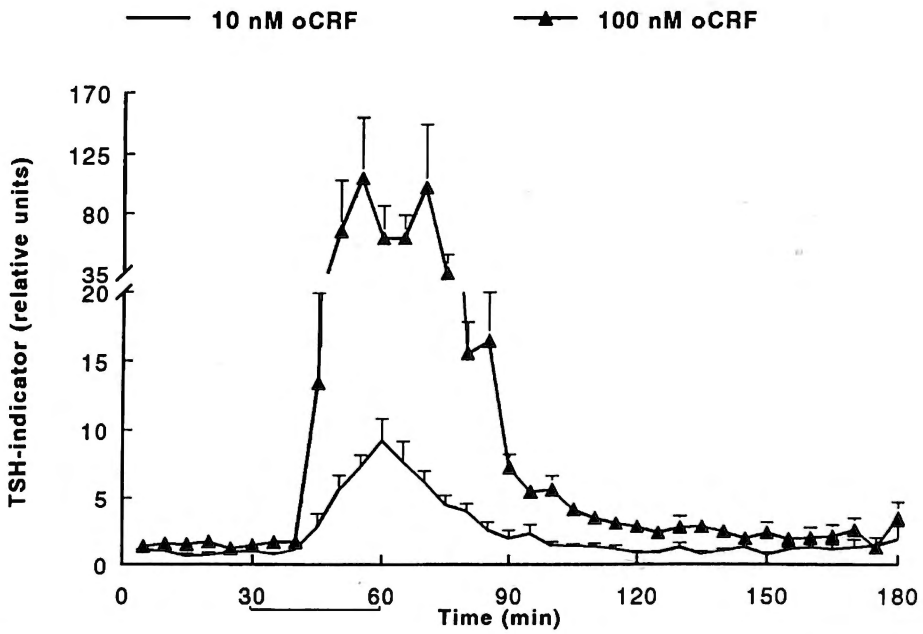

Fig. 4. — Indicative levels of *in vitro* TSH release by pituitaries of one day old chicks (Cl), stimulated for 30 min with 10 or 100 nM oCRF after a 30 min baseline registration. TSH indicator levels are calculated using a subtractive strategy (αIR-cLH). Values shown are mean ± SEM of six individuals. The horizontal line beneath the X-axis indicates the stimulation period.

DISCUSSION

The present study describes the effect of oCRF on the *in vitro* releasing activity of two tissues that are implicated in the thyroidal status of the chicken : the thyroid gland and the pituitary. oCRF is a well-known stimulator of the ACTH-release in mammals, both *in vivo* and *in vitro* (reviewed by RIVIER and PLOTSKY, 1986), and in chickens when added to dispersed adenohypophyseal cells (CARSIA *et al.*, 1986). Due to the lack of an homologous RIA for cACTH, we did not measure the amount of this hormone in our samples. CARSIA *et al.* (1986) obtained an indicative value for the ACTH concentrations in their experiment using a bioassay. Recently CRF-neurones have been localised in the avian brain (JOSZA *et al.*, 1984 ; KOVÁCS *et al.*, 1989). Also in lower vertebrates oCRF is a potent stimulator of the ACTH release. In fish and anurans this secretagogue enhanced the *in vivo* and *in vitro* ACTH secretion (FRYER *et al.*, 1983 ; CUET *et al.*, 1984 ; TONON *et al.*, 1986). *In vivo* administration of oCRF to a frog species significantly reduced the volume density of the secretory granules in ACTH-cells, taken as an indicator of short-term enhanced hormonal release (MALAGON *et al.*, 1991).

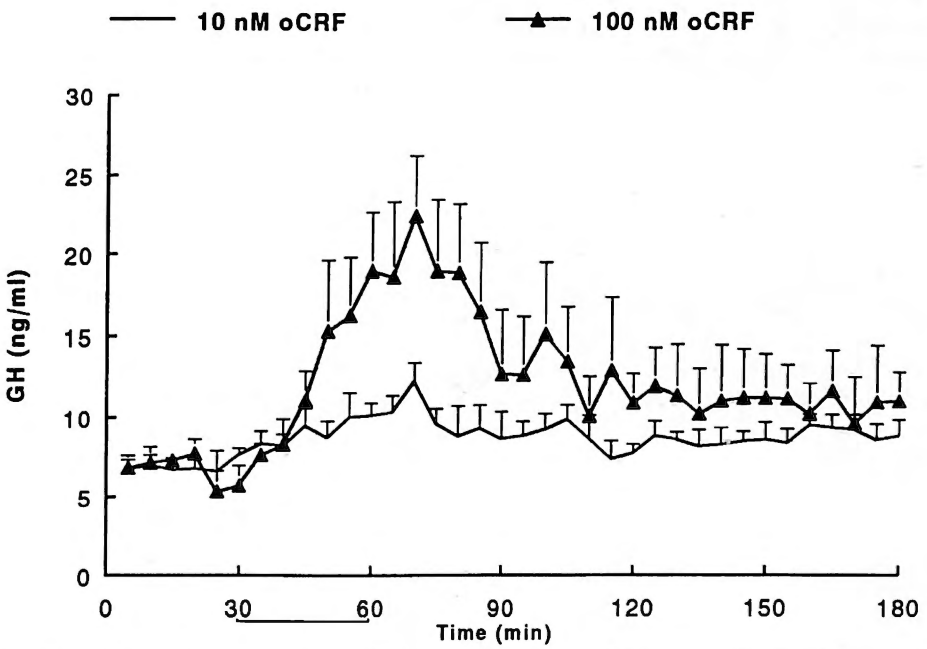

Fig. 5. — *In vitro* GH release by pituitaries of one day old chicks (Cl), stimulated for 30 min with 10 or 100 nM oCRF after a 30 min baseline registration. GH levels are measured by a homologous RIA. Values shown are mean ± SEM of six individuals. The horizontal line beneath the X-axis indicates the stimulation period.

In mammals oCRF did not change the *in vitro* TSH secretion (VALE *et al.*, 1981). To our knowledge, there are no data available concerning the effect of oCRF on the thyroidal releasing activity in mammals. Our results clearly indicate that oCRF influences the thyroidal axis in the chicken through the stimulation of TSH release as calculated with the subtractive strategy published by BERGHMAN *et al.* (1993). On the other hand, we did not see a direct effect on the thyroidal T_4 release. Our data correspond with results described in several frog species and in hatchling turtles : each time oCRF induced a rise in the *in vitro* TSH release, without influencing the thyroidal T_4 secretion (DENVER, 1988 ; DENVER and LICHT, 1989a, b ; JACOBS and KÜHN, 1992). MALAGON *et al.* (1991) observed a decrease in the volume density of the granules of the TSH-cells of a frog species after an oCRF-treatment, indicating an elevation of the TSH release. JACOBS *et al.* (1988) postulated that LH-releasing hormone (LHRH) also has a TSH-releasing activity in the frog.

oCRF also stimulated the *in vitro* release of other pituitary hormones. The GH secretion was influenced in a dose-dependent manner with the lower dose inducing almost no stimulation. Until this moment GH-releasing factor (GRF) and TRH were considered to be the main GH secretagogues in avian species (HARVEY *et al.*, 1978, 1981 ; LEUNG and TAYLOR, 1983 ; HARVEY *et al.*, 1984). Somatostatin (SRIF) plays an inhibitory role in this process (SCANES and HARVEY, 1989). In young turtles oCRF also stimulated the *in vitro* GH release (DENVER and LICHT, 1989a). RIVIER

and PLOTSKY (1986) mentioned in their review that in mammals oCRF does not have an *in vivo* (iv injections) or *in vitro* GH-releasing activity. In the rat, an intracerebroventricular (icv) oCRF-injection even caused a decrease in the GH plasma concentration (RIVIER and VALE, 1984a). Next to this *in vitro* GH-releasing activity of oCRF in newly hatched fowl, we also observed an increase in the LH release in response to the oCRF-treatment. Gonadotropin-releasing hormone (GnRH) is believed to be the main hypothalamic hormone that releases LH from the pituitary. Our results indicate that in the chicken oCRF is also a candidate for the regulation of the LH secretion. In the rat, authors did not observe any change in the LH plasma levels after an iv oCRF-injection (VALE *et al.*, 1981 ; DONALD *et al.*, 1983 ; RIVIER and VALE, 1984b), while the rhesus monkey responded with a decrease in the LH concentration (GINDOFF *et al.*, 1989). The results after an icv oCRF-injection all indicated a decrease in the LH plasma levels (RIVIER and VALE, 1984b ; PETRAGLIA *et al.*, 1987 ; MAEDA *et al.*, 1994), possibly through its inhibitory effect on the GnRH release (NIKOLORAKIS *et al.*, 1986). We did not find any reports on a LH-releasing activity of oCRF in lower vertebrates. In the turkey there are some indications available that TRH has a LH-releasing activity (WENTHWORTH *et al.*, 1976 ; FEHRER *et al.*, 1985). Although the amounts of cFSH in our samples were very low or not detectable, we can conclude that oCRF does not have an effect on the release of this pituitary hormone. To our knowledge, there are no data available in literature about this specific activity of oCRF in vertebrates.

The *in vivo* relevancy of our results are presently under investigation. The observed *in vitro* TSH-releasing potency of oCRF strengthens the data of MEEUWIS *et al.* (1989) and KÜHN *et al.* (1990), who reported an increase of the circulating thyroid hormone plasma levels after *in vivo* oCRF-treatment in normal and dwarf chicken embryos. Currently, we are focussing on the short-term effects of oCRF on the *in vivo* TSH, GH, LH and circulating thyroid hormones plasma levels in 19-day-old chicken embryos. Since GH can increase T_3 plasma concentrations through a decrease in the T_3 degradation (DARRAS *et al.*, 1992a ; 1993), we also try to estimate the impact of *in vivo* GH release after oCRF on circulating thyroid hormone levels by measuring the activity of deiodinases in several tissues.

In summary, the data presented here suggest that CRF is a potent stimulator of TSH secretion in the domestic fowl while a direct effect of CRF on the thyroid gland can be ruled out. In addition, the study showed for the first time an *in vitro* LH- and GH-releasing activity of CRF in the chicken, although less pronounced then the TSH response.

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REFERENCES

BERGHMAN, L.R., V.M. Darras, R.B. Chiasson, E. Decuypere, E.R. Kühn, J. Buyse and F. VANDESANDE (1993) — Immunocytochemical demonstration of chicken hypophyseal thyrotropes and development of a radioimmunological indicator for chicken TSH using monoclonal and polyclonal homologous antibodies in a subtractive strategy. *Gen. Comp. Endocrinol.*, **92** : 189-200.

CARSIA, R.V., H. WEBER and F.M. Jr. PEREZ (1986) — Corticotropin-releasing factor stimulates the release of adrenocorticotropin from domestic fowl pituitary cells. *Endocrinology*, **118** : 143-148.

CUET, P., A. BURLET, S. JÉGOU, G. PELLETIER, H. VAUDRY and M.C. TONON (1984) — CRF stimulates specifically the pars distalis of the frog pituitary gland. *Gen. Comp. Endocrinol.*, **53** : 438-439.

DARRAS, V.M. and E.R. KÜHN (1983) — Effects of TRH, bovine TSH and pituitary extracts on thyroidal T_4 release in *Ambystoma mexicanum*. *Gen. Comp. Endocrinol.*, **51** : 286-291.

DARRAS, V. M., A. VANDERPOOTEN A., L.M. HUYBRECHTS, L.R. BERGHMAN, E. DEWIL, E. DECUYPERE and E.R. KÜHN (1991) — Food intake after hatching inhibits the growth hormone induced stimulation of the thyroxine to triiodothyronine conversion in the chicken. *Horm. metabol Res.*, **23** : 469-472.

DARRAS, V.M., L.R. BERGHMAN, A. VANDERPOOTEN and E.R. KÜHN (1992a) — Growth hormone acutely decreases type III iodothyronine deiodinase in chicken liver. *FEBS*, **310** : 5-8.

DARRAS, V.M., T. J. VISSER, L.R. BERGHMAN and E.R. KÜHN (1992b) — Ontogeny of type I and type III deiodinase activities in embryonic and posthatch chicks : relationship with changes in plasma triiodothyronine and growth hormone levels. *Comp. Biochem. Physiol.*, **103A** : 131-136.

DARRAS, V.M., P. RUDAS, T.J. VISSER, T. R. HALL, L.M. HUYBRECHTS, A. VANDERPOOTEN, L.R. BERGHMAN, E. DECUYPERE and E.R. KÜHN (1993) — Endogenous growth hormone controls high plasma levels of 3,3',5-triiodothyronine (T_3) in growing chickens by decreasing the T_3-degrading type III deiodinase activity. *Domestic Animal Endocrinology*, **10** : 55-65.

DECUYPERE, E., C.G. SCANES and E.R. KÜHN (1983) — Effects of glucocorticoids on circulating concentrations of thyroxine (T_4) and triiodothyronine (T_3) and on peripheral monodeiodination in pre- and post-hatching chickens. *Horm. metabol. Res.*, **15** : 233-236.

DENVER, R.J. (1988) — Several hypothalamic peptides stimulate *in vitro* thyrotropin secretion by pituitaries of anuran amphibians. *Gen. Comp. Endocrinol.*, **72** : 383-393.

DENVER, R.J. and P. LICHT (1989a) — Neuropeptides influencing *in vitro* pituitary hormone secretion in hatchling turtles. *J. Exp. Zool*, **251** : 306-315.

DENVER, R.J. and P. LICHT (1989b) — Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog : Evidence for a common neuroregulation of thyroid and interrenal activity in metamorphosis. *J. Exp. Zool*, **252** : 101-104.

DONALD, R.A., C. REDEKOPP, V. CAMERON, M.G. NICHOLLS, J. BOLTON, J. LIVESEY, E.A. ESPINER, J. RIVIER and W. VALE (1983) — The hormonal actions of corticotropin-releasing factor in sheep : Effect of intravenous and intracerebroventricular injection. *Endocrinology,* **113** : 866-870.

DROUIN, J., A. DE LÉAN, D. RAINVILLE, R. LACHANCE and F. LABRIE (1976) — Characteristics of the interaction between thyrotropin-releasing hormone and somatostatin for thyrotropin and prolactin release. *Endocrinology,* **98** : 514-521.

ESTIVARIZ, F.E., M.G. CASTRO and F.C. ITURRIZA (1984) — Effect of various peptides on the release of ACTH using perfused, dispersed duck pituitary cells. *J. Steroid Biochem.,* **20** : 1542 Abs. H34.

FEHRER, S.C., J.L. SILSBY, E.J. BEHNKE and M.E. EL HALAWANI (1985) — The influence of thyrotropin-releasing hormone on *in vivo* prolactin release and *in vitro* prolactin, luteinizing hormone, and growth hormone release from dispersed pituitary cells of the young turkey (*Meleagris gallopavo*). *Gen. Comp. Endocrinol.,* **59** : 64-72.

FRYER, J., K. LEDERIS and J. RIVIER (1983) — Urotensin I, a CRF-like neuropeptide, stimulates the ACTH release from the teleost pituitary. *Endocrinology,* **113** : 2308-2310.

GINDOFF, P.R., E. XIAO, J. LUCKHAUS and M. FERIN (1989) — Dexamethasone treatment prevents the inhibitory effect of corticotropin-releasing hormone on gonadotropin release in the primate. *Neuroendocrinology,* **49** : 202-206.

HARVEY, S., C.G. SCANES, A. CHADWICK and N.J. BOLTON (1978) — The effect of thyrotrophin releasing hormone (TRH) and somatostatin (GHRIH) on growth hormone and prolactin secretion *in vitro* and *in vivo* in the domestic fowl (*Gallus domesticus*). *Neuroendocrinology,* **26** : 249-260.

HARVEY, S., R.J. STERLING and J. G. PHILLIPS (1981) — Diminution of thyrotropin-releasing hormone-induced growth hormone secretion in adult domestic fowl (*Gallus domesticus*). *J. Endocr.,* **89** : 405-410.

HARVEY, S., C.G. SCANES and J.A. MARSH (1984) — Stimulation of growth hormone secretion in dwarf chickens by thyrotrophin-releasing hormone (TRH) or human pancreatic growth hormone-releasing factor (hpGRF). *Gen. Comp. Endocrinol.,* **55** : 493-497.

IQBAL, A., E.R. KÜHN and E. DECUYPERE (1989) — Somatostatin inhibits thyroxine release from the thyroid gland of chick embryo. *Med. Sci. Res.,* **17** : 427-428.

JACOBS, G.F.M. and E.R. KÜHN (1992) — Thyroid hormone feedback regulation of the secretion of bioactive thyrotropin in the frog. *Gen. Comp. Endocrinol.,* **88** : 415-423.

JACOBS, G.F.M., M.P. GOYVAERTS, G. VANDORPE, A.M.L. QUAGHEBUER and E.R. KÜHN (1988) — Luteinizing hormone-releasing hormone as a potent stimulator of the thyroidal axis in ranid frogs. *Gen. Comp. Endocrinol.,* **70** : 274-283.

JOZSA, R., S. VIGH, A.V. SCHALLY and B. MESS (1984) — Localization of corticotropin-releasing faktor-containing neurons in the brain of the domestic fowl: an immunocytochemical study. *Cell Tissue Res.,* **236** : 245-248.

KOVÁCS, K.J., H.M. WESTPHAL and P. PÉCZELY (1989) — Distribution of glucocorticoid receptor-like immunoreactivity in the brain, and its relation to CRF and ACTH immunoreactivity in the hypothalamus of the japanese quail, *Coturnix coturnix japonica. Brain Res.,* **505** : 239-245.

KRISHNAN, K.A., J.A. PROUDMAN and J.M. BAHR (1992) — Purification and characterization of chicken follicle-stimulating hormone. *Comp. Biochem. Physiol,* **102B** : 67-75.

KRISHNAN, K.A., J.A. PROUDMAN, D.J BOLT and J.M. BAHR (1993) — Development of an homologous radioimmunoassay for chicken follicle-stimulating hormone and measurement of plasma FSH during the ovulatory cycle. *Comp. Biochem. Physiol.*, **105A** : 729-734.

KÜHN, E.R., E. DECUYPERE, A. IQBAL, D. LUYSTERBORGH and R. MICHIELSEN (1988) Thyrotropic and peripheral activities of thyrotrophin and thyrotrophin-releasing hormone in the chick embryo and adult chicken. *Horm. metabol. Res.*, **20** : 158-162.

KÜHN, E.R., L.M. HUYBRECHTS, V.M. DARRAS, R. MEEUWIS and DECUYPERE, E. (1990) — Impaired peripheral T_3 production but normal induced thyroid hormone secretion in the sex-linked dwarf chick embryo. *Reprod. Nutr. Dev.*, **30** : 193-201.

LANCE, K. and C.T. SAWIN (1979) — Effects of TSH and TRH in the turtle *Chrysemys picta*. *American Zoologist*, **19** : 964, Abs 557.

LEUNG, F.C. and J.E. TAYLOR (1983) — *In vivo* and *in vitro* stimulation of growth hormone release in chickens by synthetic human pancreatic growth hormone-releasing factor (hpGRF). *Endocrinology*, **113** : 1913-1915.

MAEDA K-I., F.R.A. CAGAMPANG, C.W. COEN and H. TSUKAMURA (1994) — Involvement of the catecholaminergic input to the paraventricular nucleus and of corticotropin-releasing hormone in the fasting-induced suppression of luteinizing hormone release in female rats. *Endocrinology*, **134** : 1718-1722.

MALAGON, M.M., A. RUIZ-NAVARRO, R. TORRENTERAS and GARCIA-NAVARRO, F. (1991) — Effects of oCRF on amphibian ACTH and TSH cells *in vivo*. a quantitative ultrastructural study. *Gen. Comp. Endocrinol.*, **83** : 487-497.

MEEUWIS, R., R. MICHIELSEN, E. DECUYPERE and E.R. KÜHN (1989) — Thyrotropic activity of ovine corticotropin-releasing factor in the chick embryo. *Gen. Comp. Endocrinol.*, **76** : 357-363.

MILNE, R.S. and J.F. LEATHERLAND (1980) — Changes in plasma thyroid hormones following administration of exogenous pituitary hormones and steroid hormones to rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.*, **66A** : 679-686.

MITCHELL, M.A., M.G. MAC LEOD and A. RUZU (1986) — The effects of ACTH and dexamethasone upon plasma thyroid hormone levels and heat production in the domestic fowl. *Comp. Biochem. Physiol.*, **85A** : 207-215.

NIKOLARAKIS, K.E., O.F.X. ALMAEDA and A. HERZ (1986) — Corticotropin-releasing factor (CRF) inhibits gonadotropin-releasing hormone release from superfused rat hypothalami *in vitro*. *Brain Res.*, **377** : 388-390.

PETRAGLIA F., S. SUTTON, W. VALE and P. PLOTSKY (1987) — Corticotropin-releasing factor decreases plasma luteinizing hormone levels in female rats by inhibiting gonadotropin-releasing hormone release into hypophyseal portal circulation. *Endocrinology*, **120** : 1083-1088.

RADKE, W.J. and R.B. CHIASSON (1977) — *In vitro* regulation of chicken thyrotropes. *Gen. Comp. Endocrinol*, **31** : 175-182.

RIVIER, C. and W. VALE (1984a) — Corticotropin-releasing factor (CRF) acts centrally to inhibit the growth hormone secretion in the rat. *Endocrinology*, **114** : 2409-2411.

RIVIER, C. and W. VALE (1984b) — Influence of corticotropin-releasing factor on reproductive functions in the rat. *Endocrinology*, **114** : 914-921.

RIVIER, C. and P.M. PLOTSKY (1986) — Mediation by corticotropin-releasing factor (CRF) of adenohypophyseal hormone secretion. *Ann. Rev. Physiol.*, **48** : 475-494.

SCANES, C.G. and S. HARVEY (1989) — Somatostatin inhibition of thyrotropin-releasing hormone- and growth hormone-releasing factor-induced growth hormone secretion in young and adult anesthetized chickens. *Gen. Comp. Endocrinol.*, **75** : 256-264.

THOMMES. R.C., J.B. MARTENS, W.E. HOPKINS, J. CALIENDO, M.J. SORRENTINO and J.E. WOODS (1983) — Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo: IV. Immunocytochemical demonstration of TSH in the hypophyseal pars distalis. *Gen. Comp. Endocrinol.*, **51** : 434-443.

TONON, M.C., CUET, P., LAMACZ, M., JÉGOU, S., J. CÔTÉ, L. GOUTEUX, N. LING, G. PELLETIER and H. VAUDRY (1986) — Comparative effects of corticotropin-releasing factor, arginine vasopressin, and related neuropeptides on the secretion of ACTH and α-MSH by frog anterior pituitary cells and neurointermediate lobes *in vitro*. *Gen. Comp. Endocrinol.*, **61** : 438-445.

VALE, W., C. RIVIER, P. BRAZEAU and R. GUILLEMIN (1974) — Effects of somatostatin on the secretion of thyrotropin and prolactin. *Endocrinology*, **95** : 968-977.

VALE, W., J. SPIESS, C. RIVIER and J. RIVIER (1981) — Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. *Science*, **213** : 1394-1397.

WENTHWORTH, B.C., W.H. BURKE and G.P. BIRRENKOTT (1976) — A radioimmunoassay for turkey luteinizing hormone. *Gen. Comp. Endocrinol.*, **29** : 119-127.

WILLIAMSON, R.A. and T.F. DAVISON (1985) — The effect of a single injection of thyrotrophin on serum concentrations of thyroxine, triiodothyronine and reverse triiodothyronine in the immature chicken (*Gallus domesticus*). *Gen. Comp. Endocrinol.*, **58** : 109-113.

WILLIAMSON. R.A. and T.F. DAVISON (1987) — Effect of increased circulating corticosterone on serum and thyroidal concentrations of iodothyronines and the responses to thyrotrophin in the immature fowl (*Gallus domesticus*). *Gen. Comp. Endocrinol.*, **65** : 65-72.

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THE UTILITY OF BIRDS AS BIOINDICATORS : CASE STUDIES IN EQUATORIAL AFRICA

by

MICHEL LOUETTE (1), LUC BIJNENS (2),
DIEUDONNÉ UPOKI AGENONG'A and ROGER CORNEILLE FOTSO (3)
(1) Royal Museum for Central Africa, B-3080 Tervuren, Belgium
(2) Faculté des Sciences, Université de Kisangani,
B.P. 2012, Kisangani, Zaire
(3) Université de Yaoundé, Yaoundé, Cameroon

SUMMARY

When site quality cannot be fully studied, due to lack of time or available specialists, the use of particular species as indicators has been proposed. However, species which indicate species-richness are needed for appreciation of biodiversity, whereas widespread stenotopic species are appropriate for habitat quality. Birds are potential bioindicators, uniting inherent biological and methodological advantages. Birds can only be bioindicators of habitat quality if they occur in the same habitat in all the sites under consideration.

Standard bird counts and captures, and standard measurements of habitat-characteristics taken along chosen transects, yield avifaunal composition and relative density in pristine and man-changed areas in equatorial Africa, in two complex areas situated in the historical forest belt (Zaire and Cameroon). The use of these data for bioindication was examined. Segregation was found to be highest in undisturbed sites within the mosaic of present-day habitats. The present study proposes, for this particular region, the use of proportions in relative density of four common species among a particular family, the bulbuls, which all are well-characterized according to habitat. Nevertheless, the authors are fully aware that this indicator cannot be extended to other regions.

Keywords : bioindicators, bird populations, monitoring, environmental change, rainforest, Africa.

INTRODUCTION

FURNESS *et al.* (1993) reviewed the use of specific bioindicators to elucidate the effects of climatic change, change due to pollution, or habitat change. However, given the rapidity with which the habitat changes, there may be insufficient time to assimilate the relevant descriptive data and assess the bioindicatory implications of that information. Hence, this raises the question as to whether there is a need for bioindicators to measure habitat change. This study considers sites in Africa

where detailed studies are almost lacking (but from which we have obtained several simple data-sets) and where local changes are often fast. Therefore, the demand for rapid decisions on the protection of sites or choice among sites is great. Consequently, the use of particular plant or animal species as bioindicatory parameters could be useful. Nevertheless, in contrast, a more detailed analytical study is preferable when time and specialists are available because, in essence, each individual organism will respond differently to secondary effects of a primary cause or, at best, it will be specific enough only to be an indicator for itself. These caveats were discussed by MORRISON (1986) and RISEBROUGH (1991), particularly in an ornithological context. Also, OLIVER and BEATTIE (1994) recently investigated the use of a set of selected organisms (mosses, spiders, ants and polychaetes) for rapid assessment of total biodiversity but concluded that « trials of all groups deemed to be useful... needed to be evaluated » and « taxonomic specialists are considered indispensable to train personnel », this makes their system far too complicated to hope for « rapid assessment ».

Birds are considered to be potential bioindicators (FURNESS and GREENWOOD, 1993). The advantages of using birds are that they are either at or near the top of the food chain. Moreover, both specialist and generalist species exist among related taxa, see below), they are easy to identify, they are often common and day-active, their ecology and behaviour is often well studied and extensive data are available, and there is an important public interest in birds (i.e. collaboration of amateur ornithologists). As well as the very characteristic species of a given ecotope, there are also some more wide-ranging species which are promising as indicators for the quality of the habitats in which they occur, assuming that observed variations in bird population parameters are directly related to a change in a factor of the environment. A further advantage of using birds might be that, due to their mobility beyond the studied transect, well-selected species can also indicate, to some extent, the quality of the surrounding area of the observation spot (cf. organisms of weak mobility) ; this should enable a better appreciation of the quality of the site. The foregoing discussion pertains essentially to the temperate regions, with at least one available textbook study linking bird population dynamics and forestry (AVERY and LESLIE, 1990).

The peculiarities needed to define an indicator are a function of the question being analyzed : unique species indicating species-richness are needed for appreciation of biodiversity, whereas stenotopic species are appropriate for habitat quality.

There is some hope that the use of Geographic Information Systems will enable faster and more accurate compilation of data files in ornithology (SHAW and ATKINSON, 1990). However, the gathering and input of census data by biologists or persons instructed by biologists will, even under ideal conditions, impose some delay and a « bottle-neck » in data input. Under present conditions, and recognizing that this subject requires more than simply notifying the presence or absence of species, this study investigates the potential of quantitative data as descriptive parameter for habitats in tropical regions.

MATERIAL AND METHODS

The authors recognize the relatively low sampling effort in both Zaire and Cameroon and the absence of replications in space and time. Despite this, and because of the lack of other available information, the field data obtained for population studies is analyzed for their bioindicatory utility :

1) Standard bird captures in mist-nets and standard measurements of habitat characteristics in the Masako nature reserve, 14 km north of Kisangani, Zaire and at sites situated at 0, 40, 152 and 333 km from Kisangani along the new Kisangani-Bukavu road (constructed through the equatorial rainforest) from February to August 1989. These sites are all situated in a habitat mosaic, but those furthest away from Kisangani possess a large surrounding buffer zone of almost intact rainforest (the largest remaining equatorial forest block in Africa). The localities along the road are composed of a first set of nets at 500 m from the road (sites 9, 11, 12 in Table 1) and other sets at 5 km from the road, beyond the heavily disturbed zone. This area holds about 350 bird species (BIJNENS and UPOKI, 1992). Table 1 gives dominance rank at each site, whereby rank = 1 if the species is the most abundant, etc. (0.5 if shared with another species).

TABLE 1

Dominance rank of selected bulbuls, showing replacement, at thirteen sites in and near Kisangani, Zaire. (NR : Masako nature reserve near Kisangani)

Site	*Good forest*			*Secondary growth*					*Edge of clearings*			*Cultivation*	
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
Distance from Kisangani	333	333	NR	152	NR	152	40	40	333	NR	40	152	0
Number of birds caught	144	138	281	95	305	63	60	56	86	175	72	100	75
Dominance Rank													
Phyllastrephus icterinus :	6	5	6	13.5	24.5	—	—	—	—	—	—	—	—
Andropadus latirostris :	1.5	1	1	2	1	1	1.5	2	1	2	—	—	—
Andropadus virens :	8	3	3	3.5	3	3.5	3	4	2	1	2	17.5	—
Pycnonotus barbatus :	—	—	—	—	—	—	—	—	9.5	25	5.5	8	4.5

2) Standard bird counts (by song recognition during fifteen minutes at the most favourable periods of the day in the months of April to August 1989 and 1991) and standard measurements of habitat-characteristics along four chosen sites, several km distant from each other, near Yaoundé, Cameroon (transects from 6 or 8 counting stations along forest paths about 3 km long). These yield avifauna composition

and abundance in a mosaic of pristine and man-changed habitats situated in the historical forest block. This area holds about 350 bird species (FOTSO, 1994). Table 2 gives the frequency index (number of contacts/ number of counts) of four selected bulbuls. Figure 1 gives the projection of the most frequent species (according to presence or absence) according to the first and the second axis in a factor correspondence analysis, maximizing correlations between habitat variables and species variables.

TABLE 2

Frequency indices of selected bulbuls, showing distribution profile and replacement along the transects at four sites near Yaoundé, Cameroon. Number of dominant bird species : Eloumden : 36 ; Kala : 56 ; Messa : 46 ; Nkol-Yeye : 47

Site	*Transect stations*								*Dominance rank*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
Phyllastrephus icterinus :									
Eloumden	0	0.3	0.9	1.0	0.6	0.7	0.8	0.1	7
Kala	0	0	0.2	0.8	0.4	0.5	0.3	0.3	15
Messa	0	0	0	0	0	0	—	—	—
Nkol-Yeye	0	0	0	0	0	0	—	—	—
Andropadus latirostris :									
Eloumden	1.4	1.2	1.2	1.2	1.2	1.2	1.2	0.5	1
Kala	0.8	0.6	0.9	1.0	1.1	0.8	1.0	1.0	2
Messa	0.1	0.3	0.8	0.6	0.3	0.5	—	—	10
Nkol-Yeye	0.5	1.0	0.2	0.1	0.7	0.4	—	—	9
Andropadus virens :									
Eloumden	1.1	1.2	0.5	0.4	0.7	0.6	0.7	0.6	4
Kala	1.2	1.1	1.0	0.9	0.7	0.9	0.9	0.9	1
Messa	1.5	1.5	1.4	1.5	1.5	1.6	—	—	1
Nkol-Yeye	1.2	1.3	1.2	1.3	1.1	1.1	—	—	1
Pycnonotus barbatus :									
Eloumden	0	0	0	0	0	0	0	0	—
Kala	0.4	0	0	0	0	0	0	0	37
Messa	1.6	1.4	1.3	1.1	0.6	0.8	—	—	3
Nkol-Yeye	1.2	1.2	1.1	0.8	0.7	0.9	—	—	2

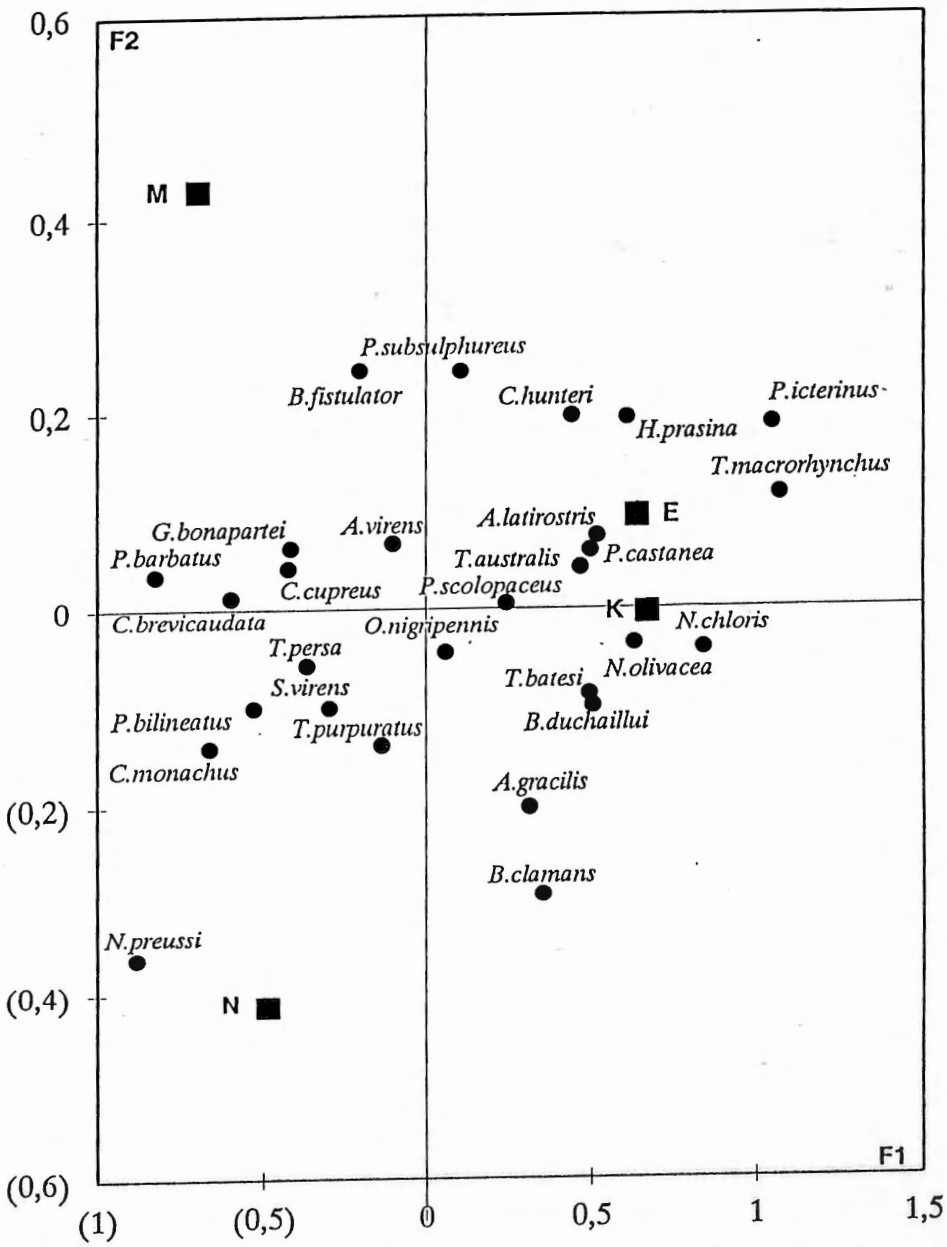

Fig. 1. — Projection of scores of the most common bird species from counts during the breeding seasons 1989-1992, on the first and the second axis in a multifactor correspondence analysis. The general position of the four counting sites (increasing deforestation from Eloumden, Kala, Messa, to Nkol-Yeye) at the forest/savanna border (Yaoundé, Cameroon) is also indicated.

The scientific names for birds used in this paper follow SIBLEY and MONROE (1990).

RESULTS

Habitat and altitude differences will be apparent for particular birds exclusive to the forest and non-forest zones ; this study is restricted to selected lowland « forests ».

Not all species of birds are prone to capture by netting and although there are certainly interspecific differences (further information in BUB, 1991), we assume that these are minimal among the low-flying small birds of mesic environments and especially in the bulbul family Pycnonotidae, which are the group of birds in our Zaire sites considered in more detail here. This family counts for 51.7 % of all birds captured at this site (UPOKI, 1992). According to the dominance rank per species in each of the thirteen Zairean stations, *Andropadus virens* Cassin 1858 is the most common bird, being present in twelve of the stations (Table 1) ; it is only absent at the city site. It is the only common bird to show a significant, positive relationship between its rank and the vegetation parameters, « Vegetation Volume Index », « Foliage Height Diversity » and the principal component in a total complex of all measured vegetation parameters ; its numbers augment with these parameters, indicating its preference for secondary vegetation in forest, because the primary forest itself has less undergrowth (BIJNENS and UPOKI, unpublished). *Andropadus virens* and its congener, *Andropadus latirostris* Strickland 1844, very often rank among the five most common species caught, but with differences according to degree of degradation (Table 1). In fact, while *A. virens* is most common in terms of being present at all but one site, *A. latirostris* is more « common » in terms of being the dominant species at most of the sites. The habitat preference and commonness is not unexpected because BROSSET and ERARD (1986) have found a situation in the forest in Gabon where proportions of these congeners vary according to site, but where it is clear that *A. latirostris* prefers less disturbed zones. Among other bulbuls appearing in the nets, according to vegetation cover, *Phyllastrephus icterinus* (Bonaparte) 1850 is present almost exclusively in prime forest whereas *Pycnonotus barbatus* (Desfontaines) 1789 is found only in disturbed habitats.

Not surprisingly, segregation of the birds in Cameroon's present-day mosaic of vegetation is most distinct, though still incomplete, in undisturbed sites. Total bird diversity augments initially with degradation. However, stability of a site is best indicated using proportions of relative density among certain genera of frugivores and insectivores (FOTSO, 1994). Figure 1 shows that the four bulbul species, already studied in the Zaire example, here are well separated also along the first axis in a multifactor correspondence analysis. Some of the other well-separated species, such as *Tauraco macrorhynchus* (Fraser) 1839, *Centropus monachus* Rüppell 1837 and *Nectarinia preussi* (Reichenow) 1892, have a restricted range in equatorial Africa and cannot be used as general habitat indicators. The ranks obtained at the four

Cameroonian study sites and the frequency values along the transects (Table 2) indicate the following habitat preferences : *Phyllastrephus icterinus* is a stenotopic forest species ; *Andropadus latirostris* prefers forest, tolerating a more heterogenous fringing zone ; *Andropadus virens* is more of a generalist, being more abundant at the edge of high-grade forest ; *Pycnonotus barbatus* avoids forest and is more abundant at the edge of degraded forest. These results corroborate the Zairean results and bring some refinement to the interpretation of the effects of border. The local conditions, such as the mosaic of very large vegetation zones, suggest segregation is to be found only in undisturbed sites.

DISCUSSION

The four selected species of bulbul show that a group of related birds can provide information on site quality, even without a detailed study of the forests themselves. The birds presence or absence and the proportion of their abundance would doubtless be useful for the appreciation of any site in this zoogeographical region in Africa. In practice, as far as application by non-specialists is concerned, recognition in the field may present a problem. Nevertheless, a mistnetting programme would enable the identification in the hand of the bulbul species discussed here ; probable exceptions are *Andropadus virens* and *Phyllastrephus icterinus*, which can be confused with congeners, not mentioned here (the other two bulbuls are easily identified in the hand).

Other quantitative bird data are rare or nonexistent for equatorial Africa (see references in BIBBY *et al.*, 1992) or have not yet been adequately analyzed (BROSSET, 1989). One study indicates the minimum size of forest fragments necessary to hold breeding pairs of birds in Malawi (DOWSETT-LEMAIRE and DOWSETT, 1984). PRODON (1992) has shown the use of correlation methods in measuring and modelling the compositional dynamics along forest successions ; this is achieved by linking the vegetation dynamics and the induced animal (i.e., bird) turnovers and computing the variations of community characteristics along successions. While this method could be employed here, it is difficult to see what would be its advantage ; we prefer to select the individual species, or a group of related species, which are most significant in a habitat comparison.

As a general, provisional conclusion, it appears that particular forest birds, if selected among species-rich taxa with finely-tuned niches, could be useful as bioindicators for rapid evaluation of site (i.e., habitat) quality in continuous habitat.

Conversely, we do not find such a use for characteristic species with restricted ranges solely. These latter certainly give information about biodiversity, but they are not potential indicators of general habitat. PRENDERGAST *et al.* (1993) have shown that habitats that are species-rich for one taxon are not necessarily species-rich for others, nor that rare species are to be found necessarily in species-rich habitats. Moreover, while presence of such birds might be indicative of site quality, this information is impossible to use generally.

Diversity, in itself, can be used to predict the structural aspects of habitats and *vice versa* (CODY, 1985). In the, often common case of complex habitat mosaics, a rapid method as tested here gives the necessary crude information. Nevertheless, we are aware that it would be dangerous to extrapolate conclusions too extensively from the results.

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REFERENCES

AVERY, M. and R. LESLIE (1990) — *Birds and Forestry*. Poyser, London. 299 pp.

BIBBY, C.J., N.D. BURGESS and D.A. HILL (1992) — *Bird census techniques*. Academic Press, London. 257 pp.

BIJNENS, L. and D. AGENONG'A UPOKI (1992) — Preliminary report of an ecological bird survey in virgin forest near Kisangani (Zaire). *Proceedings Seventh Pan-African Ornithological Congress*, Nairobi : 475-480.

BROSSET, A. (1989) — Population dynamics of birds in a northeastern Gabon forest. *Ostrich* Supp. **14** : 1-6.

BROSSET, A. and C. ERARD (1986) — *Les Oiseaux des régions forestières du nord-est du Gabon*. Société Nationale de protection de la nature, Paris. 297 pp.

BUB, H. (1991) — *Bird trapping and bird banding*. Cornell University Press, Ithaca, New York. 330 pp.

CODY, M.L. (1985) — An introduction to habitat selection in birds. *In : Habitat selection in birds*. CODY, M.L. (ed). Academic Press, Orlando, Florida : 4-56.

DOWSETT-LEMAIRE, F. and R. DOWSETT (1984) — The effect of forest size on montane bird populations. *Proceedings Fifth Pan-African Ornithological Congress*, Southern African Ornithological Society, Johannesburg : 237-248.

FOTSO, R.C. (1994) — *Dynamique des peuplements d'oiseaux dans les séries écologiques de la région de Yaoundé (Sud Cameroun)*. Doctorate thesis, Katholieke Universiteit Leuven, Belgium. 141 pp. and annexes.

FURNESS, R.W., J.J.D. GREENWOOD and P.J. JARVIS (1993) — Can birds be used to monitor the environment ? In : *Birds as monitors of environmental change*. FURNESS, R.W. and J.J.D. GREENWOOD (eds). Chapman & Hall, London : 1-41.

FURNESS, R.W. and J.J.D. GREENWOOD (1993) — *Birds as monitors of environmental change*. Chapman & Hall, London. 356 pp.

MORRISON, P. (1986) — Bird populations as indicators of environmental change. *Current Ornithology* **3** : 429-451.

OLIVER, I and A.J. BEATTIE (1994) — A possible method for the rapid assessment of biodiversity. *In : Systematics and conservation evaluation.* FOREY, P.L., C.J. HUMPHRIES and R.I. VANE-WRIGHT (eds). Clarendon Press, Oxford : 133-136.

PRENDERGAST, J.R., R.M. QUINN, J.H. LAWTON, B.C. EVERSHAM and D.W. GIBBONS (1993) — Rare species, the coincidence of diversity hotspots and conservation strategies. *Nature* **365** : 335-337.

PRODON, R. (1992) — Animal communities and vegetation dynamics : measuring and modelling animal community dynamics along forest successions. *In : Responses of forest ecosystems to environmental changes..* TELLER, A., P. MATHY and J.N.R. JEFFERS (eds). Elsevier, London & New York : 126-141.

RISEBROUGH, R.W. (1991) — Indicator species, birds, toxic contaminants, and global change. *Acta XX Congressus Internationalis Ornithologicus* : 2480-2486.

SHAW, D.M. and S.F. ATKINSON (1990) — An introduction to the use of Geographic Information Systems for ornithological research. *Condor* **92** : 564-570.

SIBLEY, C.G. and B.L. MONROE (1990) — *Distribution and taxonomy of birds of the world.* Yale University Press, New Haven & London. 1111 pp.

UPOKI, A. (1992) Les bulbuls de la réserve forestière de Masako. *Abstracts Eighth Pan-African Ornithological Congress, Bujumbura,* Burundi, 55.

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PRELIMINARY RESULTS OF LUMINESCENCE CONTROL IN ISOLATED ARMS OF *OPHIOPSILA ARANEA* (ECHINODERMATA)

by

JÉRÔME MALLEFET and MARLÈNE DUBUISSON
Université Catholique de Louvain, laboratoire de Physiologie Animale,
5 place Croix du Sud, B-1348 Louvain-la-Neuve, Belgium

SUMMARY

Luminescence induced by KCl applications on isolated arms from *Ophiopsila aranea* (FORBES) is described. Light emission is characterized by a series of flashes whose maximal intensity increases as a function of KCl concentration. None of the neuromediators tested (adrenaline, noradrenaline, 5-HT, acetylcholine, carbachol, gaba, taurine, glutamate) induced the production of light. Our study requires further experiments in order to characterize the nature of the luminescence control mechanism of *Ophiopsila aranea.*

Keywords : Echinoderm, ophiuroid bioluminescence, physiology, isolated arms.

INTRODUCTION

Ophiopsila aranea (Forbes, 1843) is a member of the class Ophiuridea living in the encrusting coralline algae zone (coralligène) in the Mediterranean Sea. The mean size of the disc is 8 mm. The very long arms (more than 10 cm) that show a pattern of bands of different pigments are known to luminesce. Early morphological studies described the luminescence sites originating from glandular cells located on the ventral and lateral plates and also in some spines of the arms next to the disc (MANGOLD, 1907 ; REICHENSPERGER, 1908 ; TROJAN, 1909). In 1952, HARVEY mentioned a yellowish green fluorescence appearing as bright points in U.V. light at the sites of luminescence. The exact nature of luminous cells remained unknown until a more recent study on *Ophiopsila californica* (Brehm and Morin, 1977) suggested a neural origin of luminous cells, termed photocytes.

In luminous ophiuroids, potassium chloride (KCl) is a classical stimulation used to induce a light emission by depolarization of the nervous elements controlling

the luminescence tissues (HERRING, 1974). For example, in *Amphipholis squamata*, the optimal concentration of KCl used to trigger light emission is 200 mM (MALLEFET *et al.*, 1989). Many different neuromediators were detected in various tissues of echinoderms (see COBB 1987, 1988) ; among them acetylcholine represents a major component of the nervous system for which some physiological activities were described. For example, pharmacological studies have identified the presence of a cholinergic system involved in the luminescence control of *Amphipholis squamata* (DEBREMAEKER *et al.*, 1993a,b).

Since no physiological data concerning the luminescence control mechanism in *Ophiopsila aranea* are available, we first tested the responsiveness of isolated articles from *Ophiopsila aranea* arms to depolarization by KCl and tried to identify a putative neuromediator that could trigger light emission.

MATERIAL AND METHODS

Specimens of *Ophiopsila aranea* were collected at the ARAGO biological station (C.N.R.S.) at Banyuls-sur-mer (France) by scuba divers working at 15-20 metres depth. The animals were transported in aerated and running natural sea water and then kept in aquaria filled with aerated and recirculating natural and artificial sea water (ASW) at 12° C. Food was provided to them three times a week.

After anaesthesia of the brittlestars by immersion in 3 % MgCl2 in ASW, arms were cut out and divided into sections of 2 to 4 articles which were then rinsed in ASW of the following composition : NaCl 400.4 mM, KCl 9.6 mM, CaCl2 9.9 mM, MgCl2 52.3 mM, Na2SO4 27.7 mM, Tris 20 mM, pH 8.3.

The articles were placed in small chambers filled with 200 µl of sea water and then stimulated by injection of air saturated solutions. Stock solutions of potassium chloride (Merck), acetylcholine (Sigma), carbachol (Janssen), adrenaline, noradrenaline (Fluka), 6-OHDA (Aldrich), 5-HT, gaba (Merck), taurine (Fluka), L-glutamate (Sigma) were dissolved in ASW just before experiments ; 200 µl of these solutions were injected in the small chambers containing the articles, using a micropipette ; light responses were followed during 10 minutes. Knowing that each isolated arm provides more than 18 articles as preparations, a total of 6 different *Ophiopsila aranea* was used in this work. Each experiment was performed on a different preparation coming from an isolated arm. Light emission was monitored with a PM270D photomultiplier connected via an amplifier to a graphic recorder. The maximum light emission (Lmax) is the parameter used to characterize *Ophiopsila aranea* light emission ; results are expressed in megaquanta per second per

article (Mq/s/art). Statistical analyses were performed using variance analysis (ANOVA 1 criteria) ; each mean value is expressed with its standard error of the mean (mean ± sem) and number of tested preparations (n).

RESULTS

KCl depolarization

Applications of KCl concentrations ranging from 100 mM to 400 mM on articles from isolated arms of 4 different *Ophiopsila aranea* always induced a light emission characterized by a series of flashes (Fig. 1) whose maximal intensity increased as a function of KCl concentration.

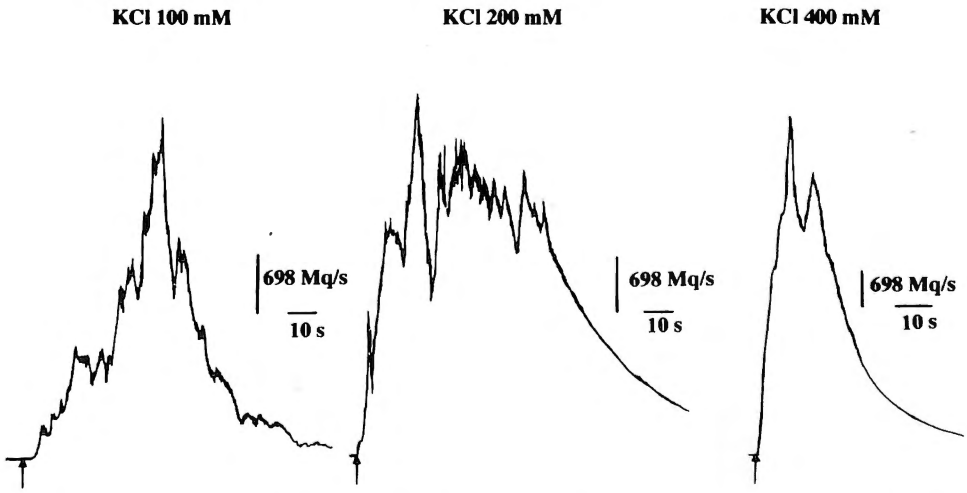

Fig. 1. — Original recordings of *Ophiopsila aranea* isolated arms in responses to different KCl concentrations ; vertical bar : light intensity of 698 Mq/s. ; horizontal bar : time scale of 10 s.

Application of KCl 100 mM triggered after 8,2 ± 0,9 s. a luminescence which reached a maximum level of 1385 ± 125 Mq/s/art (n = 72) after 37 ± 1,9 s. At 200 mM KCl, luminescence started after 1,3 ± 0,2 s. and showed a maximal value of 1733 ± 126 Mq/s/art within 32 ± 3,3 s (n = 48). In response to KCl 400 mM, light emission started 1,1 ± 0,1 s. after KCl application and developed a maximal value of 3061 ± 140 Mq/s/art after 12,8 ± 1,1 s (n = 84). The maximal light output in response to KCl 400 mM is statistically (anova 1 criteria, Fobs : 47.7, $p < 0.0001$) higher than those observed with the two other KCL concentrations tested in this study (Fig. 2).

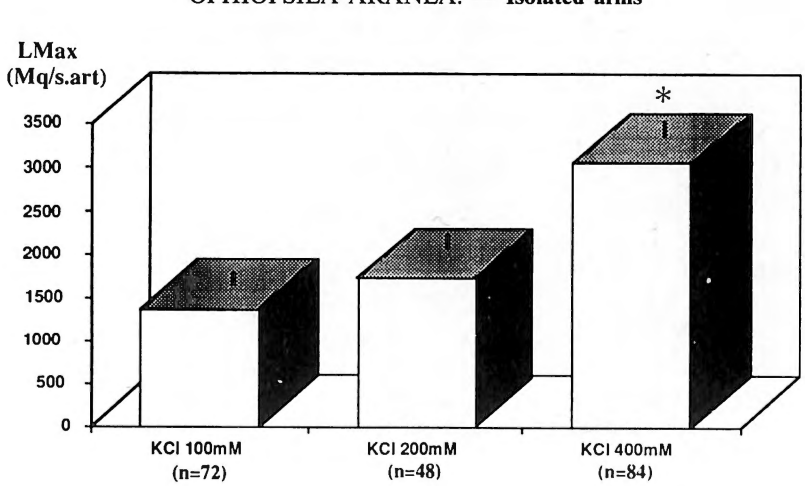

Fig. 2. — Effects of KCl concentration on light emission of isolated arms of *Ophiopsila aranea* ; (mean ± sem, n = number of preparations, * = p < 0,05).

Analysis of the maximal light responses to KCl 400 mM of articles isolated from the 5 different arms of a single animal indicates that there is no significative difference (anova 1 criteria, Fobs : 0.5, N.S.) between the arms (Fig. 3).

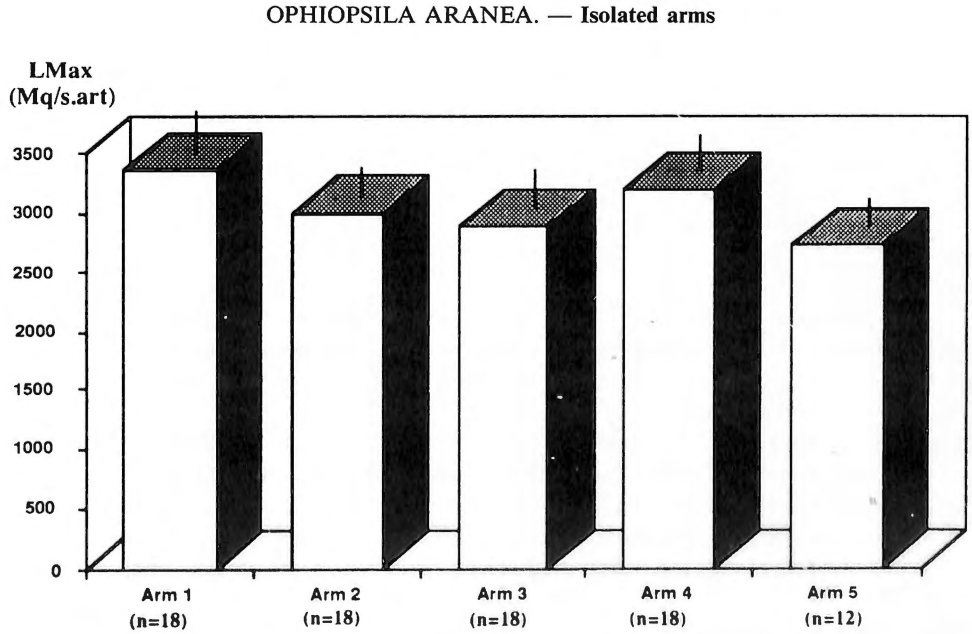

Fig. 3. — Luminescence of isolated arms from one specimen of *Ophiopsila aranea.* in response to 400 mM KCl application ; (mean ± sem, n = number of preparations, * = p < 0,05).

Pharmacology

Each neuromediator was tested on separate preparations from isolated arms of 2 different animals ; articles were bathed for 10 minutes in ASW containing the drugs. The following drugs and concentrations were used : cholinergic compounds — acetylcholine from 10^{-7}M to 10^{-3}M and carbachol at 10^{-3}M ; aminergic compounds — adrenaline, noradrenaline, 5HT and 60HDA at 10^{-3}M ; other compounds — glutamate, taurine and Gaba at 10^{-3}M. Each treatment was tested on 6 different preparations. None of the neuromediators induced the production of light from *Ophiopsila aranea* isolated articles.

DISCUSSION

KCl solutions isotonic to sea water (540 mM) were frequently used to test echinoderm's ability to luminesce (HARVEY 1952 ; HERRING 1978 ; MILLOT 1966 ; BREHM and MORIN 1977) ; in this context the 100 to 400 mM KCl concentrations used in this study are comparable to those utilised to obtain response on various isolated effector organs of echinoderms (PROTAS and MUSKE 1979). We must point out that *Ophiopsila aranea* appears to be very sensitive to KCl since the first light emission was detected at 50 mM KCl (not shown) although at this KCl concentration responses obtained were very erratic.

Comparison of light amplitudes from the 5 arms isolated from one animal, has shown that luminous capabilities are not different from one arm to another. Since we stimulated articles of the first arm before stimulating those from the next arm, this comparison also reveals that maintenance of isolated arms in ASW does not modify their luminous capabilities ; this result indicates that our experimental conditions (anaesthesia, ASW composition...) are sufficient to conserve isolated arms in good physiological conditions.

The presence of multiple flashes may suggest that KCl acts either through a mechanism that progressively activates luminous cells to trigger light emission or through depolarization of some nervous elements implicated in *Ophiopsila* luminescence control. This second hypothesis seems more plausible since it has been shown with *Ophiopsila californica* that luminescence is related to nervous activity in the radial nerve cord (BREHM 1977) ; nevertheless, the nature of the mechanism coupling nerve activity and light emission, as well as the nature of the nervous receptors are unknown.

Many different neuromediators were detected in various tissues of echinoderms (PENTREATH and COBB 1972, COBB 1987, 1988, SLOLEY and JUORIO 1990), among them acetylcholine represents a major component of the nervous system for which some physiological activities were described (SHELKONIKOW *et al.*, 1977, MORALES *et al.*, 1989). Although the present pharmacological results are preliminary, they failed to reveal any activity of this neuromediator ; this observation is in contradiction with the recent finding showing that a muscarinic cholinoreceptor is implicated in the control of another luminescent ophiuroid, *Amphipholis squamata* (DE

BREMAEKER *et al.*, 1993a,b). Our study requires further experiments in order to characterize the nature of the luminescence control mechanism of *Ophiopsila aranea*.

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REFERENCES

BREHM, P. (1977) — Electrophysiology and luminescence of an ophiuroid radial nerve. *J. exp. Biol.* **71** :213-227.

BREHM, P. and J.G. MORIN (1977) — Localization and characterization of luminescent cells in *Ophiopsila californica* and *Amphipholis squamata* (Echinodermata : ophiuroidae). *Biol. Bull.* **152** :12-25.

DE BREMAEKER, N., J. MALLEFET and F. BAGUET (1993a) — Involvement of a cholinergic control in the *Amphipholis squamata* (Echinodermata) luminescence. *Arch. int. Physiol. Bioch.* **101**(3) : 30.

DE BREMAEKER, N., J. MALLEFET and F. BAGUET (1993b) — Nature of cholinergic receptors involved in the luminescence control of *Amphipholis squamata* (Ophiuroidea). *Abstr. 8 th I.E.C.* : 16.

COBB, J.L.S. (1987) — Neurobiology of the echinodermata. In *Nervous systems in invertebrates, M.A. Ali* (ed.), 483-517. NATO ASI series A :141.

COBB, J.L.S. (1988) — Neurohumors and neurosecretion in echinoderms : a review. *Comp. Biochem. Physiol.* **91C** : 151-158.

HARVEY, N. (1952) — Echinodermata. In *Bioluminescence.* HARVEY, N. (ed.), AC Press, 472-479.

HERRING, P.J. (1974) — New observations on the bioluminescence of echinoderms. *J. Zool. Lond.* **172** : 401-418.

HERRING, P.J. (1978) — Echinoderms. In *Bioluminescence in action,* HERRING P.J. (ed.), A.C. Press : 231-236.

MALLEFET, J., F. BAGUET and M. JANGOUX (1989) — Origin of the light emission of *Amphipholis squamata* (echinodermata) to potassium chloride. *Arch. int. Physiol. Biochem.* **97** : 37.

MANGOLD, E. (1907) — Leuchtende schlangensternne und die flimmerbewegung bei *Ophiopsila. Arch. ges. Physiol. Pfügler, Bd.* CXVIII : 613-640.

MILLOT, N. (1966) — Light production. In R.A. *Boolotian* (ed.) : 487-501.

MORALES M., J. DEL CASTILLO and D.S. SMITH (1989) — Acetylcholine sensitivity of the spine test articular capsule of the sea urchin *Eucidares thibuloides. Comp. Biochem. Physiol.* **94C** : 547-554.

PENTREATH, V.W. and J.L.S. COBB (1972) — Neurobiology of echinodermata. *Biol. Rev.* **47** : 363-392.

PROTAS, L.L. and G.A. MUSKE (1979) — The effects of some transmitter substances on the tube foot muscles of the starfish, *Asterias amurensis*. *Gen. Pharma.* **11** : 113-118.

REICHENSPERGER, A. (1908) — Die drüsengebilde der ophiuren. *Zeit Wiss Zool.* **91** : 304-350.

SHELKOVNIKOV, S.A., L.A. STARSHINOVA and E.V. ZEIMAL (1977) — Two kinds of cholinoreceptors on the on-visceral muscle of some echinodermata. *Comp. Biochem. Physiol.*, **58C** : 1-12.

SLOLEY, B.D. and A.V. JUORIO (1990) — Biogenic amines in the nervous and other tissues of several species of starfish — presence of relatively high levels of tryptamine and low levels of 5 hydroxytryptamine. *Biogenic Amines.* **7** : 341-349.

TROJAN, A. (1909) — Leuchtende Ophiopsilen. *Arch. mikr. Anat.* **73** : 883-912.

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DIVERSE EFFECTS OF FOREST FRAGMENTATION ON A NUMBER OF ANIMAL SPECIES

by

ERIK MATTHYSEN, LUC LENS, STEFAN VAN DONGEN, GEERT R. VERHEYEN, LUC A. WAUTERS, FRANK ADRIAENSEN and ANDRÉ A. DHONDT
Laboratory for Animal Ecology, Department of Biology (UIA), University of Antwerp, B-2610 Wilrijk

SUMMARY

Since 1990 we have initiated a number of studies on the population ecology, population genetics and social organization of several animal species in forest fragments of varying sizes. Study species include squirrels, small passerines and butterflies. Study sites are forest fragments between 1 and 40 ha, and study plots in « continuous » forest of 100 to 1500 ha. Our results so far suggest that forest fragmentation has more profound effects on dispersal patterns and population structure than on reproductive output and survival. Effects on dispersal include at least two aspects, which are not necessarily found in the same species. First, immigration deficits may be caused by low disperser success and/or source-sink effects. Second, reduced gene flow among local populations may result in genetic differentiation and/or loss of genetic variation. Negative consequences of the latter are not obvious, but indirect effects may be important *e.g.* for local adaptation. We conclude that studies on population structure in fragmented habitats should include quantitative (dispersal distance, immi/emigration, genetic structure) as well as qualitative aspects (timing of dispersal, habitat selection).

Keywords : habitat fragmentation, gene flow, dispersal, demography.

INTRODUCTION

Habitat fragmentation has been recognized as a major threat to the survival of natural populations and the functioning of ecosystems. The reduction of large, more or less continuous habitats to small and isolated remnants affects the abundance and species composition of various taxa. Possible factors contributing to this decline include changes in predation or food availability, microclimatic effects, loss of genetic variation and lack of recolonization following local extinctions (see reviews by *e.g.* WILCOX and MURPHY, 1985, SOULÉ, 1986, OPDAM, 1991, SAUNDERS *et al.*, 1991). Given the extensive temporal and spatial scales on which these effects operate, true experimental studies are relatively scarce (LOVEJOY *et al.*, 1986, MARGULES, 1992, ROBINSON *et al.*, 1992, IMS *et al.*, 1993) and most in-

vestigators have concentrated on horizontal comparisons of historically fragmented habitats.

Since 1990 the Laboratory for Animal Ecology of the University of Antwerp has initiated a series of studies on the population ecology, population genetics and social organization of animal species from different taxa in forest fragments. The aim is to investigate whether populations in highly fragmented habitats function differently compared to continuous habitats, which mechanisms are responsible for these differences, and how these affect the status of the population. We chose to study common species, not particularly endangered, that vary in dispersal capacities, abundance and trophic level. Here we report on some of our major findings thus far, and try to present some general conclusions that can be made at this moment.

STUDY SPECIES, STUDY AREAS AND GENERAL METHODS

Our studies so far include one mammal (Eurasian red squirrel *Sciurus vulgaris* L.), four small passerines (nuthatch *Sitta europaea* L., crested tit *Parus cristatus* L., great tit *P. major* L., blue tit *P. caeruleus* L.) and one butterfly (winter moth *Operophtera brumata* L.). All species are largely restricted to mature forest (Table 1). Crested tits are restricted to coniferous forest, squirrels prefer conifers but occur in broadleaved forest as well, and the remaining species prefer broadleaved forest and reach their highest abundance in forest dominated by oak *Quercus robur* L. All species are studied in a set of patches of secondary forest or parkland (henceforth « fragments ») surrounded by agricultural land, sometimes partly by residential areas. The range of fragment sizes covaries with the abundance of the species under consideration (Table 1). Additional study sites in larger forests (> 100 ha) vary in number from one to three per species. All study sites are situated in northern Belgium, in a radius of 50 km from the city of Antwerp.

Methods are generally based on individually marking a substantial sample, if not the majority, of the population in the study sites, the only exception being the winter moth. In 1993 we started a large-scale nestbox study for great and blue tit with standard nestbox densities in 12 fragments of varying sizes, all within a few kilometers of one another. This approach provides us with direct estimates of demographic parameters, including immigration and emigration. In winter moth and tits dispersal is estimated indirectly by the study of selectively neutral genetic variation. Genetic variation is estimated by DNA fingerprinting in squirrels (WAUTERS *et al.*, 1994a) and blue tits (VERHEYEN *et al.*, in prep.), and by allozyme electrophoresis in winter moths (VAN DONGEN *et al.*, 1994). For further details see LENS and DHONDT (1994), MATTHYSEN *et al.* (1995), NOUR *et al.* (1993), VAN DONGEN *et al.* (1994) and WAUTERS *et al.* (1994a, b).

TABLE 1

Results (in part preliminary) on demography and population structure of six animal species in relation to forest fragmentation. The table indicates the level of various parameters and processes in small and isolated fragments, as compared to larger and/or less isolated fragments (or sites in continuous forest). — no difference, ?? no or insufficient information. For further information see text and references.

Species	Red Squirrel	Nuthatch	Winter moth	Crested Tit	Great Tit Blue Tit
Forest type	All	Deciduous	Deciduous	Coniferous	Deciduous
Fragment sizes	15-60 ha	2-40 ha	0.1-10 ha	10-50 ha	0.5-10 ha
Abundance	lower	lower	lower [2]	lower	—[3]
Reproduction	—	—	??	—	—
Timing	less synchronous	earlier	less synchronous ?	later [4]	earlier
Survival/condition	—	—	lower mass	—	??
Philopatry [1]	higher	—	—	—	higher
Timing of dispersal	??	delayed	??	delayed	??
Net immigration	lower	lower	??	lower	—
Gene flow	lower	—[5]	lower	??	lower [5]
Genetic variation	smaller	??	smaller	??	??
References	Wauters *et al.*, 1994a, b	Matthysen *et al.*, 1995 Matthysen, unpubl.	Van Dongen *et al.*, 1994 Van Dongen, unpubl.	Lens and Dhondt 1994 Lens, unpubl.	Verheyen *et al.*, in prep. Matthysen *et al.*, unpubl.

[1] proportion of surviving offspring that reproduce near the site of birth.
[2] not repeated in a second year (Van Dongen, unpubl.).
[3] abundance mainly determined by nestbox availability.
[4] second broods only.
[5] estimated from local recruitment rates.

RESULTS

Table 1 summarizes the major findings in the six species under study. For more details we refer to the appropriate publications. In four of six species we found lower abundances in smaller and/or more isolated forests. In the winter moth this result is apparently less robust since it was not found in all years (VAN DONGEN, unpubl.). In great and blue tits population density is largely determined by nestbox density which we controlled. High nestbox densities in all areas (8 boxes per ha or more) resulted in overall high population densities. We found very little evidence for lowered reproduction, condition or survival in relation to fragmentation. Fledgling crested tits had a lower body mass in fragments, but the effect on their fitness is not clear, apart from delayed dispersal. Surprisingly, timing of reproduction differs between fragments and large forests in most, if not all species. In squirrels and perhaps winter moths reproduction is less synchronous in forest fragments ; nuthatches, great and blue tits reproduce earlier in spring in fragments, and crested tits increase the interval between first and second broods.

Marked differences were found in dispersal patterns and/or population structure between populations in fragmented and unfragmented habitat. In general, two kinds of effects seem to exist, which are not necessarily congruent. First, in several species the degree of exchange with neighbouring populations is reduced, because of reduced emigration (*i.e.* a higher degree of philopatry) and/or reduced immigration. This implies reduced gene flow between populations and may result in genetic differentiation between local populations and loss of genetic variation. The latter was found in squirrels and winter moths. Second, a change in the immigration/emigration balance may result in a net immigration deficit. In fact, we believe that this immigration deficit may be the main reason why squirrels, nuthatches and crested tits are less abundant in forest fragments compared to larger fragments or continuous forest. Such immigration deficit can in theory be caused by two processes: either a higher loss of dispersers, or unbalanced immigration and emigration, *e.g.* net immigration in large forests and net emigration out of fragments. We have no direct evidence for either of the two, but found some qualitative differences, notably in the timing of dispersal and in habitat selection, that may give an indication on which process is responsible. In the following sections we will briefly review the results for each species.

Squirrel

Squirrel populations in forest fragments are characterized by a lower abundance, lower per capita immigration rate and a higher local survival of juveniles, compared to two large forests. Higher local survival probably indicates higher philopatry rather than true survival. The result is an apparent reduction in gene flow, since genetic variation is lower in fragments, and highly correlated with immigration rate. The increase in local survival is not sufficient to compensate for the loss of immigrants, and this may be the most likely explanation for lower population densities (WAUTERS *et al.*, 1994a). Observations on a small number of radio-

equipped juveniles show that they do not disperse far, and avoid crossing open areas (WAUTERS *et al.*, 1994b). Reduced breeding synchrony in fragments may be partly due to increased variation in the time when females reach the optimal condition for reproduction, and partly to females re-entering oestrus more rapidly after early nest loss (WAUTERS *et al.*, unpubl.).

Winter Moth

Winter moth populations in different forest fragments are significantly different in genetic composition. Moreover, genetic variation was reduced in the most isolated populations. In 1991 adult population densities were lower in more isolated fragments (VAN DONGEN *et al.*, 1994) but this relationship was not reproduced in 1993 (unpubl. results). Adult moths weighed less in genetically less variable populations (VAN DONGEN *et al.*, 1994). We currently investigate the hypothesis that because of reduced genetic variation in fragments, winter moths are less synchronized with host trees and therefore suffer in reproductive output.

Nuthatch

Juvenile nuthatches disperse over relatively large distances (several kilometers) between fragments, and philopatry is not higher in fragments compared to continous habitat. Therefore we expect no difference in gene flow rates in fragmented or unfragmented populations. However, several lines of evidence suggest that dispersal is less efficient in and among fragments (MATTHYSEN *et al.*, 1995; MATTHYSEN and D. CURRIE, unpubl.). First, few first-year birds move to a different fragment after acquiring a territory, whereas territory shifts are common in a large forest. Second, new recruits arrive later in vacant territories in summer in the fragments, although potential recruits leave their parents at the same time in fragments and a large forest. Third, recruits seem less well able to select high-quality territories during their dispersal period, and fewer suitable territories are claimed. Less efficient dispersal and delayed recruitment may imply lower disperser survival, although we have no direct evidence for the latter. This in turn may explain the lower population density in fragments.

Crested Tit

All juvenile crested tits emigrated out of their natal areas, in fragments as well as a large forest. Hence we conclude, as in the previous species, that effects of fragmentation on gene flow are probably limited. However, juvenile crested tits delay their dispersal out of fragments compared to large forest plots. The reason may be a lower body mass at fledging, or a true barrier effect (reluctance to cross open spaces). Likewise, new recruits settle at a later date (on average) in fragments, but probably for a different reason. We suggest that fragments represent second-quality habitat, and therefore receive recruits that were unable to settle in large forests (LENS and DHONDT, 1994). Perhaps as a result, social winter groups were smaller in forest fragments and had larger group territories (LENS, 1994).

Great and Blue Tit

Preliminary results suggest that reproductive success is not lower in small forest fragments compared to larger fragments. Data on food provisioning at the nest similarly show no obvious differences in provisioning rate or diet composition between small and large fragments (NOUR and CURRIE, unpubl.). Although no quantitative analysis is as yet available, large numbers of recaptures of ringed birds suggest a high local recruitment rate in the fragments compared to larger forests, suggesting a high degree of philopatry. Gene flow between study areas is high but insufficient to prevent slight genetic differentiation as detected by minisatellite DNA single locus probes (VERHEYEN *et al.*, in prep.). In a previous study, DHONDT *et al.* (1990, 1991) found lower population densities and lower reproductive success in forest and park fragments around Ghent compared to study plots in and near a large forest in Antwerp. However, these results cannot be directly compared to the fragments in this study where nestbox density is lower.

DISCUSSION

The effects of habitat fragmentation – apart from the loss of suitable habitat – are manyfold and have been addressed in an increasing number of studies. Some effects are now well documented, *e.g.* changes in predation level (*e.g.* ANDRÉN and ANGELSTAM, 1988, NOUR *et al.*, 1993), increases in the number of edge species (RANNEY *et al.*, 1981, JANZEN, 1983) and disappearance of species with high area requirements (*e.g.* ASKINS *et al.*, 1987, BIERREGAARD and LOVEJOY, 1989). These effects can be characterized in general as changing aspects of habitat quality. In this respect we find surprisingly little effect on reproduction and survival.

Nevertheless we document lower population densities of most species in smaller or more isolated forest fragments. It is noteworthy that all of these species have been confronted with fragmentation of their habitat for the past centuries, if not longer. Despite the lack of effects on reproduction and survival, our results do indicate possible routes for demographic effects. Notably, the winter moth which is an important food source during the reproductive period of tits and nuthatches (PERRINS, 1991), is affected by fragmentation: abundance seems to be lower and is probably more variable in fragments, and male moths weigh less. As the latter reflects reduced larval growth, this means that less biomass is available at the higher trophic levels. We currently investigate to what extent the food situation for the birds is really affected by the fragmentation-sensitive population structure of the winter moth.

Another suite of effects of habitat fragmentation can be characterized as changes in population structure through changes in dispersal patterns. As fragmentation proceeds, dispersal from one habitat fragment to another becomes more difficult. Many studies, especially theoretical investigations, have addressed the threats to the small populations resulting from the fragmentation of formerly large populations. The basic idea is that local populations become separated so widely that their demography and genetic dynamics become more independent of one another, which

may eventually lead to local extinctions and/or loss of genetic variation. For instance, metapopulation theory (GILPIN and HANSKI, 1991) predicts that smaller and/or more isolated populations have a higher chance to go extinct without being recolonized in the future, leading to a lower equilibrium level of extant populations, or even extinction of the entire metapopulation.

In our studies we find evidence that populations of squirrels and winter moths (and to some degree, blue tits) are structured into local populations with limited gene flow between them. Whether and how this population structure explains lower abundances is less clear, however. In squirrels, lower genetic variation is not correlated with lower reproductive success. In winter moths few data on fitness are available, and the effect on fitness may be rather complex through reduced synchronization.

However, effects on dispersal patterns may be more complex than reduced gene flow rates. In nuthatches and crested tits the average dispersal distance, and therefore the exchange between local populations, remains large in fragmented habitats. Nevertheless, some qualitative changes in dispersal are apparent: timing, habitat selection, and the immigration/emigration balance. It seems possible that these qualitative changes are accompanied by, or result in, a higher mortality of dispersers. Such an effect can decrease the viability of the entire fragmented population without apparent changes in dispersal distances or gene flow. It is noteworthy that nuthatches and crested tits share a yearround-territorial social system which forces young birds to disperse early in search of vacancies (MATTHYSEN *et al.*, 1995, LENS and DHONDT, 1994). In the squirrel both effects may operate: reduced dispersal, but also lower success of individuals that disperse.

Changes in habitat quality may also interfere with the dispersal pattern. In the crested tit there is a suggestion that fragments are second-quality habitat and therefore receive fewer immigrants. This possibility seems less likely in the squirrel where dispersal distances are short anyway (WAUTERS *et al.*, 1994b).

In conclusion, we suggest that effects of habitat fragmentation can be a complex mixture of changes in habitat quality and changes in dispersal patterns. Habitat selection and disperser success may be key factors in determining the fitness of a population in highly fragmented habitat. Therefore dispersal needs to be studied in both its qualitative and quantitative aspects. Once the population structure of a species is affected, this can have far-reaching consequences on their interactions with other species. For instance, winter moths with insufficient genetic variation may be incapable of synchronizing with their host trees ; on the other hand, gene flow in tits may be too high to allow local adaptation to different food conditions (DHONDT *et al.*, 1990).

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REFERENCES

ANDRÉN, H. and P. ANGELSTAM (1988) — Elevated predation rates as an edge effect in habitat islands: experimental evidence. *Ecology,* **69** : 544-547.

ASKINS, R.A., M.J. PHILBRICK and D.S. SUGENO (1987) — Relationship between the regional abundance of forest and the composition of forest bird communities. *Biol. Conserv.,* **39** : 129-152.

BIERREGAARD, R.O. and T.E. LOVEJOY (1989) — Effects of forest fragmentation on Amazonian understory bird communities. *Acta Amazonica,* **19** : 215-241.

DHONDT, A.A., F. ADRIAENSEN, E. MATTHYSEN and B. KEMPENAERS (1990) — Nonadaptive clutch sizes in tits. *Nature,* **348** : 723-725.

DHONDT, A.A., B. KEMPENAERS and J. DE LAET (1991) — Protected winter roosting sites as a limiting resource for blue tits. *Proc. XX Int. Ornith. Congr.,* 1436-1443.

GILPIN, M. and I. HANSKI (1991) — *Metapopulation dynamics : empirical and theoretical investigations.* Academic Press, London : 336 pp.

IMS, R.A., J. ROLSTAD and P. WEGGE (1993) — Predicting space use responses to habitat fragmentation: can voles *Microtus oeconomus* serve as an experimental model system (EMS) for capercaillie grouse *Tetrao urogallus* in boreal forest? *Biol. Conserv.,* **63** : 261-268.

JANZEN, D.H. (1983) — No park is an island: increase in interference from outside as park size decreases. *Oikos,* **41** : 402-410.

LENS, L. (1994) — *Factors influencing crested tit Parus cristatus social organization.* Unpublished Ph. D. thesis, University of Antwerp : 178 pp.

LENS, L. and A.A. DHONDT (1994) — Effects of habitat fragmentation on the timing of Crested Tit Parus cristatus natal dispersal. *Ibis,* **136** : 147-152.

LOVEJOY, T.E., R.O. BIERREGAARD, A.B. RYLANDS, J.R. MALCOLM, C.E. QUINTELA, L.H. HARPER, K.S. BROWN, A.H. POWELL, G.V.N. POWELL, H.O.R. SCHUBART and M.B. HAYS (1986) — Edge and other effects of isolation on Amazon forest fragments. In : SOULÉ, M.E. (ed.), *Conservation Biology : the science of scarcity and diversity.* Sinauer Associates, Sunderland, Mass. : 257-285.

MARGULES, C.R. (1992) — The Wog-Wog habitat fragmentation experiment. *Envir. Conserv.,* **19** : 316-325.

MATTHYSEN, E., F. ADRIAENSEN and A.A. DHONDT (1995) — Dispersal distances of Nuthatches *Sitta europaea* in a highly fragmented landscape. *Oikos* , **72** : 375-381.

NOUR, N., E. MATTHYSEN and A.A. DHONDT (1993) — Artificial nest predation and habitat fragmentation: different trends in bird and mammal predators. *Ecography,* **16** : 111-116.

OPDAM, P. (1991) — Metapopulation theory and habitat fragmentation: a review of holarctic breeding bird studies. *Landsc. Ecol.,* **5** : 93-106.

PERRINS, C.M. (1991) — Tits and their caterpillar food supply. *Ibis,* **133 (Suppl. 1)** : 49-54.

RANNEY, J.W., M.C. BRUNER and J.B. LEVENSON (1981) — The importance of edge in the structure and dynamics of forest islands. In: BURGESS R.L. and D.M. SHARPE (eds.), *Forest island dynamics in man-dominated landscapes,* Springer, New York : 67-95.

ROBINSON, G.R., R.D. HOLT, M.S. GAINES, S.P. HAMBURG, M.L. JOHNSON, H.S. FITCH and E.A. MARTINKO (1992) — Diverse and contrasting effects of habitat fragmentation. *Science,* **257** : 524-526.

SAUNDERS, D.A., R.J. HOBBS and C.R. MARGULES (1991) — Biological consequences of ecosystem fragmentation — a review. *Conserv. Biol.,* **5** : 18-32.

SOULÉ, M.E. (1986) — *Conservation biology: the science of scarcity and diversity.* Sinauer Associates, Sunderland, Mass : 584 pp.

VAN DONGEN, S., T. BACKELJAU, E. MATTHYSEN and A.A. DHONDT (1994) — Effects of habitat fragmentation on population structure of the winter moth. *Acta Oecol.,* **15** : 193-206.

WAUTERS, L.A., Y. HUTCHINSON, T.D. PARKIN and A.A. DHONDT (1994a) — The effects of habitat fragmentation on demography and the loss of genetic variation in the red squirrel. *Proc. Roy. Soc. Lond. B,* **255** : 107-111.

WAUTERS, L.A., P. CASALE and A.A. DHONDT (1994b) — Space use and dispersal of red squirrels in fragmented habitat. *Oikos,* **69** : 140-146.

WILCOX, B.A. and D.D. MURPHY (1985) — Conservation strategy. the effects of fragmentation on extinction. *Am. Nat.,* **125** : 879-887.

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LINDANE RAISES CATFISH ELECTRODETECTION THRESHOLD

by

ROBERT C. PETERS (1), WIM J.G. LOOS (1), MARGREET HOBBELEN (1), FRANKLIN BRETSCHNEIDER (1), and JOOP L.M. HERMENS (2)
Utrecht University,
(1) Department of Comparative Physiology, Padualaan 8, 3584 CH
(2) RITOX, Yalelaan 2, 3508 TD
NL-Utrecht, The Netherlands

SUMMARY

The detection threshold of the electric sense in catfish, *Ictalurus nebulosus*, was assessed psychophysically in order to investigate the effects of the organic toxicant lindane. In tapwater the detection threshold for ac-stimuli of 1 Hz at 18° C was 8.5 μV/cm. In 0.1 % DMSO in tapwater the threshold was 14 μV/cm. In 50 μg/l lindane dissolved in 0.1 % DMSO in tapwater the threshold went up to 1000 μV/cm. After clearing, the threshold returned to 36 μV/cm. The results demonstrate that catfish ampullary electroreceptors are susceptible to exposure to sublethal doses of organic pesticides. Since ampullary electroreceptor organs are known to respond also to heavy metal pollution, the electric sense seems a promising system to monitor the cumulative pollution of aquatic habitats.

Keywords : Electroreception, electroreceptor organs, lindane, conditioning, psychophysics, behaviour, pollution, BEWS, biomonitor, *Ictalurus nebulosus*.

INTRODUCTION

Ampullary electroreceptor organs of the freswater catfish *Ictalurus nebulosus* are cutaneous sense organs, the receptor cells of which are directly exposed to the aquatic environment. Toxicants administered via the water reach the receptor cells within minutes or seconds. (BRETSCHNEIDER *et al.*, 1979 ; KRAMER, 1984 ; PETERS *et al.*, 1989, 1991 ; ROTH, 1971 ; ZHADAN and ZHADAN, 1975 ; ZWART, 1988). Earlier it has been demonstrated that functioning of electroreceptor organs depends to a great extent on the ionic composition of the aquatic environment. The presence of for instance Cu-ions in a concentration of 1 ppb or less causes a ten-fold increase in the detection threshold of the electric sense (NEUMAN *et al.*, 1991). Previously there were no data available on the effects of organic toxicants on ampullary electroreceptor organs. We performed the following experiment to investigate whether the electric sense of catfish is susceptible to an organic toxicant like lindane.

MATERIALS AND METHODS

Animals

Two catfish, *Ictalurus nebulosus,* with lengths of about 21 cm were kept in glass tanks, dimensions, 100 × 30 cm, water height 10 cm during the course of the experiment. Initially the tanks were filled with copper-free tap water, conductivity 0.22 mS/cm (4.5 kOhm.cm) and pH = 8.2 at 18° C. The conductivity increased to 0.27 mS/cm in the course of one week, due to excretion by the fish, and to feeding. The fish were subjected to a 12 h light, 12 h dark regime, and tested during the dark periods which corresponds to their normal activity pattern. Once a week the water was refreshed. The temperature of the water varied less than 2° C during the measurements. Food, minced beef with gelatin and agar-agar, was administered during the tests through peristaltic pumps at either end of the tank. Food was used as a positive reinforcer (reward).

Protocol

The complete experiment consisted of collecting threshold curves at a temperature of 18° C during a period of 7 weeks. The detection thresholds were measured at a frequency of 1 Hz, twice per night. Each single threshold session consisted of 100 trials. During week 1 control values of the sensitivity limit were assessed. In week 2 the fish were exposed to 0.1 % DMSO in order to test if the solvent itself affects electroreceptor functioning. Control thresholds were measured in clear tap water again in week 3. In week 4 the fish were taken from the tank, and lindane was added to a final concentration of 50 µg/l to test the rate of change of concentration of lindane during a week. During this week water samples were taken every 24 hrs after administration, and analysed by means of gas chromatography. The analyses were carried out on a Carlo Erba type Mega 5360 GC equipped with a ECD400 detector, split injector and a 15 m capillary fused silica column with 0.25 mm DB-5 stationary phase. The fish were put in the tank again in week 5 to give them the opportunity to regain a stable performance. 50 µg/l Lindane in 0.1 % DMSO was added to the tank water in week 6. Lindane loaded water was replaced by clear water again and control threshold values were measured in clear water in week 7. The concentration of 50 µg/l lindane was chosen because it is the LC50 at 4 days exposure for *Ictalurus melas* (JOHNSON and FINLEY, 1980). Lindane concentrations in the river Rhine amounted to 0.01 - 0.4 µg/l from 1969 to 1974 (HERBST, 1991).

Stimulation, training, and psychophysics

The stimulation procedure and statistics of the psychophysical approach are described in detail elsewhere (NEUMAN *et al.*, 1991 ; PETERS *et al.*, 1995).

RESULTS

The average concentration of lindane during the pretrial period in week 4, and the test period in week 6 is shown in Fig. 1. The concentration changes drastically in both cases, but remains stable enough from day 1 to 5 to to fulfill the requirements of this experiment. After day 5 the concentration falls to about 10 % of the initial value.

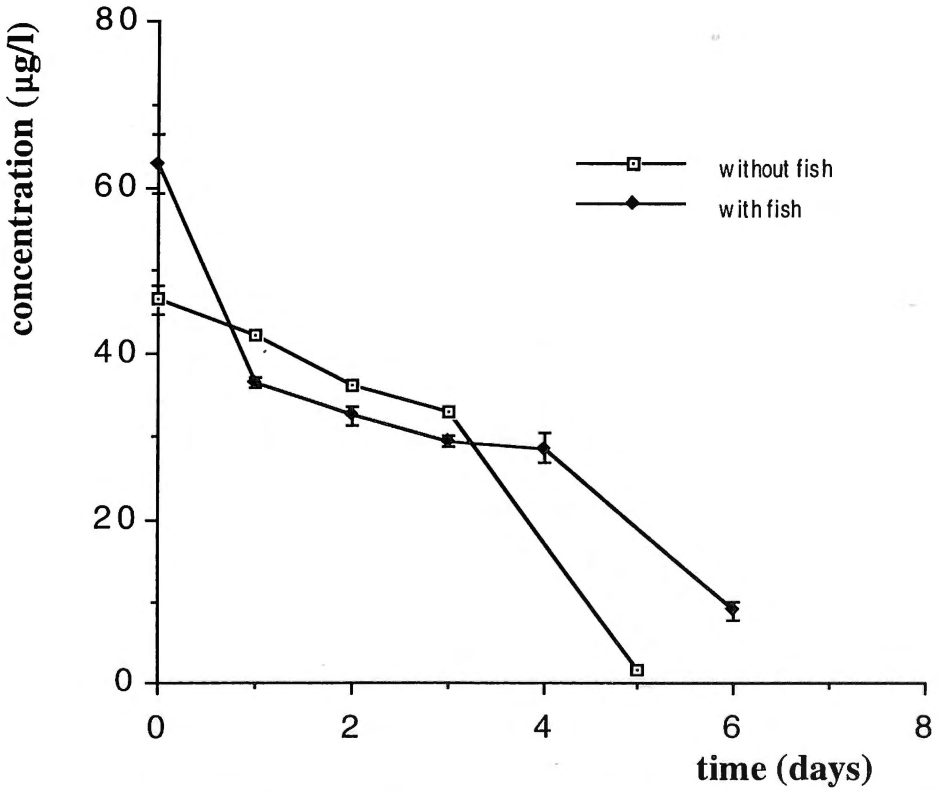

Fig. 1. — Concentration of Lindane in absence of fish during week 4, and in the presence of fish during week 6.

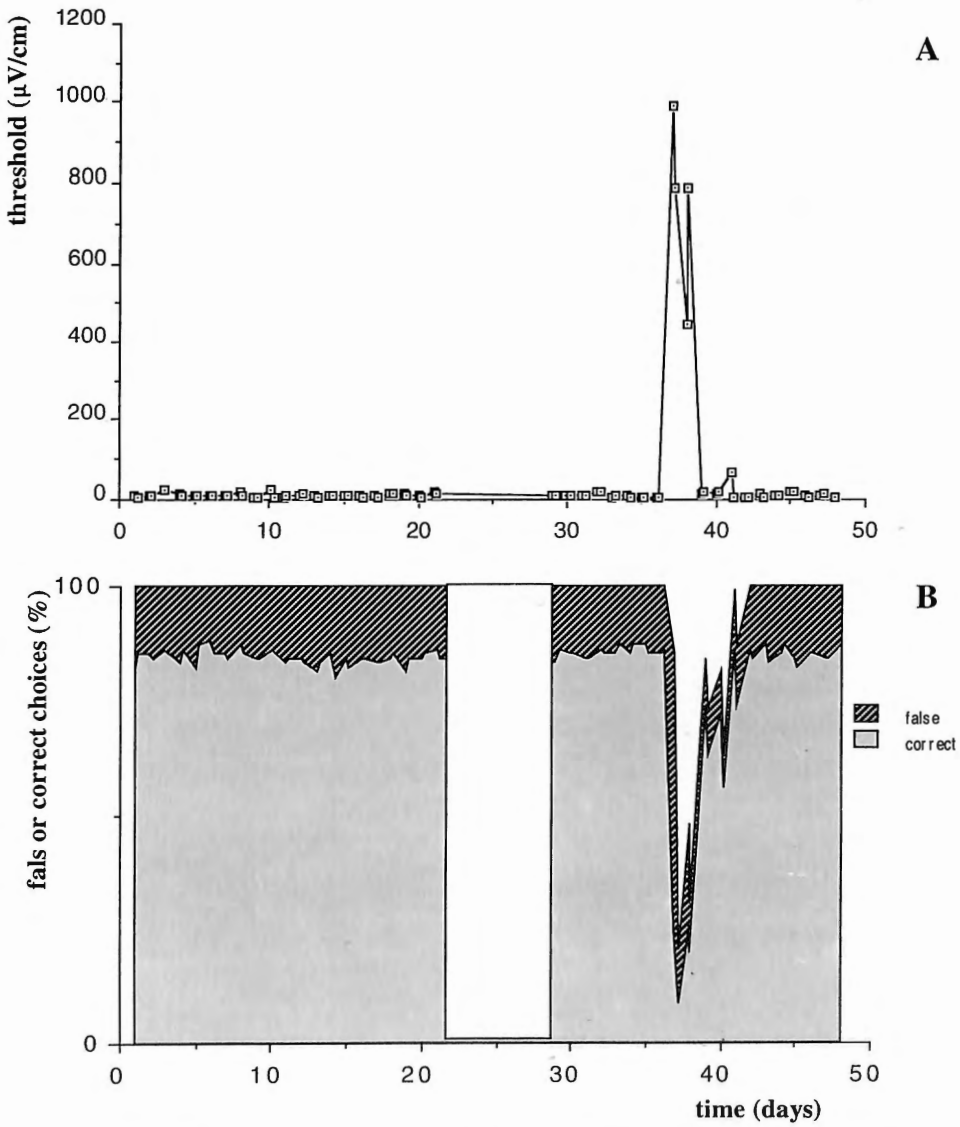

Fig. 2. — Threshold and performance of fish #1. a. Detection threshold in µV/cm, at 18° C and 0.22 mS/cm. In week 4 the fish was taken out of the tank in order to test the stability of the (50 µg/l) lindane concentration in 0.1 % DMSO test solution. The fish was exposed to the test solution in week 6; b. Percentage correct choices, false choices, and nogos. During application of the test solution the number of nogos increases dramatically, but the ratio between correct and false choices shows that the fish still performs at threshold level.

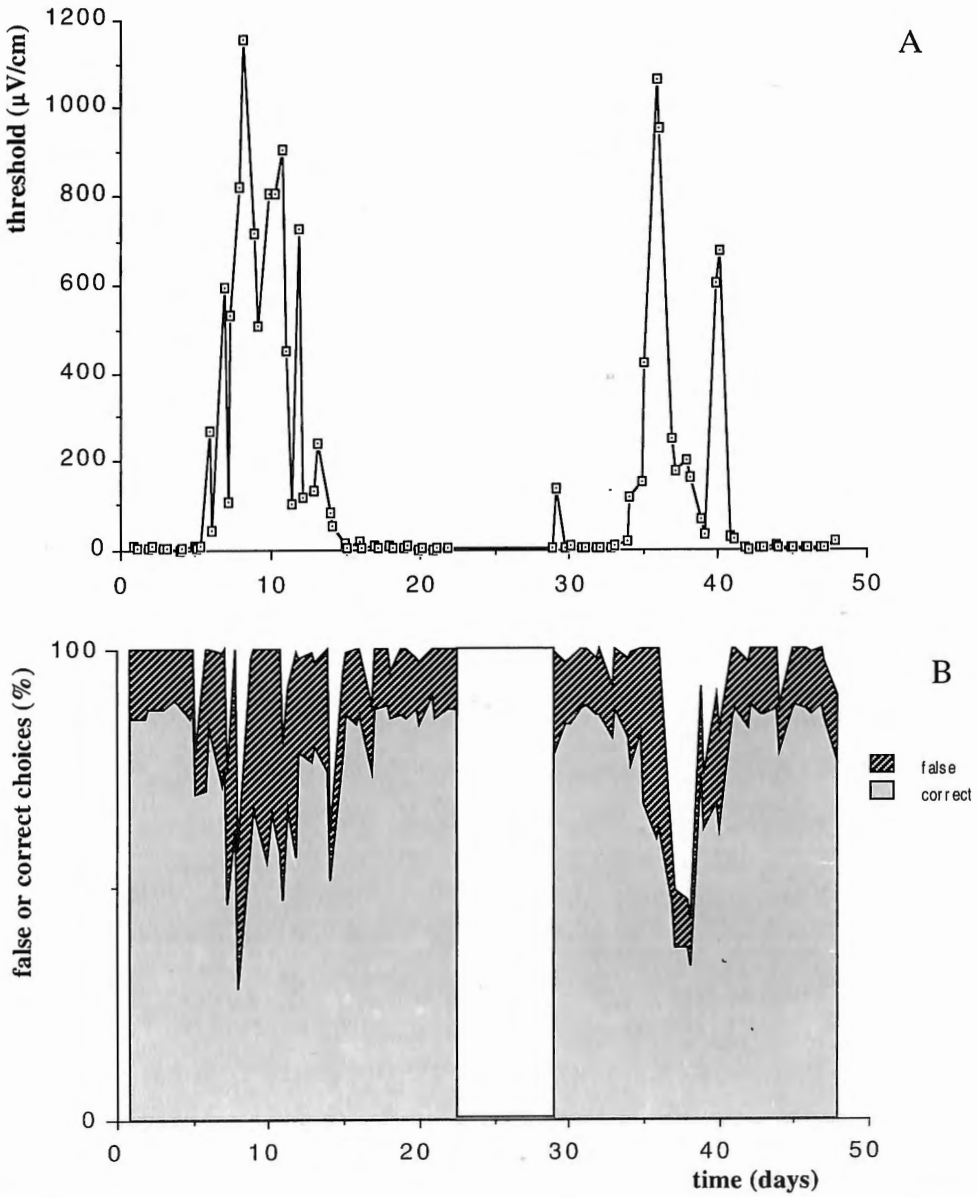

Fig 3. — Same as Fig. 2 for fish #2. On the whole this particular specimen performed less stably than fish 1 (cf. Fig. 2).

Figs 2 and 3 present the electrodetection thresholds and the percentages of correct choices, false choices and nogos in the course of the experiment. One of the fish (Fig. 2) performed extremely well, whereas the other (Fig. 3) performed rather variably. In the well performing specimen the mean theshold remained at 8.5, 14, 14, and 8.5 μV/cm respectively in the first four weeks, whereas only during the test period, week 6, the threshold went up to a value of about 1000 μV/cm. Afterwards the threshold returned to 36 μV/cm again. The percentage of correct choices remained constant at 85 % throughout the experiment, and only during the test period did the number of nogos increased temporarily. The other specimen had more difficulties in performing reliably. Already in the first week the threshold started to climb and resulted in an average value of 272 μV/cm. Also a relatively large number of nogos occurred, even before DMSO or lindane was administered to the water. During DMSO administration the threshold went up to 466 μV/cm, and returned to 24.5 μV/cm in week 3. On the other hand, during weeks five, six and seven, the response pattern strongly resembled that of its companion, although the rise in threshold began before lindane was added. In weeks 5, 6, and 7 the mean threshold values were 8.5, 507, and 36 μV/cm respectively. There is a response to lindane, but the general level of performance is less stable. It is striking that in the best performing subject (Fig. 2) the threshold and the performance in general is not affected by the relatively high concentration of DMSO (0.1 %).

DISCUSSION

The concentration measurements convinced us that the actual concentration of lindane during the experiments was sufficiently constant to allow conclusions about possible effects of the pollutant on electroreceptive behaviour. As for the performances of the two fish we have to be more careful. The best performing fish (Fig. 2) prompts the conclusion that DMSO has no influence on the electroreceptive abilities, whereas lindane has a drastic effect. The data of the other specimen support this finding to a certain extent, but the overall variability makes the results less conclusive. On the other hand, earlier psychophysical experiments on the electrosensitivity of catfish have also shown that sometimes, without any apparent cause, the threshold can increase spontaneously.

We conclude from this pilot experiment that the electric sense of catfish is not only susceptible to heavy metals (NEUMAN et al., 1991) but also to an organic toxicant : lindane. It is not inconceivable that electroreception will also suffer from organic compounds other than lindane. Since it has been shown that ampullary electroreceptors are also sensitive to the composition of the water, we propose that the electric sense of catfish can serve as a biomonitor system which responds to sublethal doses of toxicants. Because there is a strong physiological resemblance between the electric sense and the lateral line system, we expect that the susceptibility of the electric sense is representative for the sensitivity of the lateral line system as well. We think that the sensory systems in the integument of aquatic organisms are well suited to monitor the quality of environmental water. Elec-

troreceptor organs are particularly attractive because the stimulus, an electric current, can be very easily and reliably applied.

REFERENCES

BRETSCHNEIDER, F., G. KROESBERGEN and F.B. BEIJNINK (1979) — Functioning of catfish electroreceptors : relation between skin potential and receptor activity. *J. Physiol. Paris,* **75** : 343-347.

HERBST, M. (1991) — *Environmental health criteria 124 : lindane.* HWO, Geneve.

JOHNSON, W.W. and M.T. FINLEY (1980) — *Handbook of acute toxicity of chemicals to fish and aquatic invertebrates.* Resourc. Publ. 137, Fish Wildl.Serv., U.S.D.I., Washington.

KRAMER, B. (1984) — Tranquilizer reduces electric organ discharge frequency in a teleost fish. *Naturwiss.,* **71** : 99.

NEUMAN, I.S.A., C. VAN ROSSUM, F. BRETSCHNEIDER, P.F.M. TEUNIS and R.C. PETERS (1991) — Biomonitoring : cadmium deteriorates electro-orientation in catfish. *Comp. Biochem. Physiol.,* **100**C : 259-262.

PETERS, R.C., F. BRETSCHNEIDER, W.J.G. LOOS and I.S.A. NEUMAN (1991) — Electro-reception and aquatic biomonitoring. In : *Bioindicators and Environmental Management.* JEFFREY, D.W. and B. MADDEN (eds.). Academic Press, London : 427-433.

PETERS, R.C., W.J.G. LOOS, F. BRETSCHNEIDER and P.S. HEIJMEN (1995) — Psychophysical assessment of catfish electrosensitivity as a function of temperature and frequency. *Neth. J. Zool.* (in press).

PETERS, R.C., R. ZWART, W.J.G. LOOS and F. BRETSCHNEIDER (1989) — Transduction at electro-receptor cells manipulated by exposure of apical membranes to ionic channel blockers. *Comp. Biochem. Physiol.,* **94**C, 663-669.

ROTH, A. (1971) — Zur Funktionsweise der Elektrorezeptoren in der Haut von Welsen (*Ictalurus*) : Der Einfluss der Ionen im Suesswasser. *Z. vergl. Physiol..* **75,** 303-323.

ZHADAN, G.G. and P.M. ZHADAN (1975) — Effect of sodium, potassium and calcium ions on electroreceptor function in catfish. *Neurophysiol. Kiew,* **7** : 403-410.

ZWART, R. (1988) — Electrophysiological changes and histochemical demonstration of intracellular cadmium in catfish electroreceptors after exposure to cadmium. *Neth. J. Zool.,* **38** : 215.

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PREVAILING INDOOR CLIMATE CLASSIFICATION TO PREDICT HOUSE DUST MITE ABUNDANCE IN DUTCH HOMES

by

GERD SCHOBER, IVONNE VERSTAPPEN, MARIËLLE C.L. SNIJDERS and JOHANNA E.M.H. VAN BRONSWIJK
Interuniversity Task Group 'Home and Health',
Center for Biomedical & Health-care Technology,
Eindhoven University of Technology,
P.O.Box 513, NL-5600 MB Eindhoven, The Netherlands.

SUMMARY

In Europe 10-15 % of the human population is sensitized to allergens of house dust mites (Pyroglyphidae). Population development of house dust mites is primarily influenced by water activity (a_w) of the mite habitat. The availability of H_2O (water-activity and relative humidity) in the niches of mites may correlate with the absolute humidity of the room air. Absolute humidity also plays a role in technical indoor climate classifications of Dutch and Belgian buildings. To investigate the effects of dry and humid room conditions on mite abundance, dust samples were taken in 14 living-rooms and bedrooms in the Netherlands. Mite numbers in floor and furniture dust from dry rooms (class II) did not exceed allergologically relevant no-sensitization thresholds of 10 mites/g of floor dust and 100 mites/g of dust from furniture. Climate classes might be different in the various spaces of a dwelling. Assessment of the indoor climate class should be done in the bedrooms as well as in the living-rooms. Results suggest that the technical classification of indoor climate is useful for future building design or when managing home sanitation.

Key words : indoor climate classification, mite abundance, living-room, bedroom, building design, home sanitation.

INTRODUCTION

In Europe 10-15 % of the human population is sensitized to allergens of house dust mites (KORT, 1994). These allergens can provok clinical symptoms such as asthma, rhinitis, bronchitis or atopic dermatitis. Significant correlations between house dust mite exposition or allergen content of dust and clinical symptoms have been demonstrated. Provisional no-sensitization thresholds for house dust mites or allergen exposure have been formulated (VAN BRONSWIJK, 1988 ; PLATTS-MILLS *et al.*, 1985 ; KORSGAARD, 1983). Exposure levels above these values (*e.g.* 10 mites/g of

floor dust or 100 mites/g of furniture/mattress dust) significantly increase the disease risk for developing mite asthma.

Development of house dust mites is primarily influenced by water-activity (a_w) of the mite habitat. By measuring the relative air humidity adjacent to a substrate the water-activity (a_w) of a substrate may be assessed. The availability of water (water-activity and relative humidity) in the micro-niches of mites may correlate with the absolute humidity of the room air.

Absolute humidity also plays a role in technical indoor climate classifications of Dutch and Belgian buildings (TAMMES and VOS, 1984 ; VAN HEES, 1986 ; PONCELET and HENS, 1985 ; SCHOBER, 1989), consisting of 4 classes, representing very dry spaces (class I) to very humid spaces (class IV). This classification is based upon water vapour pressure differences between indoor and outdoor environment in relation to outdoor temperature.

In this study, indoor climate classification has been investigated in relation to mite abundance in house dust samples.

MATERIAL AND METHODS

Preliminary field trials were performed in the Netherlands in the surroundings of the cities of Eindhoven, Enkhuizen, Utrecht and Zeist. Dust samples from floors and furniture of living-rooms as well as from floors of bedrooms were collected by a household vacuumcleaner (Hoover S 2222, power : 700 W, with a small nozzle type A). The carpeting and furniture of the rooms were sampled twice (with a time interval of 1 month) for 1 min/m^2. Mite analysis of the dust samples occurred in the laboratory using a flotation method (VAN BRONSWIJK, 1981). In the living-rooms indoor climate was registrated by thermohygrographs (type 252 Ua and type 253 W. Lambrecht, Göttingen, Germany). No climate registrations were performed in the bedrooms since in the tradition of building technology the climate class of the living-room is valid for the entire house. Outdoor climatic measurements at the nearest weather station were obtained from the Royal Dutch Meteorologic Institute (KNMI, De Bilt, The Netherlands). The readings of a 4-5 week period were used to calculate mean absolute air humidity values (in g water/m^3 air) and the partial water vapour pressure (Pa). From these values technical indoor climate classes of each of 14 living-rooms were calculated from the 4-5 week average of differences in vapour pressure indoor and outdoor, in relation to outdoor temperatures. Both « Dutch calculations » (VAN HEES, 1986) and « Belgian calculations » (PONCELET and HENS, 1985) were performed. The classification systems for Dutch and Belgian buildings evolved independently. When comparing both systems they show the same classification for the outdoor air temperature range from 0 to 20 degrees C (representative outdoor air temperatures for both countries).

RESULTS

The results of mite abundance and climate registrations are presented in Tables 1 and 2.

Table 1 presents the results of climate classification and geometric means of number of pyroglyphid mites/g of dust from floors and furniture of living-rooms. Mite numbers in floor and furniture dust samples from dry living-rooms do not exceed the generally accepted no-sensitization thresholds of 10 mites/g dust for floors and 100 mites/g dust for furniture. This coincides with the limit between indoor climate class II and III in the Dutch as well as the Belgian classification system with one possible exception. On the other hand living-rooms with a climate class of IV are always mite ridden, while those of class III show noxious concentrations with again one possible exception.

TABLE 1

Technical Dutch and Belgian indoor climate classes and geometric means of numbers of pyroglyphid mites/g of dust in floor dust and furniture dust of 14 living-rooms.

Climate class (*) Dutch	Climate class (*) Belgian	Home number	Mites/g of floor dust	Mites/g of furniture dust	Exceeding no-sensit. threshold
II	II	1	11	71	yes/no
II	II	3	1	30	no
II	II	4	2	8	no
II	II	5	9	58	no
II	II	6	1	39	no
II	I-II	8	5	33	no
II	II	14	— (**)	8	no
III	III	10	146	16	yes/no
III	III	12	83	130	yes
III	III	13	— (**)	— (**)	—
IV	IV	2	63	122	yes
IV	IV	7	41	132	yes
IV	IV	9	25	127	yes
IV	IV	11	42	94	yes

no-sensitization thresholds : floor dust = 10 mites/g dust
furniture dust = 100 mites/g dust

(*) I = very dry rooms
II = dry rooms without water vapour production in the rooms
III = humid rooms with moderate water vapour production
IV = humid rooms with high water vapour production and moisture problems

(**) = no textile floors / no textile covered furniture

TABLE 2

Geometric means of numbers of pyroglyphid mites in floor dust of 14 bedrooms and technical Dutch indoor climate classes.

Mites/g of floor dust	Home number	Climate class living-room Dutch	Climate class living-room Belgian	Expected climate class bedroom (*) Dutch & Belgian
0	6	II	II	II
16	5	II	II	III-IV
20	11	IV	IV	III-IV
25	9	IV	IV	III-IV
32	8	II	I-II	III-IV
40	10	III	III	III-IV
43	13	III	III	III-IV
50	1	II	II	III-IV
63	2	IV	IV	III-IV
79	3	II	II	III-IV
158	4	II	II	III-IV
251	7	IV	IV	III-IV
—	12 (**)	III	III	—
—	14 (**)	II	II	—

no-sensitization threshold = 10 mites/g gust

(*) I = very dry rooms
II = dry rooms without water vapour production in the rooms
III = humid rooms with moderate water vapour production
IV = humid rooms with high water vapour production and moisture problems

(**) = no textile floors covering

Table 2 presents the results of the bedroom samples. Almost all mite numbers exceed the no-sensitization threshold. However, no climate registrations took place in these bedrooms. The table includes climate classifications of the living-rooms. In a separate column expected climate classifications of bedrooms according to mite numbers are included. In general bedrooms proved to be more 'humid' than living-room measurements indicated.

DISCUSSION

To reduce mite exposure in the homes of mite allergic patients thresholds have been formulated as regards survival and population development of house dust mites. For survival in a dry climate *Dermatophagoides* species require a minimum

relative humidity of 45 % at 20° C room temperature (VAN BRONSWIJK, 1981). Egg production starts when relative humidity exceeds 60 % (LARSON, 1969). Population growth then initiates an increasing allergen production with maximum levels at 75 to 85 % relative humidity (WHARTON, 1976).

Our results indicate that rooms in Dutch buildings with a low mite exposition for (mite) allergic patients should belong to climate class II of either the Belgian or the Dutch system. These rooms should be considered 'safe' rooms for the allergic patient.

Mite numbers in almost all bedrooms exceeded the no-sensitization threshold. When comparing the mite results of the living-room samples with those of the bedrooms we concluded that most bedrooms are more humid than the living-rooms of the same house. Usually Dutch bedrooms are only poorly heated during winter. Climate classes may be different in the various spaces of a building. For that reason a classification of the indoor climate of a dwelling should include at least measurements in the living-room and in the bedrooms.

From Table 1 it can be concluded that high indoor air humidity (climate class IV) does not necessarily result in highest mite numbers since excessive humidities of the indoor air are not in favour of house dust mites growth. Other aspects, besides humidity, such as the quality of a substrate could become important when interpreting results. Recently the protein content of floor dust has been found to be a relevant parameter for mite development (KOREN and ECKHARDT, personal communication).

In conclusion the results of our preliminary field trials suggest that the technical classification of the indoor climate is useful in future building design, to construct dry buildings, or when managing home sanitation to reduce house dust mite exposition in the homes of allergic patients.

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REFERENCES

BRONSWIJK, J.E.M.H. VAN (1981) — *House dust biology for allergists, acarologists and mycologists.* NIB Publishers Zeist : 316 pp.

BRONSWIJK, J.E.M.H. VAN (1988) — Allergie gegen Hausstaubmilben. *Sozialpädiatrie in Praxis und Klinik,* **10** : 876-882.

HEES, R.P.J. VAN (1986) — *Vochtproblemen in bestaande woningen.* Stichting Bouwresearch, Rotterdam : 185 pp.

KORSGAARD, J. (1983) — Preventive measures in mite asthma. A controlled trial. *Allergy,* **38** : 93-102.

KORT, H.S.M. (1994) — A structured approach to allergen avoidance in dwellings with special emphasis on the ecosystem of humid indoor walls and room partitions. Thesis Technical University Eindhoven : 115 pp.

LARSON, D.G. (1969) — The critical equilibrium activity of adult females of the house dust mite Dermatophagoides farinae (Hughes). Thesis Ohio State University : 35.

PLATTS-MILLS, T.A.E., P.W. HEYMAN, M.D. CHAPMAN, T.F. SMITH and S.R. WILKINS (1985) — Mites of the genus Dermatophagoides from the houses of asthmatic and other allergic patients in North America : development of a radioimmunoassay for allergen produced by D. farinae and/or D. pteronyssinus. *International Archives of Allergy and Applied Immunology,* **77** : 163-165.

PONCELET, P. and H. HENS (1985) — Inside climate in dwellings ; a statistical evaluation. (IEA 14, Workshop on Condensation Problems), Leuven : 24 pp.

SCHOBER, G. (1989) — Absolute indoor air humidity and the abundance of allergen producing house dust mites and fungi in the Netherlands. In : C.J. Bieva, Y. Courtois and M. Govaerts (Eds.). Proceedings : Present and future of indoor air quality. *Excerpta Medica,* Amsterdam : 363-370.

TAMMES, E. and B.H. VOS (1984) — *Warmte- en vochttransport in bouwconstructies.* Kluwer, Deventer/Antwerpen : 419 pp.

WHARTON, G.W. (1976) — House dust mites. *Journal of Medical Entomology,* **12** : 577-621.

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POPULATION DYNAMICS OF CEREAL LEAF BEETLES, *OULEMA MELANOPUS* L. AND *O. LICHENIS* VOET (*COLEOPTERA : CHRYSOMELIDAE*), ON WHEAT FIELDS IN SOUTHERN BELGIUM

by

DIDIER STILMANT
DEPARTMENT OF ECOLOGICAL AND BIOGEOGRAPHICAL SCIENCES,
CATHOLIC UNIVERSITY OF LOUVAIN, 4-5 PLACE CROIX-DU-SUD,
B-1348 LOUVAIN-LA-NEUVE (BELGIUM)

SUMMARY

In order to determine the pest status of cereal leaf beetles in Southern Belgium, the population densities of *Oulema melanopus* (L., 1758) and *O. lichenis* (Voet, 1806) were studied at Ciney and Louvain-la-Neuve during 1993 and 1994. During the two-year study period *Oulema* spp. populations had never reached the economic threshold of 2.5 larvae per tiller. The levels of the *Oulema* populations were significantly different between the 2 sites in 1994 ; Louvain-la-Neuve supporting greater densities than Ciney. Phenological differences between the two locations were postulated to be the result of two phenomena : (1) the early attainment of the physiological time of development, expressed in degree days, necessary for immature stage development, in Louvain-la-Neuve, the warmest location, and (2) the early attainment of the cold sum necessary to end the adult diapause in Ciney, the coldest site, however this second argument stays as a hypothesis that must be tested.

Keywords : Oulema melanopus, Oulema lichenis, population dynamics, winter wheat pests.

INTRODUCTION

Oulema melanopus (L., 1758) is distributed in the whole Palearctic region (BALACHOWSKY and MESNIL, 1935) and in 1962 it was accidentally introduced into Michigan. From there it spread rapidly from an area ranging from Pennsylvania to Winsconsin and from Kentucky to Michigan and Ontario (GALLUN *et al.*, 1966 ; TUMMALA *et al.*, 1975). *O. lichenis* (Voet, 1806) is more common in Europe. The color of the pronutum, red for the former and metal blue for the latter, makes it possible to distinguish them easily (BALACHOWSKY and MESNIL, 1935).

They both feed on small grains, with a preference for oat and barley *versus* wheat by *O. melanopus* and wheat *versus* barley by *O. lichenis*. However, only the

first species has the reputation of being an economic pest, mainly in continental-type climate areas, where a short spring is followed by a dry summer (BALACHOWSKY and MESNIL, 1935). In some years, estimated reductions of grain yield as high as 70 % were reported in Central Europe (KNECHTEL and MANOLACHE, in WILSON *et al.*, 1969) and in Michigan (GUYER, in GALLUN *et al.*, 1966). Little is known about their density and there is no mention if these species reach the economic threshold in Belgium. The economic threshold level is 2.5 larvae per tiller, in the Paris Basin (Anonymous, 1982), and 0.5 larvae per tiller, in Switzerland (HAUSAMMAN, personnal communication), at the wheat growth stage 50 to 59 on the Zadoks scale (ZADOKS *et al.*, 1974). The lower threshold in the second area is due to climatic conditions more favorable to the pest, in the more continental-type climate. Therefore the purpose of this work was to analyse the phenology and the abundance of these species in two different biogeographical areas in Southern Belgium.

MATERIAL AND METHODS

This study was conducted during 1993 and 1994, on two sites in the southern part of Belgium, Ciney, in the Condroz region, and Louvain-la-Neuve, in Brabant-Wallon. During the first year two fields of winter wheat, cultivar Estica (field C 1) and cultivar Torino (field C 2), at Ciney and one winter wheat field, cultivar Estica (field L 2), and one spring wheat (field L 1) at Louvain-la-Neuve, were sampled. In 1994, three winter wheat fields, with cultivars Sideral (field C 1), Clan (field C 2) and Estica (field C 3) at Ciney and Clan (fields L 1 and L 3) and Estica (field L 2) at Louvain-la-Neuve, were sampled.

The number of *Oulema* spp. adults, eggs, larvae and *O. lichenis* pupae were counted weekly, from the beginning of May till the end of July, *in situ*, on 100 tillers taken on two transects. The *O. melanopus* pupae formed in the ground were not sampled.

The means of the number of eggs, larvae and pupae were compared by using ANOVA2 — fixed model after a log (x+1) transformation.

RESULTS

During the two-year study, *Oulema spp.* populations never reached the economic threshold of 2.5 larvae per tiller at the stage 50 to 59 on the Zadoks scale (Anonymous, 1982). The maximum observed was 0.2 larvae per tiller per week in 1994 in the fields L 1, L 2 and C 3.

In 1993, the levels of the pest population were not significantly different between the two sites ($F_{(eggs)}(1,12) = 1,62$ NS ; $F_{(larvae)}(1,13) = 0,98$ NS). However,

in 1994, the incidence of *Oulema* populations was more important in Louvain-la-Neuve than in Ciney ($F_{(eggs)}(1,28) = 12,15^{***}$; $F_{(larvae)}(1,28) = 12,80^{***}$, $F_{(pupae)}(1,20) = 21,08^{***}$).

The major differences in the phenology of these species occurred between fields of winter wheat (Fig. 2) and spring wheat (Fig. 1), but these results must be considered with care as only one field of each kind was taken into account. On spring wheat the maximum oviposition was recorded, at growth stages 54-55, three weeks later than on winter wheat, at growth stage 39. Likewise, the maximum larval incidence on spring wheat occurred, at growth stage 55-65, one to two weeks later than on winter wheat, at growth stage 54-55.

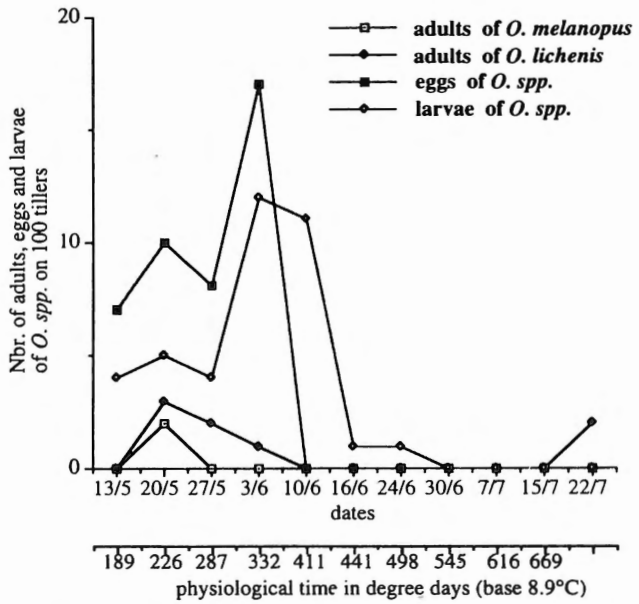

Fig. 1. — Phenology of *O. melanopus* and *O. lichenis* on spring wheat, Louvain-la-Neuve, 1993.

Small differences appeared between sites of winter wheat (Figs 2-3). In 1994, the peak of *Oulema* spp. oviposition occurred at Ciney, the colder area, one week before Louvain-la-Neuve. Nevertheless this delay is negligible for the larval peaks that were synchronized in both sites, and pupation began one week earlier at Louvain-la-Neuve. In both sites, the maximum number of eggs was observed one week after the peak of *O. lichenis*, except at Ciney in 1994 where both peaks were simultaneous.

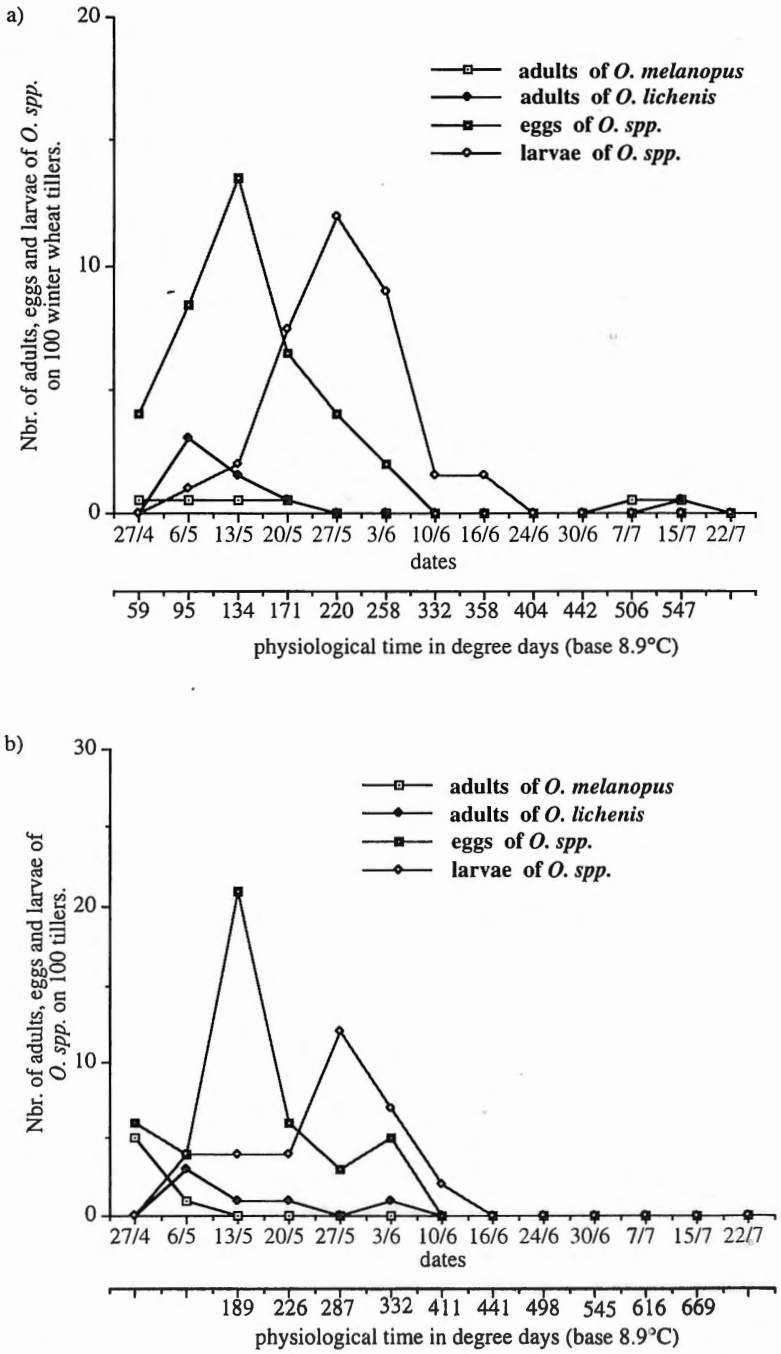

Fig. 2. — Phenology of *O. melanopus* and *O. lichenis*, on winter wheat in 1993. (a) Ciney (mean on 2 fields) (b) Louvain-la-Neuve.

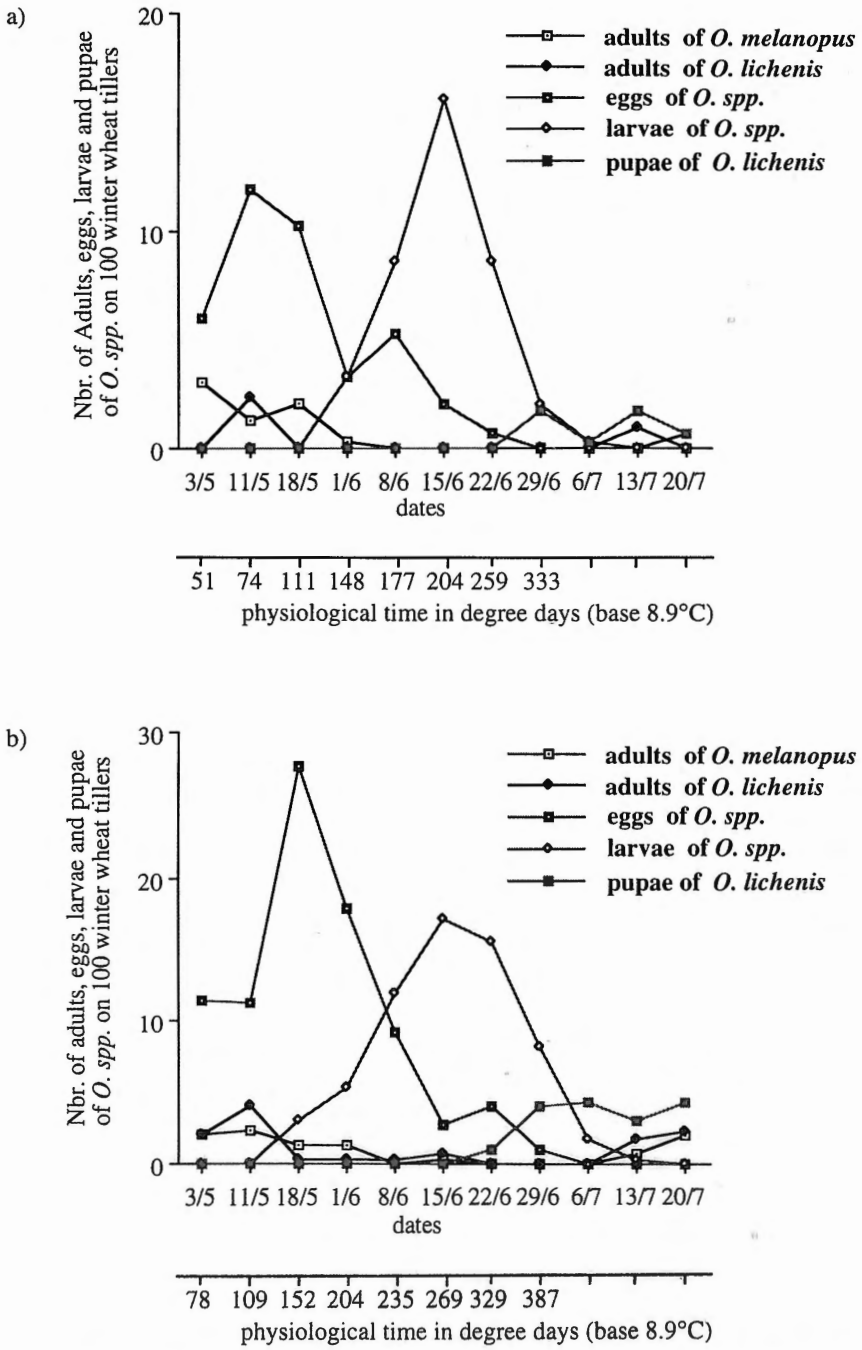

Fig. 3. — Phenology of *O. melanopus* and *O. lichenis*, on winter wheat in 1994. (a) Ciney (mean on 3 fields) (b) Louvain-la-Neuve (mean on 3 fields).

The timing of these events can be expressed in degree days (Table 1) based on the development threshold temperature that is estimated to be 8.9° C for all immature stages of *O. melanopus* (YUN, 1967, in TUMMALA *et al.*, 1975).

TABLE 1

Phenological timing expressed in degree days
(base 8.9° C)

	Peak of egg density	*Time between the two peaks*	*Peak of larvae*
Ciney 1993	133.8	86.6	220.4
Louvain-la-Neuve 1993	188.9	98.3	287.2
Ciney 1994	73.5	130.0	203.5
Louvain-la-Neuve 1994	151.8	117.2	269.0

DISCUSSION

In Belgium, Cereal Leaf Beetles remain marginal pests in winter wheat fields, as their densities are below the economic threshold. However, no data are available concerning other small grain cereal crops. Indeed, as *O. melanopus* prefers oat and barley, it would be interesting to know its density on these crops. According to WILSON *et al.* (1969), the loss of oat grain yield due to this pest ranges from 148 to 266 kg/ha at the density of one larva per tiller.

YUN (1967, in TUMMALA *et al.*, 1975) estimated that physiological time of development of the eggs, larvae and pupae of *O. melanopus* was 82, 116 and 216 degree days respectively. This explains why in 1994, at Louvain-la-Neuve, pupal formation was earlier than at Ciney, although the oviposition began later in the first site. Lengths of larval development were, in 1994, 1.4 to 1.6 times greater than expected. This may be due to the cooler spring of 1994 (see the degree days scales of the two seasons in the same site).

In 1994, the earliest oviposition at Ciney, the cooler area, may be explained by the early attainment of the cold sum, necessary to end the adult diapause. Adults emerged earlier and could oviposit. However this argument stays as a hypothesis that must be tested.

The low *Oulema* spp. density observed might be due to the climatic situation. It might also be due to a great activity of their different parasitoids or to the use of resistant varieties of winter wheat. An egg and three larval parasitoid species were recorded in Europe on this pest (STEHR, 1968). The character which plays a role in the varietal resistance is the length and the density of trichomes. These trichomes act by isolating the eggs in the air ; improving thereby their desiccation ;

and, because they can not be digested, they kill the young larvae by penetrating the midgut epithelium (GALLUN *et al.*, 1966 ; PAPP *et al.*, 1992).

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REFERENCES

Anonymous (1982) — Criocères des céréales. Fiche ACTA, **179**.

BALACHOWSKY, A. and L. MESNIL (1935) — *Les insectes nuisibles aux plantes cultivées ; leurs mœurs, leur destruction.* Vol I. Etablissements Busson, Paris, 1137 pp.

GALLUN, R.L., R. RUPPEL and E.H. EVERSON (1966) — Resistance of small grains to the cereal leaf beetle. *Journal of Economic Entomology*, **59 (4)** : 827-829.

PAPP, M., J. KOLAROV and A. MESTERHAZY (1992) — Relation between pubescence of seedling and flag leaves of winter wheat ant its significance in breeding resistance to cereal leaf bettle (Coleoptera : Chrysomelidae). *Environmental Entomology*, **21 (4)** : 700-705.

STEHR, F.W. (1968) — Establishment in the United States of *Tetrastichus julis*, a larval parasite of the cereal leaf beetle. *Journal of Economic Entomology*, **63 (6)** : 1968-1969.

TUMMALA, R.L., W.G. RUESINK and D.L. HAYNES (1975) — A discret component approach to the management of the cereal leaf beetle ecosystem. *Environmental Entomology*, **4 (2)** : 175-18.

WILSON, M.C., R.E. TREECE, R.E. SHADE, K.M. DAY and R.K. STIVERS (1969) — Impact of cereal leaf beetle larvae on yields of oats. *Journal of Economic Entomology*, **62 (3)** : 699-702.

ZADOKS, J.C., T.T. CHANG and C.F. KONZAK (1974) — A decimal code for the growth stages of cereals. *Weed Research*, **14** : 415-421.

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ULTRASTRUCTURAL ORGANISATION OF THE EPIDERMAL GLANDS IN THE INTEGUMENT OF THE SEA SPIDERS *NYMPHON GRACILE* LEACH 1814, *ACHELIA LONGIPES* HODGES 1864, AND *PYCNOGONUM LITTORALE* (STRÖM, 1762) (CHELICERATA, PYCNOGONIDA)

by

PHILIPPE THIRY (1), PHILIPPE COMPÈRE (1), JEAN-CLAUDE BUSSERS (2) and GERHARD GOFFINET (1)

(1) Laboratoire de Biologie générale et de Morphologie ultrastructurale
(2) Laboratoire de Morphologie, Ecologie et Systématique animales,
Institut de Zoologie, Université de Liège,
quai Ed. Van Beneden 22, B-4020 Liège (Belgium)

SUMMARY

The present paper describes the ultrastructural organisation of the epidermal glands in three pycnogonid species : *Nymphon gracile, Achelia longipes*, and *Pycnogonum littorale*. These glands are distributed over the whole body surface and lodged in cuticular holes. They are composed of at least five cells : two secretory cells, one enveloping cell, and two (or more) accessory cells. The presence of nerve extensions in contact with the enveloping cell strongly suggests that the glands are under nervous control. They may play a defensive role.

Keywords : pycnogonids, integument, epidermal glands, ultrastructure.

INTRODUCTION

Epidermal glands, also called « dermal glands », are a well-known feature of the integument in insects (NOIROT and QUENNEDEY, 1974) and crustaceans (DOUGHTIE and RAO, 1982 ; COMPÈRE, 1990), among which their morphology and precise location vary widely. Functionally, the secretions of these glands are recognised to play various roles, notably in social relationships, sexual behaviour, defence, and the formation of some cuticular layers. Epidermal glands are very numerous in the integument of sea spiders or pycnogonids, a class of marine chelicerates, where they are distributed over the whole body surface and, peculiarly, are lodged in holes within the cuticle. Although the glands are mentioned by several authors (FAGE, 1949 ;

KING, 1974 ; DAVENPORT *et al.*, 1987 ; FAHRENBACH, 1994), their ultrastructural and cellular organisation has not been described.

MATERIAL AND METHODS

Individuals of three pycnogonid species, *Nymphon gracile, Achelia longipes*, and *Pycnogonum littorale*, were collected at the Marine Station of Wimereux (France). The tibias and femurs of the walking legs as well as the trunk were excised from living animals and fixed by immersion for 72 h at 20° C in 2.5 % glutaraldehyde buffered with diluted sea water adjusted to pH 7.4 according to MILLONIG (1976). The samples were then decalcified for 72 h at 4° C in 0.2 M EDTA at pH 8.0, rinsed in filtered sea water, and post-fixed in 1 % OsO_4. After washing in distilled water and dehydration through a graded ethanol series, the samples were embedded in epoxy resin according to the standard procedure.

For observations in transmission electron microscopy, ultrathin sections were cut either perpendicularly or parallel to the cuticle surface, then stained with uranyl acetate and lead citrate. They were observed in a JEOL JEM 100-SX electron microscope at 80 kV accelerating voltage.

For scanning electron microscopy, ethanol-dehydration was followed by critical point drying in CO_2. After coating with gold-palladium in a sputtering apparatus (BALZERS SCD 030), the samples were examined in a JEOL JSM-840A scanning electron microscope at 20 kV accelerating voltage.

RESULTS

As shown in our various micrographs and schematised in Fig. 1, the fine structure and cellular organisation of the epidermal glands are quite similar in the three species. The glands are formed of epidermal cells lodged in large holes in the cuticle (Fig. 2). They are connected to the outer cuticular surface through a short excretory duct lined by a thin cuticle. At the cuticular surface, two symmetrical cuticle lips located in small, circular depressions form the edges of the epidermal gland openings. These openings are relatively difficult to observe owing to the presence of numerous epibiotic organisms (Fig. 3).

As a rule, each gland is composed of five cells : two secretory cells, one enveloping cell, and two (or more) accessory cells.

The secretory cells are of two types, distinguishable on the basis of the electron-density of the secretory granules filling their apical cytoplasm. One type contains large, electron-lucent granules whilst the second exhibits smaller, moderately electron-dense granules. Both types of secretory cells are connected to the excretory duct , their apices bearing a few, scarce microvilli.

The enveloping cell surrounds the secretory cells ; it isolates them from each other and from the other epidermal cells (Figs 3 and 4). Distally, between the apex of the secretory cells and the lower end of the excretory duct, it forms a sort of

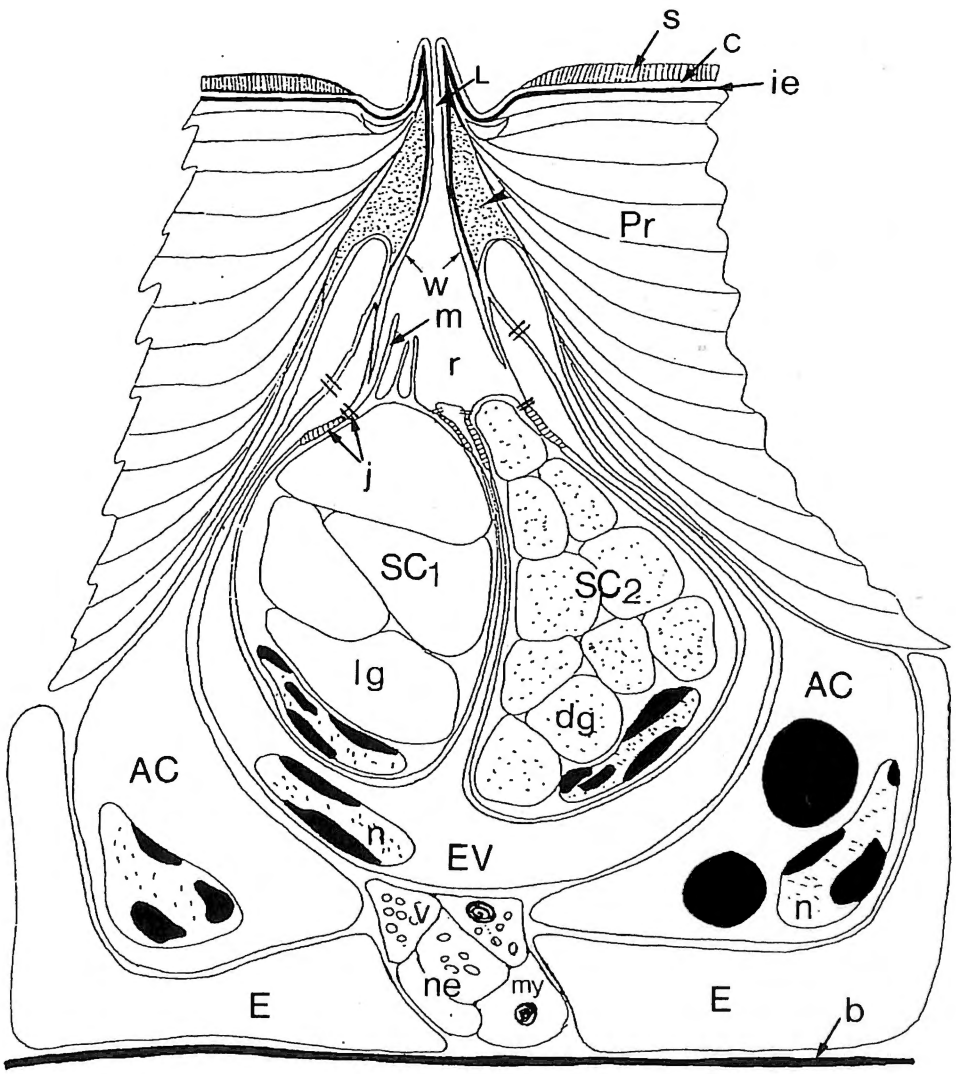

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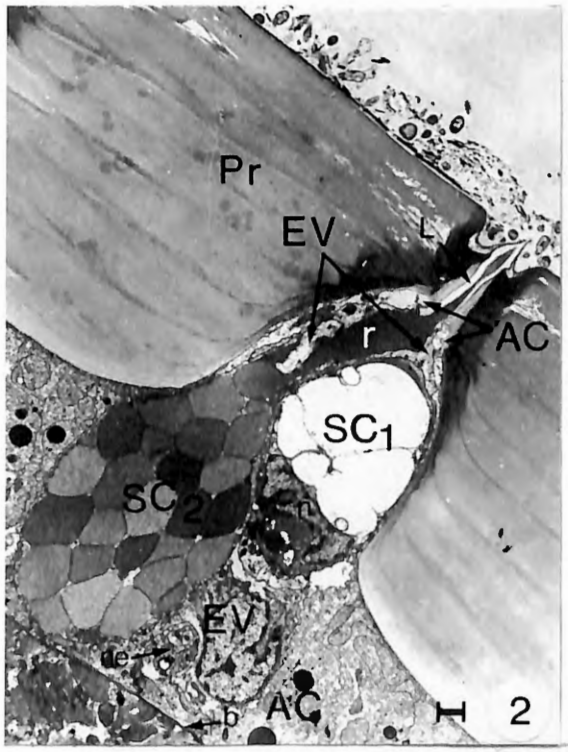
Fig. 1. — Diagram showing the cellular organisation of a pycnogonid epidermal gland. AC, accessory cells ; b, basal lamina ; c, cuticulin layer ; dg, electron-dense granules ; E, epidermal cells ; EV, enveloping cell ; ie, inner epicuticle ; j, intercellular junctions including a zonula adherens and a septate desmosome ; L, excretory duct lumen ; lg, electron-lucent granules ; m, microvilli ; my, myelin sheath ; n, nucleus ; ne, nerve extensions ; Pr, procuticle ; r, collection reservoir ; s, surface coat ; SC1, type-1 secretory cell ; SC2, type-2 secretory cell ; v, synaptic-like vesicles ; w, cuticular wall of the excretory duct ; arrow head, modified procuticle.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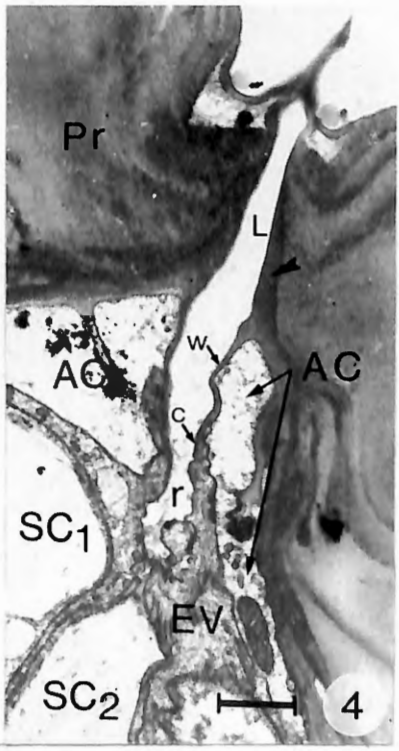
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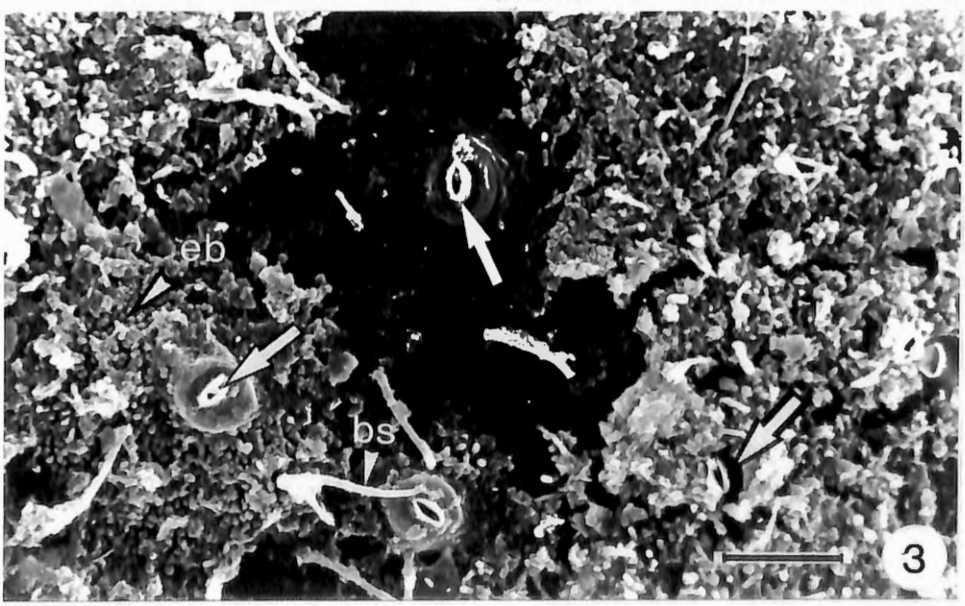
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reservoir that collects the secretion products. Basically, the enveloping cell is in contact with nerve extensions showing synaptic-like vesicles and myelin sheaths.

The accessory cells are slightly modified epidermal cells lining the cuticular wall of the hole and surrounding the cuticular sheath of the excretory duct.

The cuticle of the excretory duct is composed of three different layers : the cuticulin layer, the inner epicuticle, and the procuticle (Figs 1 and 4), layers previously identified in the sclerite cuticle of *P. littorale* (COMPÈRE *et al.*, 1993). Toward the lower end of the duct, the procuticle and epicuticle gradually become thinner, whilst the cuticulin layer subsists and still appears to line the duct through the enveloping cell where it appears abruptly to end.

DISCUSSION

On the basis of their organisation, the epidermal gland cells of pycnogonids can be classified as class 3 secretory cells, described by NOIROT and QUENNEDEY (1974) in insects and arachnids.

In agreement with these authors' definition of class 3 gland cells, the epidermal gland cells of pycnogonids have no direct contact with the cuticle and are connected to the external medium by an excretory duct that runs through a specialised cell, sometimes forming a collection reservoir. The enveloping cell may correspond with the specialised canal-forming cells of insect glands, the canal cell and the intermediate cell.

While the principal originality of pycnogonid epidermal gland cells is their location inside cuticle holes, they also differ in the following respects from those of insects : (1) the excretory duct does not issue from a microvillous terminal apparatus invaginated into the secretory cells ; (2) the enveloping cell differs from canal or intermediate cells in that it completely surrounds the two secretory cells.

Our results support the hypothesis, proposed by FAHRENBACH (1994), that the epidermal glands of pycnogonids play a defensive role. Such a role would fit with

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Fig. 2. — General view of an epidermal gland in the leg femur of a female *Nymphon gracile* Leach. AC, accessory cells ; b, basal lamina ; EV, enveloping cell ; ie, inner epicuticle ; L, excretory duct lumen ; n, nucleus ; ne, nerve extensions ; Pr, procuticle ; r, collection reservoir ; SC1, type-1 secretory cell ; SC2, type-2 secretory cell. Scale bar = 1 µm.

Fig. 3. — Cuticle surface of the femur of a female *Nymphon gracile* Leach. eb, epibiotic organisms ; bs, bipartite sensillum ; arrows, openings of the epidermal gland ducts. Scale bar = 10 µm.

Fig. 4. — Detail of the apex and excretory duct of an epidermal gland in the tibia of a female *Nymphon gracile* Leach. AC, accessory cells ; c, cuticulin layer ; EV, enveloping cell ; L, excreting duct lumen ; Pr, procuticle ; r, collecting reservoir ; SC1, secretory cell of the type 1 ; SC2, secretory cell of the type 2 ; w, cuticular wall of the excreting duct ; arrow head, modified procuticle. Scale bar = 1 µm.

the following facts : (1) the presence of nerve extensions in contact with the enveloping cell strongly suggests that these glandular formations are under nervous control, presumably enabling the animals to discharge their secretion product when disturbed ; (2) the epidermal glands are distributed over the whole body surface ; since they do not seem to be involved in the deposition of cuticular material, a defensive role might explain this distribution ; (3) when disturbed, *P. littorale* was found by TOMASCHKO (1994) to defensively secrete a mixture of eight ecdysteroids. This discharge of ecdysteroids significantly deterred from feeding the common shore crab *Carcinus maenas* (L.), recognised as a general predator in the habitat of this pycnogonid species.

We may thus reasonably hypothesise that the peculiar epidermal glands of pycnogonids are involved in secreting ecdysteroids or other substances rendering these vulnerable animals inedible by predators. This hypothesis, however, must be taken with caution and remains to be confirmed. Mimetism is another possible means by which pycnogonids might defend themselves (FAGE, 1949).

ACKNOWLEDGEMENTS

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REFERENCES

COMPÈRE, P. (1990) — Fine structure and elaboration of the epicuticle and pore canal system in tergite cuticle of the land isopod *Oniscus asellus* during a molting cycle. In P. JUCHAULT and J.P. MOCQUARD : *Biology of terrestrials isopods III.* Poitiers : 169-175.

COMPÈRE, PH., PH. THIRY, J.-C. BUSSERS and G. GOFFINET (1993) — Ultrastructure of the integument of the sea spider *Pycnogonum littorale* (Ström) (Pycnogonida). *Belgian J. Zool.*, **123** (suppl. 1) : 11-12.

DAVENPORT, J., N. BLACKSTOCK, D.A. DAVIES and M. YARRINGTON (1987) — Observations on the physiology and integumentary structure of the Antartic pycnogonid *Decolopoda australis*. *J. Zool.* (Lond.), **211** : 451-465.

DOUGHTIE, D.G. and R.K. RAO (1982) — Rosette glands in the gills of the Grass Shrimp, *Palaemonetes pugio*. Comparative morphology, cyclical activity and innervation. *J. Morphol.*, **171** : 41-67.

FAGE, L. (1949) — Classe des Pycnogonides. In P.P. GRASSÉ : *Traité de Zoologie, Tome VI, Chélicérates*. Masson, Paris : 906-941.

FAHRENBACH, W.H. (1994) — Microscopic anatomy of Pycnogonida : 1. cuticle, epidermis and muscle. *J. Morphol.*, **222** : 33-48.

KING, P.E. (1974) — British sea spiders. Arthropoda : Pycnogonida. Key and notes for the identification of the species. In Linnean Society of London : Synopsis of the British Fauna n° 5. Academic Press, London, New York : 67 pp.

MILLONIG, G. (1976) — Laboratory manual of biological electron microscopy. SAVIOLO, M., Vercelli : 67 pp.

NOIROT, C. and A. QUENNEDEY (1974) — Fine structure of insect epidermal glands. *Annu. Rev. Entomol.*, **19** : 61-80.

TOMASCHKO, K.H. (1994) — Defensive secretion of ecdysteroids in *Pycnogonum littorale* (Arthropoda, Pantopoda) — *Z. Naturforsch.*, **49c** : 367-371.

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KINEMATICS OF THE ESCAPE HEAD RETRACTION IN THE COMMON SNAKE-NECKED TURTLE, *CHELODINA LONGICOLLIS* (TESTUDINES : PLEURODIRA : CHELIDAE)

by

JOHAN VAN DAMME, PETER AERTS and FRITS DE VREE
University of Antwerp (U.I.A.), Department of Biology,
Universiteitsplein 1, B-2610 Antwerp

SUMMARY

Cervical movements during the fast escape head retraction in the pleurodiran turtle Chelodina longicollis were studied by means of x-ray cinematography. Radio-opaque markers were inserted near the cervical joints to allow calculation of joint rotations between the successive vertebrae expressed as a function of time and head position. Head retraction as a percentage of the extended neck configuration and angular and linear velocities were also calculated. A combination of muscular organisation and kinematics shows that the neck is divided into two functional regions, anterior and posterior to the biconvex fifth cervical vertebra respectively. Head retraction proceeds in two phases. During the first phase the animal retracts the head very fast underneath the carapace but leaves the neck partially exposed. This phase (with exception for C3−2 and C5−4) shows no significant differences in the timing of the peak-velocities. During the second phase rotations occur mainly in the proximal joints leading to the maximally retracted configuration. Left and right head retractions are mirror images. Retractions never start from a completely extended neck configuration. Initial angles always occur in C6−5 (joint between vertebrae 5 and 6) and C8−7. These joints are also the major bending sites for full retraction of the neck. Peak-velocities of these joints strongly correlate with each other but also with head retraction. When expressed as functions of head position, the rotation patterns of the proximal joints are particularly stereotyped. It is hypothesized that both initial angels and stereotypical retraction patterns are required to allow a fast (escape) retraction powered by a simple motor pattern.

Keywords : neck, kinematics, reptiles, Chelodina.

INTRODUCTION

Reptiles, birds and mammals possess a distinct cervical region which gives the head great mobility with respect to the trunk. The benefit to the animal is manifold : *e.g.* mobile prey can be captured easily, the environment can be scanned

effectively and the head can be retracted close to the trunk, protecting it against negative environmental stimuli (GANS, 1992).

Over the past decade, there has been considerable advance in the knowledge of the kinematics of the avian cervical system (ELSHOUD and ZWEERS, 1987 ; KOOLOOS and ZWEERS, 1989 ; ZWEERS *et al.*, 1977 ; ZWEERS, 1982 ; HEIDWEILLER *et al.*, 1992). The kinematics of the cervical system of turtles, however, has not yet been studied in great detail.

In turtles, the potential for morphological differentiation is largely influenced by the presence of the bony shell. Few changes occur at the level of the trunk, but body parts outside the bony shell are very susceptible to functional changes. Therefore, it is not surprising that the neck of testudinians has shown several conspicuous modifications during the course of turtle evolution (WILLIAMS, 1950 ; HOFSTETTER and GASC, 1969). Starting from a nearly rigid cervical region (with unspecialized cervical vertebrae), two basic patterns of mobile neck configurations evolved independently (GAFFNEY, 1975). Cryptodiran turtles retract the head by bending their neck in the vertical plane. Pleurodirans or side-necked turtles withdraw the head by bending the neck in the horizontal plane ; they fold it back between the dorsal and ventral rim of the bony shell. The neck remains partially exposed in the outer carapacial chamber in front of the pectoral girdle.

In all species of recent pleurodiran turtles, the cervical system can be described as an open kinematic chain of ten links : the body, the head and eight cervical vertebrae. This system has, within certain limits, 27 degrees of freedom, and more than 50 mono- and polyarticular muscles (SHAH, 1963) are present to steer a variety of movements in which speed (*e.g.* escape retraction, feeding strikes) as well as accuracy (*e.g.* feeding strikes) are important. It is obvious that such a system must move the constituent links in concert, which presumably requires a high level of neuromotor control. Anatomical studies of the neck muscles and vertebrae of several turtle species are available (*e.g.* BOJANUS, 1819, 1821 ; VAILLANT, 1881 ; OGUSHI, 1913 ; VALLOIS, 1922 ; WILLIAMS, 1950 ; GEORGE and SHAH, 1954 ; GEORGE and SHAH, 1955 ; HOFSTETTER and GASC, 1969 ; SCANLON, 1982). However, only WEISGRAM and SPLECHTNA (1992) have discussed the integration of morphology and kinematics of the neck system.

This paper is the first in a series dealing with the functional anatomy and cervical kinematics of the Common snake-necked Turtle, *Chelodina longicollis*. The neck in these turtles is very long. This allows the head to be displaced through an extensive volume of space, effectuating a whole range of movements. Slow movements in any direction are performed while scanning the environment. The head can be projected and retracted very rapidly during feeding strikes (see VAN DAMME *et al.*, in prep.). The displacement range usually remains small. The long neck also permits the animal to maintain the trunk deep below the surface, and to extend the neck for breathing (snorkelling ; VAN DAMME *et al.*, in prep.).

The fast escape head retraction has a protective function and must be feasible at any time and from any starting position. In contrast to feeding and snorkelling movements, fast escapes induce the largest joint rotations of the movement reper-

toire in *Chelodina*, and will be discussed in the present paper. Retractions following upon a feeding strike are excluded as they form an integrated part of the feeding behaviour and remain always limited to a much smaller displacement range. They will be dealt with in a subsequent paper.

The fast escape retraction of the head is analyzed by means of cineradiography. These data permit discussion of the dominant kinematical patterns associated with rapid cervical retraction. It also permits hypothesizing about the functional significance of specific initial neck configurations of this mechanism.

MATERIAL AND METHODS

Three live adult specimens of *Chelodina longicollis* were used for the experiments : Chelo #1 (female, 0.73 kg, 0.18 m carapace length, 0.12 m neck length), Chelo #2 (male, 0.52 kg, 0.15 m carapace length, 0.11 m neck length), Chelo #3 (female, 0.70 kg, 0.18 m carapace length, 0.12 m. neck length). The animals were obtained with the help of the Antwerp Zoo, and were housed in a glass aqua-terrarium on a 12h light/dark cycle. The water temperature was kept at 28°C. Twice a week the turtles were fed mainly with meat (carved heart muscle), mice and small invertebrates (crickets, grasshoppers).

Head retraction movements (=cervical movements in the horizontal plane) of Chelo #1 and Chelo #2 were recorded cineradiographically (dorsoventrally) using a Siemens Tridoros Optimatic 800 x-ray generator equipped with a Sirecon-2 image intensifier. An integral Arriflex 16 mm camera recorded movements on 7231 Eastman negative film at 50 frames per second. Tube voltage was 50 kV (400mA), the exposure time of the x-ray flashes 1 ms and the distance between tube and image intensifier 1 m. In order to keep the animal above the image amplifier the animals were restrained by means of a body-shaped corset made of thermoplastic material (Orthoplast ; Johnson and Johnson Orthopaedics). To improve the accuracy of the image analysis, radio-opaque lead markers pinched out of a 1mm thick lead sheet were inserted under the periosteum by hypodermic needles in Chelo #1 and Chelo #2 under radiographic control. Because Chelo #3 belonged to the Antwerp zoo, no invasive experiments were allowed on this animal. The animals were anaesthetized with an intramuscular injection of 147 mg/kg Ketamine hydrochloride. The markers were inserted in 12 locations ; one dorsally on the caudal part of the skull, one near each of the cervical joints (dorsal on the posterior zygapophysis of the vertebrae), one on the first dorsal vertebra, and two into the anterior part of the carapace (Fig. 1A). The position of these markers was checked on dorsoventral and lateral radiographs. For Chelo #2 the marker at the level of joint C2−1 was not inserted properly, and therefore could not be used in the analyses (*i.e.* joint angles S−C1 and C2−1 are missing for this specimen). Sequences were projected frame by frame on a Hipad Digitizer (Houston Instruments) connected to a 80386-DX IBM-compatible computer. The position of the markers was digitized and converted to a turtle bound frame defined by the carapace markers (ref 1 and ref 2 on Fig. 1A).

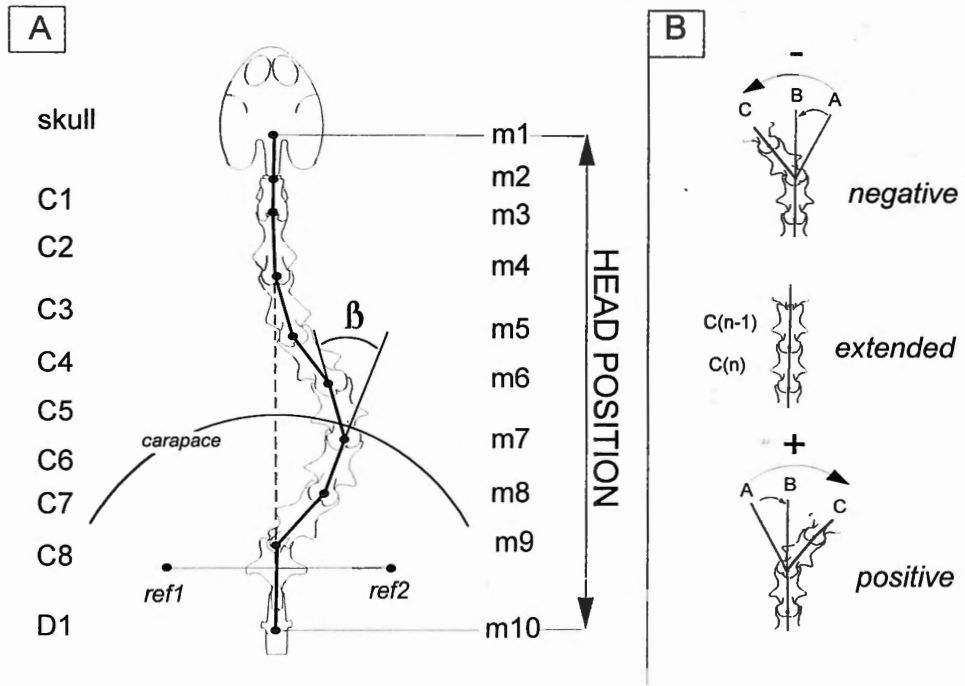

Fig. 1. — *Chelodina longicollis* — A. Position of the radio-opaque markers (black dots, m1 – m10) on a schematic representation of a neck configuration from which a retraction can start. The black solid line represents a stick diagram as constructed based on the position of these markers. The length of the dashed line gives the head position. ref 1 and ref 2 are the carapace markers. β represents a starting angle (*e.g.* C6 – 5). — B. Terminology of joint rotation. Starting from the extended configuration (middle), counter clockwise rotations (above) result in a negative joint angle. Clockwise rotations (below) result in a positive joint angle. Distance A-C represents the total joint excursion. Distance A-B the contralateral joint excursion.

The degree of head retraction is defined by the rectilinear distance between the cranial-most (m1) and the caudal-most (m10) marker (Fig. 1A), and is expressed as the percentage of the maximum possible distance between these two markers. Thus, a completely extended neck coincides with a head position of 100 %, whereas maximal retraction equals 25 %. Left and right head retractions stands for movements in which the final retraction of the head occurs to that particular side of the body. Twenty head retractions were analyzed in detail (5 left and 5 right trials for each specimen). Left and right movements were compared within and between both specimens. For each movement, stick diagrams were constructed in order to obtain a schematic representation of the observed cervical movement (Fig. 3B).

Special attention was paid to changes in angles between successive vertebrae (*i.e.* joint angles), both as a function of time and as a function of the degree of head retraction. These changes are defined as rotations about a vertical axis of ver-

tebra n − 1 with respect to the more caudal one (vertebra n ; see Fig. 1B). Starting from the extended position (= 0 degrees of lateral flexion) a positive joint angle is defined as a displacement of the anterior vertebra to the right (clockwise rotation, Fig. 1B).

Slow head displacements were induced by luring the animal with a prey item. When the head was positioned approximately in the midsagittal plane, a fast escape head retraction was initiated by suddenly touching the tip of the snout with a plastic tube. In most cases however, retraction began from an obviously bent neck, and all joints showed a starting angle. When during a retraction a joint angle crosses the extended configuration of the joint, the starting angle is defined as contralateral (with respect to the joint angle at maximal head retraction), and the rotation up to the fully extended position is called a contralateral change of joint angle. Retraction movements were never observed to start from the 100 % head position, because the cineradiographs of an extended neck always revealed initial angles being present in a few specific joints (see further).

Angular velocities (rad.s^{-1}) in the joints and the linear velocity of the head (percentage.s^{-1}) were obtained by differentiation of the smoothed displacement data of the cineradiographical recordings (WOOD, 1982). This procedure emphasizes the relationships in timing and relative importance of the centres of motion (= joints). A Quintic spline routine (supplied by G.A. WOOD, Department of Movement Studies, University of Western Australia) was used, and the accuracy of fit (least-square criterion) was adjusted until the velocity profile was free of any biologically irrelevant oscillations (*i.e.* oscillations in the acceleration profiles occurring at frequencies above the ones expected from the displacement pattern itself). For each trial, peak-velocities and the corresponding times at which these peaks occurred were determined for the joints and the head (Table 1). Kinematic variables were statistically analyzed as follows. Times of peak-velocities among joints were compared within trials using a Friedman Two-way Anova. Pairwise comparison of the relative times of the joints with the head were analyzed with Wilcoxon Matched-pairs Signed-rank tests (NORUSIS, 1986). To determine multivariately which peak-velocities of joints were correlated with the head, a canonical correlation analysis (SAS v6.07) was carried out.

Static radiographs (50 kV) were made of all three specimens with the neck forced to maximal retraction. Figure 5 is based on such a radiograph of Chelo #3.

Vertebrae are indicated by capital C or D (cervical and dorsal vertebrae respectively) followed by their serial number. C1 is closest to the head. Joints are labelled by the number of the adjacent vertebrae : C(n) − (n − 1), with n the number of the more proximal (caudal) vertebra and (n − 1) the number of the more distal (cranial) one. For example, the joint between vertebra 5 and 6 is defined as « joint C6 − 5 ».

RESULTS

Morphology

The cervical column in all recent turtles consists of eight elongated vertebrae (C1 − C8) and nine joints. Fig. 2 (part A, B, C) illustrates the terminology of the

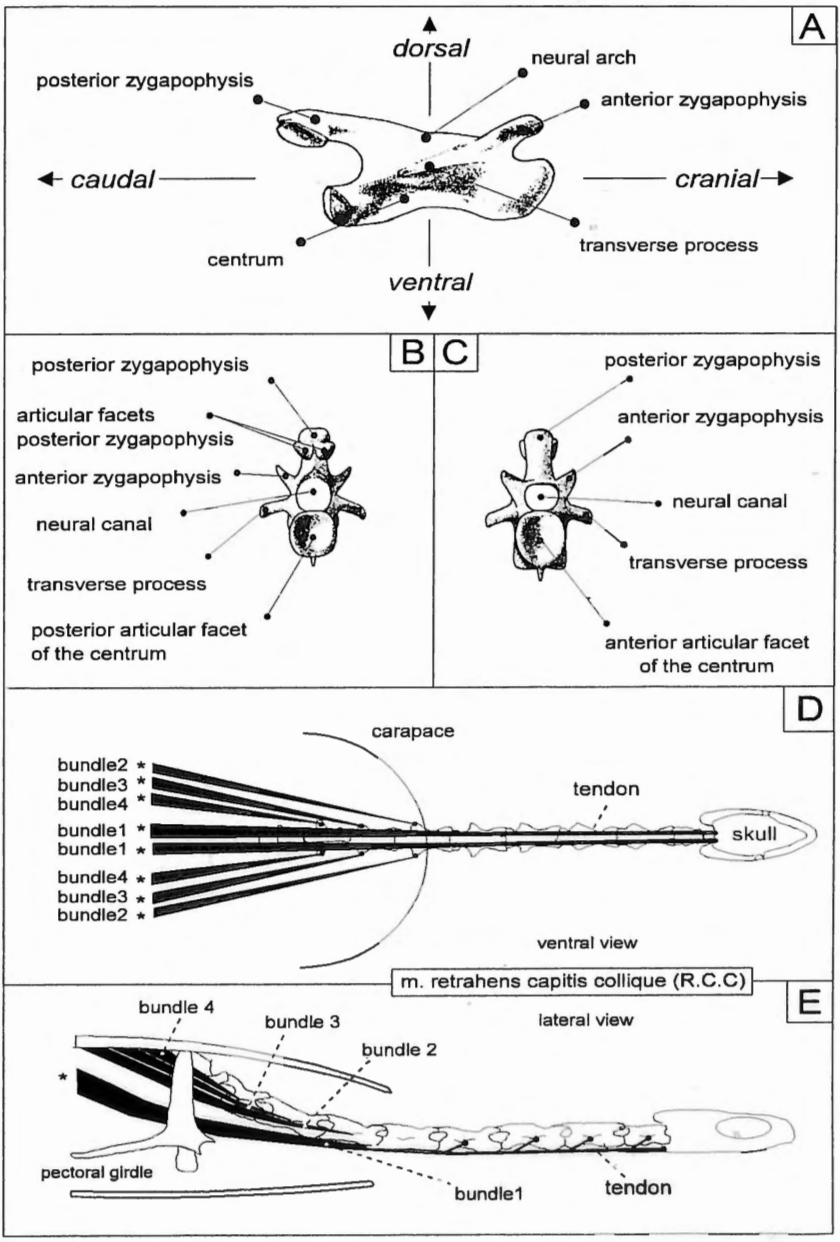

Fig 2. — *Chelodina longicollis* — Upper half : Terminology of the most important structural elements of a cervical vertebra (*e.g.* C7). A. lateral view, B. posterior view, C. anterior view (terminology after ROMER and PARSONS, 1977). — Lower half : organisation of the m. retrahens capitis collique. D (ventral view) and E (dorsal view). « * » indicates that the place of origin is not shown (see description for more details). Each bundle is numbered according to its site of insertion in the neck. Bundle 1 has the rostral most insertion.

most important structural elements of a cervical vertebra (*e.g.* C7) in *Chelodina*. The neural spine is reduced to a inconspicuous longitudinal rim. The anterior zygapophyses are clearly separated from each other. Their articulation facets are mainly oriented in the medio-dorsal direction. The posterior zygapophyses are fused to each other except those of C1, but the articulation facets remain separated and face mainly ventrally and slightly laterally. The elevation of the posterior zygapophysis gradually increases from distal to proximal. The transverse processes are strongly developed, especially those on the more proximal vertebrae (for a more detailed description of the vertebrae of Chelodina see VAILLANT, 1881). Vertebral centra can be biconvex (C5, C8 : two condyles), biconcave or amphicoelous (C1, C7 : two cotyles), procoelous (C6 : anterior cotyle, posterior condyle) or opisthocoelous (C2, C3, C4 : anterior condyle, posterior cotyle).

The muscular organisation of the neck in *Chelodina* is very complex. There are short monoarticular and longer polyarticular muscles. The most important muscle powering fast head retraction (based on position and morphology) is the m. retrahens capitis collique (Figs 2D, 2E). It consists of four well developed muscle bundles on each side of the trunk. Bundle 1 (RCC_1) is the longest muscle observed in the whole turtle body. It originates from the lateral sides of the eighth dorsal vertebra immediately anterior to the pelvic girdle, and from the carapace adjacent to this vertebra. The muscle bundle runs ventrally to the other bundles of the RCC-complex and the cervical vertebrae, lying dorsal to the oesophagus and trachea. At the level of C5 the muscle fibres of RCC_1 insert on a large tendon which extends anteriorly to the base of the skull attaching to the basioccipital. Small tendon slips split off from this large tendon and insert on the transverse processes of the first four cervical vertebrae. Bundle 2 (RCC_2) originates on the ventral side of the neural plate of the carapace, adjacent to D5. It runs anteriorly, parallel and dorsal to bundle 1 and inserts by means of a large, flat tendon on the transverse processes of C6. Bundle 3 (RCC_3) originates from the ventral side of the neural plate adjacent to the fourth dorsal vertebra. The tendon inserts on the lateral side of C7. The muscle fibres of the smallest bundle (bundle 4, RCC_4) originate at the level of D3 and insert on the transverse processes of C8.

Kinematics

Detailed description of an escape retraction movement

A representative example of a left head retraction movement of Chelo #1 is illustrated in Figure 3. Starting from a nearly extended neck configuration (90 % position, Fig. 3A), retraction takes about 0.6 s. The displacement range is large, but retraction is still incomplete (retracted head position equals 35 %, versus 25 % for maximal retraction). The actual movement is represented by the stick figures of Fig. 3B (1) (time interval : 20 ms.). Figure 3B (2) shows the path of the joints during the retraction. The four proximal joints displace latero-caudally to the right. The more distal (=cranial) joints move mainly in caudal direction (=in the direction of the carapace). Because the spatial displacement of a joint represents the summed

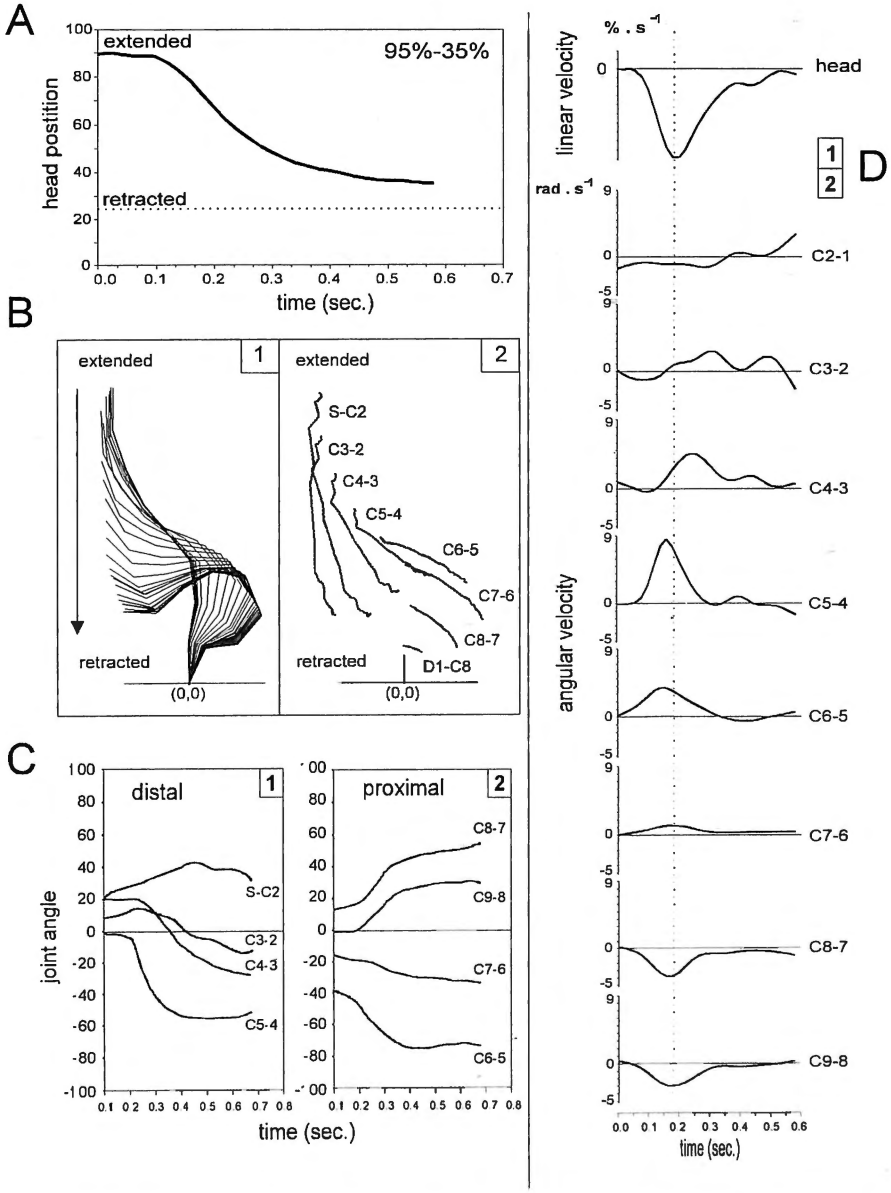

Fig 3. — *Chelodina longicollis* : Kinematics of a left head retraction movement. — A. head position in function of time. — B. stick diagrams, B1. sticks illustrating schematically the retraction movement, B2. path followed by individual joints. The arrow indicates the direction of movement. — C. changes in joint angle as function of time. C1. anterior neck region. C2. posterior neck region. — D. velocity profiles of head retraction (D1) expressed in percentage head position.s^{-1}, and of changes in joint angles (D2) expressed in rad.s^{-1}. Dashed line coincides with maximal head velocity.

effect of all rotations in the more proximal joints, the lengths of the paths increase from proximal to distal. Figure 3C gives the changes of joint angle as a function of time. Because the retraction movement initiates from an already partly retracted head position (90 %), starting angles (*i.e.* diverging from zero) exist in several joints. In the posterior part of the neck, the largest starting angles occur in C6−5 (negative) and C8−7 (positive). All joints, with exception of the first (C2−1) and the last two (D1−C8 and C8−7) show changes of joint angle in the negative direction. In the distal part of the neck the fastest changes in joint angle occur in C5−4 (see also the velocity profile of C5−4 in Figure 3D (2)). In the proximal part of the neck, rotations in C7−6 remain restricted.

The velocity profiles of the head retraction and joint rotations are illustrated in Fig. 3D (1) and 3D (2) respectively. The peak velocity of head retraction equals -281 %.s^{-1}, which accords to an actual velocity of -0.35 m s^{-1} (retractions result in negative velocities). The angular velocity profiles of the first two joints (C2−1 and C3−2) are rather flattened and irregular. From C4−3 on, a distinct peak in the profile is apparent which tends to coincide with the peak velocity of head retraction. From C4−3 to C7−6 the peak velocities are positive (clockwise rotation). In the last two joints the peak velocities are negative (counter clockwise rotation). The greatest peak amplitude is observed in C5−4 (approximately 9 rad.s^{-1}). Velocities in C7−6 remain very low throughout neck retraction.

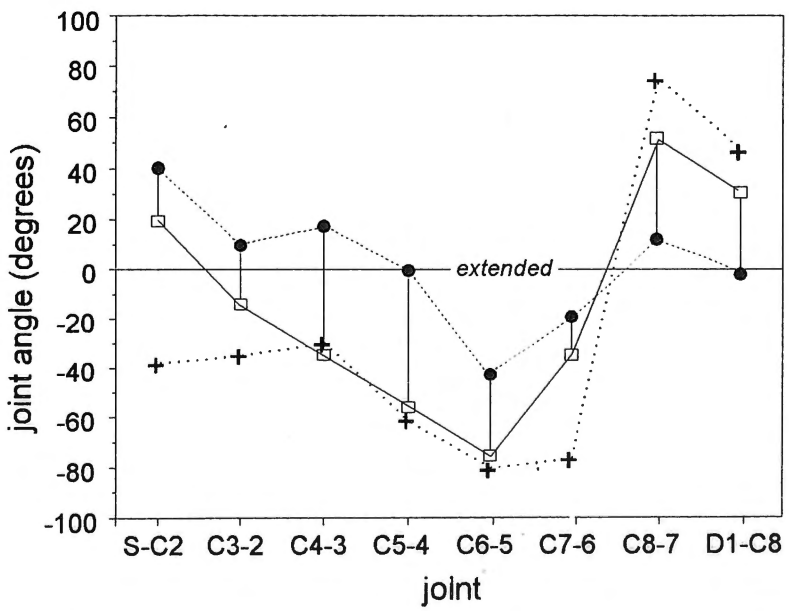

Fig. 4. — *Chelodina longicollis* : Kinematics of a left head retraction movement. Filled dots : starting angles. Filled squares : final angles for this particular sequence. Plus signs : final joint angles occurring in a complete left head retraction.

Fig. 4 summarizes the range of motion in the different joints for this specific left head retraction movement. Differences between the final joint angles observed in this trial (open boxes in Fig. 4), and the final joint angles occurring at maximal head retraction (« + » in Fig. 4) are observed in the three proximal most joints (especially C7−6) and the two anterior most joints (C2−1 and C3−2).

Neck configuration at maximal retraction

Fig. 5 illustrates the final neck configuration when head and neck are completely retracted (example given for a right head retraction of Chelo #3). This figure is based on a static radiograph of a living specimen forced to retract its head maximally. The values of the final joint angles are indicated. Left and right configurations are mirror imaged (final joint angles for a right retraction : S−C1=0° ; C2−1=37° ; C3−2=35° ; C4−3=26° ; C5−4=47° ; C6−5=77° ; C7−6=70° ; C8−7=−92° ; D1−C8=−58°). Irrespective the sign, the largest joint angles appear in the most proximal part of the neck, especially in C8−7 and C6−5 (« * » in Fig. 5). Little variation is observed in the joint angles of this final neck configuration between the three specimens.

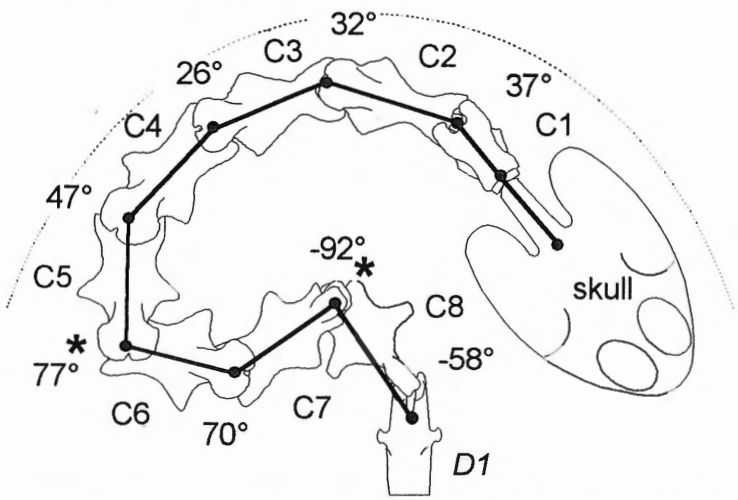

Fig. 5. — *Chelodina longicollis* : Neck configuration with the head completely retracted (*e.g.* for a right head retraction). C1−C8 : cervical vertebrae. D1 : first dorsal vertebra (fused with the carapace). Asterisks : largest final joint angles. Dotted line represents the outer limits of the carapace.

General considerations of the escape head retraction in Chelodina longicollis

The next description is based on the entire data pool of the analyzed escape head retraction movements in both specimens of *Chelodina* (Chelo #1 and Chelo #2 ; 20 sequences in total). Fig. 6A of Chelo #1 shows the rotation ranges combined for all analyzed sequences. This is done for the left (N=5) and right

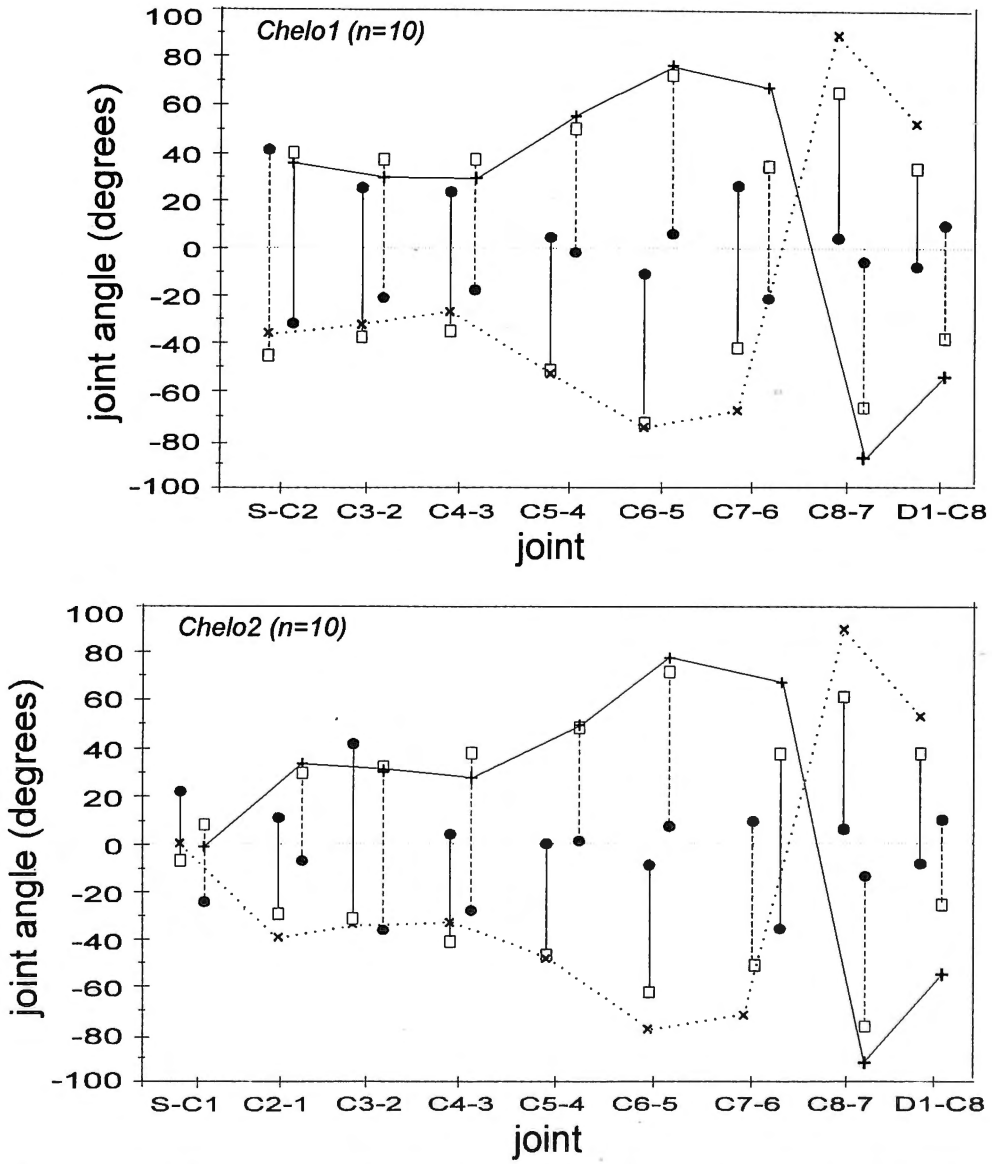

Fig. 6. — *Chelodina longicollis* : Observed ranges of joint rotations during head retraction in Chelo #1 (A) and Chelo #2 (B). Dotted lines : summation of the movement range for 5 (n = 5) right head retraction movements. Plus signs : joint angles for a complete right head retraction (see also Fig. 5). Solid lines : summation of the movement range for 5 left head retraction movements. Crosses : joint angles for a complete left head retraction. Black dots : starting values. Open squares : final values.

(N = 5) retractions separately. Figure 6B presents the same information for Chelo #2. The patterns for left and right head retractions are clearly mirror images of each other. The more proximal joints attain a larger final joint angle than the more distal joints. The range of motion in the successive joints varies with the configuration of the neck at the beginning of the retraction movement. Nevertheless, some joints show little (C5 − 4) or no (C6 − 5 and C8 − 7) contra-lateral joint excursions. As mentioned earlier, the differences between the observed final joint angles (open boxes in Figs. 6A, 6B) and those at maximal head retraction (« + » and « × » signs in Figs. 6A, 6B) are most conspicuous for the proximal joints (especially C7 − 6).

Plotted against head position, the joint angles of all the analyzed sequences appear to show similar movement patterns. Superimposing these patterns for all sequences results in the construction of displacement envelopes (Fig. 7), which reveal the range of angular rotations in each joint throughout the escape head retraction (see discussion). The width of an envelope at any specific degree of head retraction expresses the range of angles to which the involved joint is restricted when passing through that particular head-position during retraction. Narrow envelopes thus suggest stereotyped movement patterns, whereas broad envelopes indicate that an animal has considerable kinematical flexibility. Figure 7 shows that envelopes are much narrower for the proximal joints than for the distal ones (*i.e.* freedom increases towards the head). Moreover, for all joints the rotational freedom decreases with increasing retraction (see discussion).

Peak velocities and correlated timing are presented for all analyzed trials in Table 1. Joints S − C1 and C2 − 1 are not included as they could not be measured for Chelo #1 (see Materials and Methods). The bold figures refer to the observed maxima. The fastest head retraction observed in Chelo #1 was 2.68 m s^{-1} and that for Chelo #2 was 19.66 m s^{-1} (in the table head velocity is expressed in % s^{-1} to facilitate comparisons). Table 1 further shows a tendency towards coinciding timing of the velocity peaks (see also Fig. 6D). The statistical treatment and interpretation is presented in the discussion.

DISCUSSION

Morphologists have investigated and described the cervical system of turtles for about two centuries. The main conclusions were related to the morphological difference between the pleurodiran and cryptodiran neck systems. WILLIAMS (1950) studied the shape of the central articulations between the neck vertebrae in a large number of turtle skeletons. Without taking into account ligamentous connections, zygapophyses and neck musculature this author constructed a diagram representing the two major types of neck movements. He found that joints C6 − 5 (mostly) and D1 − C8 (always) are procoelous in pleurodiran and cryptodiran turtles, and related this to their importance as centres of movement. SCANLON (1982) described the anatomy of the neck of the western painted turtle *Chrysemys picta belli* (Cryptodira) and made predictions concerning patterns of neck movements based upon morphological data and radiographs. This author concluded that predictions about

kinematics of neck movements based on morphological data do not always agree with the observed *in vivo* movements. WEISGRAM and SPLECHTNA (1990) compared the anatomy of the cervical vertebrae in a cryptodiran turtle (*Testudo hermanni*) and in a short necked pleurodiran turtle (*Pelomedusa subrufa*). On the basis of ligamentous preparations and radiographs of these preparations fixed in the retracted position, they determined the maximal range of motility in each joint. Their study suggests that all cervical joints in *Pelomedusa* show the same range of lateral mobility. They conclude that joints C4−3 and C8−7 are the important centres of motion in the neck of *Pelomedusa*. In *Testudo* the cervical joints C3−2 and C8−7 have the largest potential for carrying out movements. Based on these results, WEISGRAM and SPLECHTNA (1990) modified the diagram of WILLIAMS (1950).

Whereas all these studies discuss the functioning of the testudinian neck entirely on the basis of anatomical and static configurational data, WEISGRAM and SPLECHTNA (1992) assessed changes in the neck joints during feeding movements of the Australian side-necked turtle *Chelodina novaeguinaeae* based on video recordings combined with radiographs. They noted that the three caudal-most cervical joints (D1−C8, C8−7, C7−6) appear to be of major importance. However, no data were presented on head retraction.

Several practical problems are inherent to (ciné-)filming or video-taping neck movements of turtles. The bony shell obscures large parts of the neck, whereas the loose connection between the skin and the underlying parts hampers linkage of the external movements to the precise movements of the neck skeleton. X-ray cinematography allows one to compensate for these problems, although the image can still be blurred (especially in the posterior neck region) due to the presence of the bony shell. The implantation of radio-opaque markers in the neck, as used in the present study, further improves analysis of the intervertebral movements.

Interpretation of the kinematics

Fig. 7 shows the displacement envelopes of the cervical joints in the neck for head retraction movements either to the left or to the right. The narrower the envelope, the less the variation is : the rotations in the involved joint as a function of the degree of head retraction are more stereotyped (see above). An increasing stereotypy from distal to proximal is observed (Fig. 7). This phenomenon can be related to the differentiation of the neck musculature. The proximal vertebrae have highly developed transverse processes (Fig. 2A) which reflect the insertion of the massive muscle bundles of the proximal neck musculature (*e.g.* m. R.C.C bundles 2, 3, 4 ; Figs 2D, 2E). This part of the neck is responsible for the largest part of the retraction (Fig. 5). The distal vertebrae receive the insertion of split muscle bundles. Such an increased splitting of the musculature allows smaller, more subtle joint rotations in the distal joints. Therefore, the anterior part of the neck plays an essential role in head orientation (including dorsoventral movements). Fig. 7 also shows that the stereotypy increases for all joints with the degree of head retraction. This means that a small retraction starting from a more or less extended position can be performed in a more versatile way, compared to a retraction over an identical range, but beginning with the neck already partly retracted.

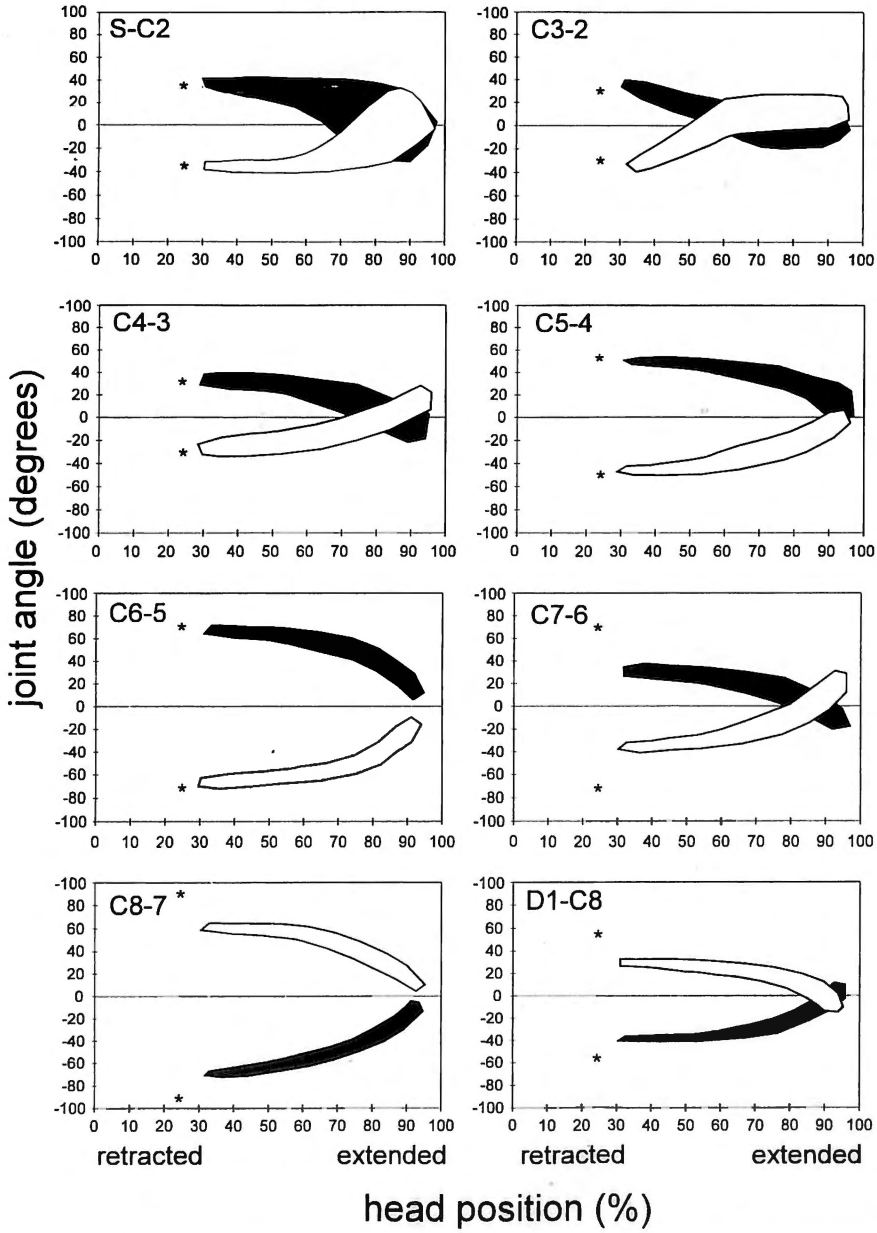

Fig 7. — *Chelodina longicollis* : Displacement envelopes (=changes in joint angle as function of head position) of cervical joint rotations in Chelo #1. Black envelopes : right head retractions (n=5). White envelopes : left head retractions (n=5). Asterisks : final joint angle when the neck is completely retracted.

Besides this stereotypical pattern of changes in joint angle, kinematics of left and right head retraction within each turtle are mirror imaged (Fig. 6). Both facts support the presence of a fixed and simple neuromotor pattern to drive the fast and complete head retraction (for instance in an escape response), because then fast and accurate folding of the neck is assured.

From the kinematic analysis it follows that head retraction never starts from the fully extended neck configuration (*e.g.* Fig. 3), even when externally the neck appears to be extended. Several joints (C6−5 and C8−7 always, C5−4 often) show initial angles. When the animal is heavily anaesthetized, the neck will be completely extended only if a considerable external force is applied. Initial angles can either be positive or negative, and this is determined by the preceding slow neck movements (head movements from left to right and *vice versa* necessitates such changes). Nevertheless, the symmetrical structure of vertebrae and muscles suggests that the completely extended neck configuration is physiologically feasible.

One could think of a possible damage of the neck musculature due to the insertion of the radio-opaque markers, or of an occasional malformation of the neck. However, identical configurations are observed in both specimens, before as well as after surgery. Therefore, it seems likely to assume that initial angles in an externally extended neck are biologically relevant and coincide with the configuration that minimizes the potential (i.e. elastic) energy in the entire neck system. Energy might be stored in all strained structures crossing the joints (muscles, tendons, joint capsules,...). It has to be noticed, however, that extracapsular ligaments, as described in the avian cervical system (LANDOLT and ZWEERS, 1985) are not present in turtles.

The biological significance of this might be that in this way fast escape head retraction can always be driven by one fixed bilateral symmetrical motor pattern. Due to the presence of initial angles the subsequent angular displacement is determined to a large extent, without necessitating subtle motor control for each individual joint. Retraction starting from a completely extended situation requires a more complex and flexible motor control, and is obviously much more sensitive to erroneous folding of the neck. This can be exemplified by a chain of interconnected rigid links (simulating vertebral elements and head) held in a vertical position, the lower end movably connected to a horizontal rigid support (the body). If this chain is left free, gravity will act on it as a simple, very stereotyped force generator. Starting with all links in line, the manner in which it will fold is indefinite. However, the introduction of a few initial angles between some of the links will reduce the variability of the folding pattern drastically. If initial angles of opposite sign are introduced, the folding pattern will mirror image its counterpart, although the force generator remains unchanged. This hypothesis fits in with the conclusions on stereotyped movements drawn from the displacement envelopes and the configurations of maximal retraction (see above). Based on the morphology of the RCC_1 (its length and the rather ventral and medial course of its tendon in the neck), it is this bundle that powers the fast head retraction. This hypothesis is now being tested by means of electromyography. Preliminary results indeed indicate that left and right RCC_1 muscle bundles show a bilateral synchronous activity during

the fast escape head retraction, independent from the position from which the movement starts (unpublished observations).

Velocity profiles and head retraction

Concerning the timing of joint rotations, three basic types of movement pattern can be proposed. First, the joint rotations could follow a pattern that implies a sequential timing of changes in joint angle from proximal to distal. Secondly, the timing of joint rotations might show a random pattern, but this is difficult to associate with the observed stereotypy. Thirdly, joint rotations can be synchronized, which should nicely integrate with the concept of the fixed motor pattern (see above). To tackle this, the time variables of head retraction and joint rotations (based on peak-velocity ; see Table 1) for each trial were statistically analysed using a Friedman Two-way Anova (NORUSIS, 1986). This test reveals a ranking in timing of joint rotations (Chi-Square : 24.9 ; D.F. : 6 ; P = 0.0004 ; n = 20 ; Table 2). However, this time ranking differs from the geometric ranking (*i.e.* position in the neck : no bike chain principle), and only two of the joints (C3 − 2 and C5 − 4 : *i.e.* the extremes of the ranking) show a significant difference in timing relative to the head (Wilcoxon Matched-pairs Signed-rank test, Table 2). This means that all cervical joints (except C3 − 2 and C5 − 4) and the head tend to peak together. The peak velocity in joint C3 − 2 occurs significantly later than the peak velocity of the head. This is because, during retraction, the head often remains partly oriented in anterior direction. Only at the very end of the retraction phase, this joint increases its flexion. Peak velocity in C5 − 4 precedes that of head retraction significantly. This might be related to the insertion morphology of RCC_1 (on all vertebrae in front of this joint ; see Fig. 2B).

HEIDWEILLER *et al.* (1992) described the kinematics of cervical movements during upstroke in 1 to 4 week old chickens as a « bike chain » pattern (*i.e.* rotations in neighbouring joints succeed each other in time). This bike chain pattern could be related to movement principles that minimize the rotational forces or maximize the rotation efficiency. These authors also further postulate that the bike chain pattern might also be a consequence of the construction of the cervical column.

A sequential timing of joint rotations from proximal to distal as described in chickens is absent in *Chelodina*. In turtles, such a system might be advantageous when the neck must be withdrawn through a narrow orifice. This bike chain pattern is indeed observed in long necked cryptodiran turtles with an extremely flattened carapace, such as *Trionyx* (unpublished observations). However, in *Chelodina* the outer carapace chamber has a broad slit-like aperture and provides sufficient space for simultaneously rotating joints. The latter may be beneficial when retraction speed must be optimized. One can indeed observe that the head is first withdrawn very fast under the carapace, but that the neck remains partly exposed. Only in a second phase, smaller changes occur, positioning head and neck in their final, maximal retracted configuration. The final positioning involves changes in joint angle mainly in the posterior joints (especially C7 − 6).

Correlating the peak velocity of head retraction with these of the cervical joints may permit the recognition of important centres of motion in the neck of *Chelodina*. Notice however that linear and angular velocities are related to each other. However, the linear velocity of the head is the result of joint rotations (see VAN INGEN SCHENAU, 1989). Joints showing a significant correlation with the head are believed to be the main effectors of the retraction. Joints which do not correlate are more independent in their speed profiles and probably have primarily a steering function. A canonical analyses was carried out on the peak-velocity data of Table 1. The results are presented in Table 3. Based on an univariate approach high correlations between the head and four specific joints (C4−3, C6−5, C8−7, C9−8) are found. Based on the multivariate approach, the new canonical variable (see standardized canonical coefficients in Table 3) is significantly correlated with the head (Likelihood Ratio : 0.08784 ; F=17.8 ; DF=7,12 ; P=0.0001). High correlations are found for only two joints ; C6−5 and C8−7. These are the major bending points in the neck judging their final angles (see Fig. 9), and are precisely the joints which always show an initial angle (see above). The significant correlation found for C4−3 and D1−C8 in the univariate approach indicates that a relationship must be present between D1−C8 and C4−3 and one or more other cervical joints. Table 4 shows the correlation matrix between the different joints (canonical analyses based on peak-velocity data). Joints C9−8 and C4−3 correlate significantly with the two major bending points in the neck C6−5 and C8−7. As expected, there is a high pairwise correlation between these two as well.

The m. retrahens capitis et collique

The Retrahens muscle complex is one of the most striking muscles in the neck and body of *Chelodina*. The RCC_1 runs from the base of the skull to the last dorsal vertebrae at the level of the pelvic girdle. In other words, the length of the muscle almost equals this of the entire animal. This is because, besides force, contraction speed as well as contraction range are very important for this muscle. Putting more contractile units (sarcomeres) in series increases the speed of the muscle (*e.g.* Josephson, 1975). Moreover, given a certain shortening distance, the longer the muscle fibres are (= more sarcomeres), the closer to the optimum of the length-tension curve they can maintain while working, thus ensuring a continuously high force output. To avoid non-uniform distribution of the contractile state (= sarcomere length = number of cross bridges) over the length of the fibre, leading to contracting fibre segments extending others, a serial arrangement of shorter muscle fibres, combined to a polysegmental and polyneural innervation would be expected (GANS and DE VREE, 1987). Such an arrangement is indeed found in the short necked cryptodiran turtle *Pseudemys (Trachemys) scripta* (CALLISTER *et al.*, 1992).

TABLE 1

Chelodina longicollis : *Absolute values of peak-velocities (upper left value in each cell) and corresponding time from initiating of retraction (lower right value in each cell) for each joint of 20 head retractions. Top half represents 10 head retractions of Chelo #1. Bottom half represents 10 head retractions of Chelo #2). Bold numbers : maximal peak-velocity observed for each joint in all trials (Head in %/s^{-1}, joints in rad.s^{-1}, time in sec.)*

	chelo1	*chelo1*	*chelo1*	*chelo1*	*chelo1*	*chelo1*	*chelo1*	*chelo1*	*chelo1*	*chelo1*
HEAD	281 0.18	107 1.06	93 0.62	141 0.68	358 0.22	341 0.22	350 0.70	**450** 0.82	380 1.02	160 0.20
C3−2	2.58 0.32	2.76 1.06	1.67 0.54	0.56 0.84	1.69 0.26	3.56 0.24	4.01 0.80	4.19 0.80	**8.20** 1.00	4.36 0.20
C4−3	4.59 0.24	1.29 1.02	1.19 0.40	2.11 0.72	3.75 0.26	4.59 0.16	7.33 0.70	**20.93** 0.82	17.62 0.94	4.54 0.16
C5−4	6.84 0.16	10.54 1.02	3.70 0.50	1.88 0.68	5.09 0.22	5.02 0.20	6.98 0.68	**7.33** 0.78	4.71 0.98	2.09 0.16
C6−5	3.73 0.14	0.49 1.06	4.12 0.56	1.92 0.66	5.02 0.20	1.57 0.12	6.98 0.72	**8.72** 0.78	5.23 0.98	4.01 0.18
C7−6	1.24 0.18	0.51 1.02	2.44 082	0.49 0.72	3.73 0.18	**5.16** 0.12	2.62 0.72	2.97 0.80	1.31 1.02	2.09 0.18
C8−7	3.77 0.18	0.40 1.01	2.60 0.54	1.26 0.64	4.61 0.18	3.11 0.16	6.11 0.70	**7.15** 0.82	6.98 1.00	3.14 0.22
D1−C8	3.02 0.18	0.52 0.90	1.06 0.60	1.62 0.66	2.08 0.24	1.33 0.16	2.62 0.68	**4.01** 0.86	3.84 1.08	2.44 0.18
	chelo2	*chelo2*	*chelo2*	*chelo2*	*chelo2*	*chelo2*	*chelo2*	*chelo2*	*chelo2*	*chelo2*
HEAD	470 0.24	458 0.18	**591** 0.26	305 0.30	190 0.7	230 0.26	280 0.62	510 0.68	580 0.76	70 0.5
C3−2	**7.03** 0.28	6.84 0.20	9.18 0.30	1.67 0.68	5.58 0.70	3.49 0.30	7.85 0.66	8.90 0.70	4.71 0.76	5.06 0.50
C4−3	9.77 0.22	11.20 0.16	23.64 0.26	3.33 0.26	4.45 0.68	13.61 0.24	10.21 0.60	**24.34** 0.68	17.27 0.78	5.23 0.48
C5−4	4.10 0.22	2.46 0.16	**7.83** 0.24	5.16 0.30	6.45 0.72	4.88 0.26	3.49 0.64	6.98 0.64	4.36 0.78	3.84 0.48
C6−5	4.57 0.24	3.72 0.18	**13.40** 0.24	3.31 0.28	3.84 0.76	5.23 0.24	5.58 0.62	9.07 0.64	11.51 0.76	3.05 0.54
C7−6	0.91 0.22	0.40 0.22	3.16 0.26	0.51 0.28	1.92 0.72	0.87 0.26	**3.31** 0.62	1.74 0.70	1.92 0.78	1.31 0.48
C8−7	5.44 0.22	5.09 0.18	**10.61** 0.26	0.72 0.32	3.66 0.72	3.84 0.26	4.88 0.58	8.72 0.70	9.94 0.82	1.74 0.58
D1−C8	3.00 0.24	3.37 0.18	**5.02** 0.26	1.33 0.32	1.92 0.18	3.49 0.26	2.62 0.62	4.36 0.70	4.01 0.80	2.09 0.56

TABLE 2

Chelodina longicollis : *Statistical analysis of the relative time of peak-velocities. Middle column : Ranking of the timing of joint rotations based on a Friedman Two-way Anova of the time variables. Last column : Pairwise comparison of the relative times within trials (Wilcoxon matched-pairs signed-ranks tests. P= probabilities for coinciding timing of peak-velocity of head retraction and joint rotation)*

Joint	*mean-ranking (Friedman Anova)*	*P (Wilcoxon)*
C5−4	2.72	0.0061
C6−5	3.22	0.0783
C4−3	3.30	0.3942
C7−6	4.07	0.9547
HEAD	4.20	—
C8−7	4.40	0.9721
C9−8	4.72	0.2489
C3−2	5.55	0.0115

TABLE 3

Chelodina longicollis : *Statistical analysis of the peak-velocities. Column A and B : Univariate approach. A. correlations between each joint and the head. B. correlation between each joint and the new canonical variable. Column C. Multivariate approach (taking in to account correlations between the joints). Standardized canonical coefficient for each joint in the new canonical variable (bold numbers : high correlations)*

	Univariate		*Multivariate*
Joint	*R*	*Correlations between variables and the canonical variable*	*Standardized canonical coefficients*
C3−2	0.584	0.6114	−0.142
C4−3	**0.708**	**0.7407**	0.036
C5−4	0.518	0.5424	−0.040
C6−5	**0.798**	**0.8359**	**−0.580**
C7−6	0.245	0.2565	−0.114
C8−7	**0.924**	**0.9678**	**1.637**
C9−8	**0.816**	**0.8547**	0.011
	A	**B**	**C**

TABLE 4

Chelodina longicollis : *Pairwise correlations between peak-velocities of the different cervical joints (bold numbers : $P<0.001$)*

	C3−2	*C4−3*	*C5−4*	*C6−5*	*C7−6*	*C8−7*	*C9−8*
C3−2	1						
C4−3	0.58	1					
C5−4	0.36	0.43	1				
C6−5	0.52	**0.77**	0.65	1			
C7−6	−0.00	0.29	0.46	0.55	1		
C8−7	0.63	**0.78**	0.64	**0.94**	0.42	1	
C9−8	0.60	**0.83**	0.58	**0.82**	0.25	**0.88**	1

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REFERENCES

BOJANUS, L.H. (1819) — Anatome Testudinis Europaeae, Volume 1. Vilnae, Luthuania. Republished in 1970, *Facsimile reprints in Herpetology*, nr. 26, Society for the study of amphibians and reptiles, Ohio.

BOJANUS, L.H. (1821) — Anatome Testudinis Europaeae, Volume 2. Vilnae, Luthuania. Republished in 1970, *Facsimile reprints in herpetology*, nr. 26, Society for the study of amphibians and reptiles, Ohio.

CALLISTER, R.J., R. CALLISTER and E.H. PETERSON (1922) — Design and control of the head retractor muscle in a turtle, *Pseudemys (Trachemys) scripta*. II. Efferent innervation. *J. Comp. Neurol.*, **325** : 422-434.

ELSHOUD, G.C.A. and G.A. ZWEERS (1987) — Avian Cranio-cervical Systems part III : Robot Kinematics for Cervical Systems. *Acta Morphol. Neerl.-Scand.*, **25** : 235-260.

GAFFNEY, E.S. (1975) — A Phylogeny and Classification of the Higher Categories of Turtles. *Bull. Am. Nat. Hist.*, **155** : 389-436.

GANS, C. and F. DE VREE (1987) — Functional bases of fibre length and angulation in muscle. *J. Morph.*, **192** : 63-85.

GANS, C. (1992) — Why develop a Neck ? In : Berthoz, Vidal and Graf (eds.), *The Head-Neck Sensory Motor System*, Oxford University Press : 17-21.

GEORGE, J.C. and R.V. SHAH (1954) — The myology of the head and neck of the common Indian pond turtle, *Lissemys punctata granosa* (Schoepff). *J. Anim. Morph. Physiol.*, **1** : 1-12.

GEORGE, J.C. and R.V. SHAH (1955) — The myology of the head and neck of the Indian tortoise, *Testudo elegans*. *J. Anim. Morph. Physiol.*, **2** : 1-13.

HEIDWEILLER, J., A.H.J. VAN DER LEEUW and G.A. ZWEERS (1992) — Cervical kinematics during drinking in developing chickens. *J. exp. Zool.*, **262** : 135-153.

HOFSTETTER, R. and J.P. GASC (1969) — Vertebrae and ribs of modern reptiles. In : Gans and Parsons (eds.), *Biology of Reptilia*, vol. 1, Academic Press : 201-231.

JOSEPHSON, R.K. (1975) — Extensive en intensive Factors determining the performance of striated muscle. *J. exp. Biol.*, **194** : 135-154.

KOLOOS, J.G.M. and G.A. ZWEERS (1989) — Mechanics of drinking in the Mallard (*Anas platyrhynchos* ; Anatidae). *J. Morphol.*, **199** : 327-347.

LANDOLT, R. and G.A. ZWEERS (1985) — Anatomy of the muscle-bone apparatus of the cervical system in the mallard (*Anas platyrhynchos*). *Neth. J. Zool.*, **34** (4) : 611-670.

NORUSIS, M.J. (1986) — SPSS/PC$^+$ for the IBM PC XT/AT. Chicago, Illinois : SPSS Inc.

OGUSHI, K. (1913) — Anatomische Studien an der japanischen dreikralligen Lippenschildkröte (*Trionyx japonicus*). *Morph. Jb.*, **46** : 299-562.

ROMER, A.S. and T.S. PARSONS (1977) — *The vertebrate body*. W.B. Saunders Company, 624 pp.

SCANLON, T.C. (1982) — Anatomy of the neck of the Western Painted turtle (*Chrysemys picta belli* Gray ; Reptilia, Testudinata). Unpublished Ph.D. dissertation. The University of Michigan : University Microfilms International, 283 pp.

SHAH, R.V. (1963) — The neck musculature of a Cryptodire (*Deirochelys*) and a Pleurodire (*Chelodina*) compared. *Bull. Mus. Comp. Zool.*, **129** : 343-368.

VAILLANT, M.L. (1881) — Mémoire sur la disposition des vertèbres cervicales chez les chéloniens. *Ann. Sci. nat. Zool. Paleont.*, (6) **10** (7) : 1-106.

VALLOIS, H.V. (1922) — Les transformations de la musculature de l'épisome chez les vertébrés. *Arch. Morph. gen. exp.*, **13** : 1-538.

VAN INGEN SCHENAU, G.J. (1989) — From rotation to translation : constraints on multijoint movements and the unique action of bi-articular muscles. *Human Movement Science*, **8** : 301-337.

WEISGRAM, J. and H. SPLECHTNA (1990) — Intervertebral movability in the neck of two turtle species (*Testudo hermanni, Pelomedusa subrufa*). *Zool. Jb. Anat.*, **120** : 425-431.

WEISGRAM, J. and H. SPLECHTNA (1992) — Cervical movements during feeding in *Chelodina novaeguinaeae* (Chelonia, Pleurodira). *Zool. Jb. Anat.*, **122** : 331-337.

WILLIAMS, E.E (1950) — Variation and selection in the cervical central articulations of living turtles. *Bull. Amer. Mus. nat. Hist.*, **94** (9) : 505-562.

WOOD, G.A. (1982) — Data smoothing and differentiation procedures in Biomechanics. *Exercise and Sports Sciences Reviews*, **10** : 308-362.

ZWEERS, G.A. (1982) — Drinking of the pigeon (*Columba livia L.*). *Behaviour*, **80** : 274-317.

ZWEERS, G.A., A.F.Ch. GERRITSEN and P.J. VAN KRANENBURG-VOOGD (1977) — Mechanics of feeding of the Mallard. *Contr. Vert. Evol.*, **3** : 1-110.

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EPIDERMAL GROWTH FACTOR (EGF) IN THE QUAIL OVARY

by

LUC VAN NASSAUW[1], RONALD DE DEURWAERDER, ANTHRANILLA LEEUWESTEYN, FERNAND HARRISSON and MARC CALLEBAUT

RUCA, Laboratory of Human Anatomy and Embryology, Groenenborgerlaan 171, B-2020 Antwerpen

[1]Senior Research Assistant of the Belgian National Fund for Scientific Research

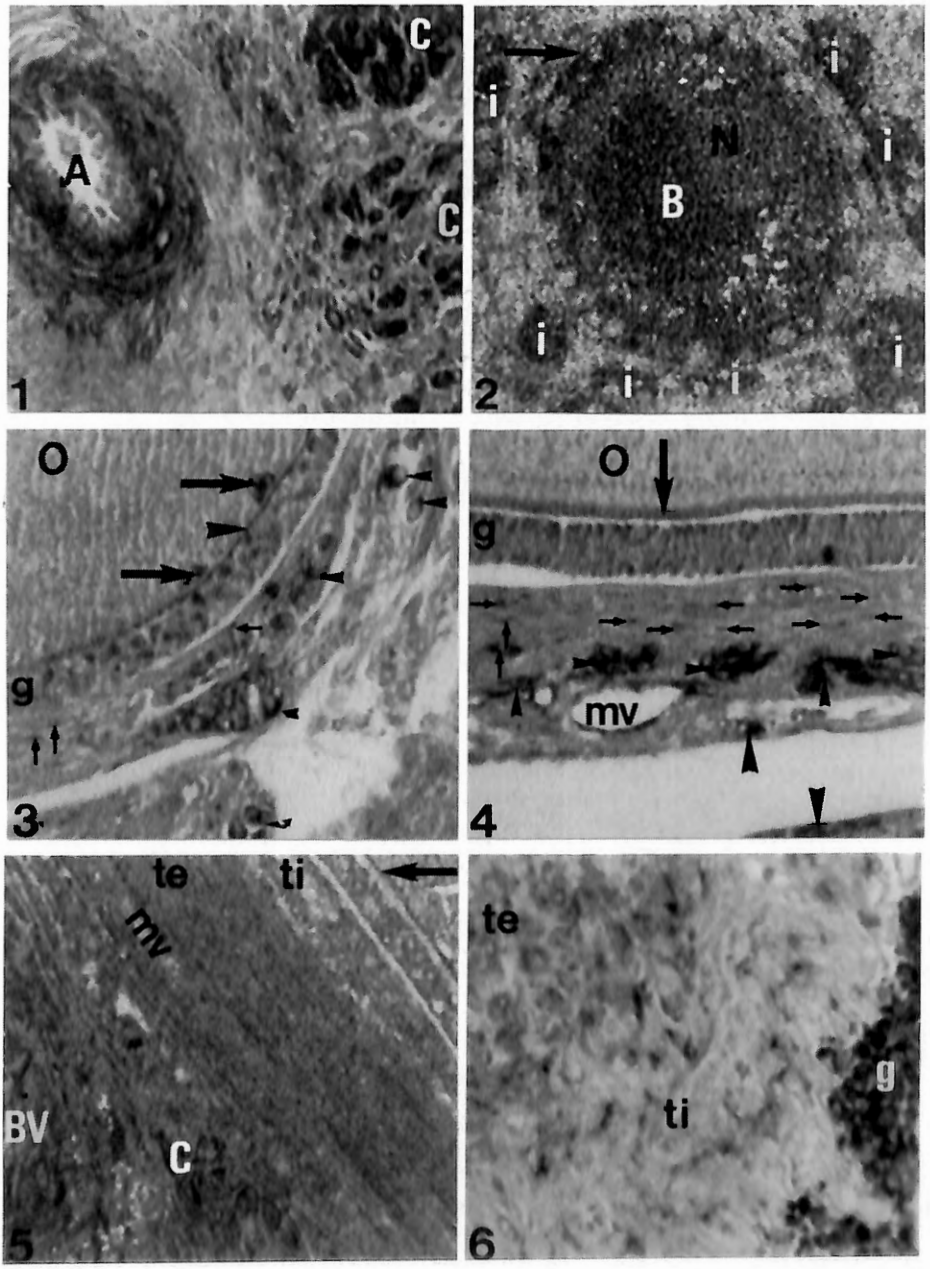
SUMMARY

Using immunocytochemical techniques for the localization of epidermal growth factor (EGF) in the quail ovary, we observed a major amount of EGF in smooth muscle cells of blood vessels and of chordae, in interstitial cells, in granulosa cells of small follicles, in the Balbiani complex of prelampbrush oocytes, in nerve cells, and in the cells of granulosa and theca externa of postovulatory follicles. In general, the staining intensity of granulosa cells decreased during folliculogenesis, and increased after ovulation. In the oocyte, immunoreactivity was shifted from the Balbiani complex to the zona radiata during development. These results support the hypothesis that EGF primarily acts on less differentiated follicles. It is also suggested that EGF can modulate ovarian contractile processes.

Keywords : Epidermal growth factor, quail, ovary.

INTRODUCTION

Ovarian folliculogenesis is a dynamic and complex process, which is regulated by the interplay of several factors, including growth factors. The present study focuses on EGF, a small single-chain polypeptide (MW 6043) originally detected during a search for nerve growth-promoting factors in the mouse (LEVI-MONTALCINI and COHEN, 1960). It is best recognized for its mitogenic activity. Distribution and role of EGF in the ovary have mainly been investigated in mammals (reviewed by MULHERON and SCHOMBERG, 1993). In this study, we have localized EGF in the quail ovary.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MATERIAL AND METHODS

Female adult Japanese quails (*Coturnix coturnix japonica L.*) were killed by decapitation. Their ovaries were fixed in EACH fixative (PERRY-O'KEEFE *et al.*, 1990), Carnoy's fluid, methacarn (PUCHTLER *et al.*, 1970), or Bouin's fixative. After tissue processing, the tissue blocks were embedded in paraffin or in ImmunoBed (TM) (Polysciences Inc., Warrington, PA), a plastic embedding medium. We used commercially available antibodies : rabbit polyclonal antibodies directed against mouse EGF (SIGMA Chemical Co., St.-Louis, MO) or against human EGF (Santa Cruz Biotechnology Inc., Santa Cruz, PA), and a mouse monoclonal antibody directed against human EGF (Oncogene Science Inc., Uniondale, NY). EGF was localized in paraffin sections using the unlabelled antibody peroxidase-anti-peroxidase technique. Immunoreactivity (IR) was revealed by the method of GRAHAM AND KARNOVSKY (1966) or of SHU *et al.* (1988). In semi-thin plastic sections, EGF was localized using the immunogold-silver staining procedure. The oocytes and follicles were classified in stages according to CALLEBAUT (1973).

Method specificity was controlled by incubation with primary antibody preabsorbed with recombinant human EGF (Santa Cruz Biotechnology Inc.). Antibody specificity was tested using immunoblotting.

RESULTS

The three primary antibodies yielded the same results. IR was predominantly found in smooth muscle cells of blood vessels and of chordae (Fig. 1), in granulosa cells of prelampbrush follicles (Fig. 2), in interstitial cells (Figs 2-4), in nerve cells, in the Balbiani complex of prelampbrush oocytes (Fig. 2), in the zona radiata of developing oocytes (Figs 4, 5), and in granulosa cells and cells of the theca externa of postovulatory follicles (Fig. 6). The staining intensity of granulosa cells decreased during follicular maturation, and increased after ovulation. In the oocyte, IR was shifted from the Balbiani complex to the zona radiata during oocyte development. IR was also detected in some cells of atretic follicles, of the superficial epithelium, of the tunica albuginea, and of the theca of developing follicles (Figs 3-5). In one

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Figs 1-6. — Micrographs of the quail ovary (× 446). — 1. Medulla : IR in smooth muscle cells of blood vessels (A : artery) and of chordae (C). — 2. Cortex : IR in granulosa (arrow) of prelampbrush follicles, in interstitial cells (i), and in the Balbiani complex (B). N : nucleus. — 3. Early lampbrush follicle : IR in interstitial cells (small arrowheads), in thecal cells (small arrows), in granulosa (g), in cortical ooplasm (arrowhead), and in cells (arrows) engulfed by the oocyte (O). — 4. Stalked follicle : IR in zona radiata (arrow), in granulosa, in thecal cells (small arrows), in cell clusters in thecal periphery (small arrowheads), in smooth muscle cells of the middle venous layer (mv), and in branches of chordae (arrowheads). — 5. Mature preovulatory follicle : IR in wall of blood vessels (BV), in chordae, in thecal cells, and in zona radiata (arrow). ti : theca interna ; te : theca externa. — 6. Postovulatory follicle : IR in granulosa cells, in cells of theca interna, and in contracted cells of theca externa.

of the ovaries, positively stained engulfed cells in the ooplasm of developing oocytes, were observed (Fig. 3).

DISCUSSION

In a previous study, ONAGBESAN *et al.* (1993) demonstrated the presence of an EGF-like peptide in the theca of preovulatory follicles of the hen. In the present study, we revealed that EGF can be found in several ovarian cell types. We noticed that in some mammals, EGF is also detected in interstitial cells (GÖRITZ *et al.*, 1994 ; KANNO *et al.*, 1994), in thecal and granulosa cells (ROY and GREENWALD, 1990 ; MARUO *et al.*, 1993 ; GÖRITZ *et al.*, 1994 ; KANNO *et al.*, 1994), and in oocytes (KASSELBERG *et al.*, 1985 ; ROY AND GREENWALD, 1990 ; MARUO *et al.*, 1993). Moreover, ROY and GREENWALD (1990) showed in the hamster that staining intensity in granulosa cells fades during folliculogenesis.

A few *in vitro* experiments, examining the role of EGF in the hen ovary, were performed (reviewed by PEDDIE *et al.*, 1993). It was found that EGF stimulates proliferation of the granulosa and theca. EGF attenuates gonadotropin action and inhibits steroidogenesis. It prevents premature differentiation of granulosa cells. The effects of EGF are decreasing with follicular maturity.

These results and data support the hypotheses that EGF primarily acts on less differentiated follicles (MULHERON and SCHOMBERG, 1993), and that EGF potentially produced by thecal interstitial cells (paracrine) or granulosa cells (autocrine) acts on granulosa cells *in vivo* (JOHNSON, 1994).

Relying on the presence of EGF in ovarian smooth muscle cells, abundantly present in the avian ovary (VAN NASSAUW *et al.*, 1994), the existence of ovarian contractility (VAN NASSAUW *et al.*, 1994), and the data concerning contractile effects of EGF (HOLLENBERG *et al.*, 1989 ; PETITCLERC *et al.*, 1994), it is suggested that EGF may modulate ovarian contractile processes. Finally, the presence of positively stained engulfed cells in the ooplasm of developing oocytes is a rare event, the meaning of which remains unclear.

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REFERENCES

CALLEBAUT, M. (1973) — Correlation between germinal vesicle and oocyte development in the adult Japanese quail (*Coturnix coturnix japonica*) : a cytochemical and autoradiographic study. *J. Embryol. exp. Morph.*, **29** : 145-157.

GÖRITZ, F., K. JEWGENOW, F. KLIMA, C. PITRA and H.H.D. MEYER (1994) — Epidermal growth factor (EGF) and epidermal growth factor receptor (EGF-R) in ovary of domestic cat (*Felis catus*). *A.R.T.A.*, **6b** : 77-78.

GRAHAM, R.C. Jr. and M.J. KARNOVSKY (1966) — The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney : ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.*, **14** : 291-302.

HOLLENBERG, M.D., I. MURAMATSU, H. IIOH, P. PATEL, S.-G. YANG and K. LEDERIS (1989) — Contractile actions of epidermal growth factor-urogastrone in isolated smooth muscle preparations from guinea pig stomach : structure-activity relationships and comparison with the effects of human transforming growth factor-alpha. *J. Pharmacol. Exp. Ther.*, **248** : 384-390.

JOHNSON, A.L. (1994) — Gonadotropin and growth factor regulation of avian granulosa cell differentiation. In : *Perspectives in Comparative Endocrinology* : edited by National Research Council of Canada. National Research Council of Canada, Canada : 613-618.

KANNO, Y., S. KOIKE and T. NOUMURA (1994) — Immunohistochemical localization of epidermal growth factor in the developing rat gonads. *Zool. Sci.*, **11** : 83-87.

KASSELBERG, A.G., D.N. ORTH, M.E. GRAY and M.T. STAHLMAN (1985) — Immunocytochemical localization of human epidermal growth factor/urogastrone in several human tissues. *J. Histochem. Cytochem.*, **33** : 315-322.

LEVI-MONTALCINI, R. and S. COHEN (1960) — Effects of the extract of the mouse submaxillary salivary glands on the sympathetic system of mammals. *Ann. Rev. N. Y. Acad. Sci.*, **85** : 324-341.

MARUO, T., C.A. LADINES-LLAVE, T. SAMOTO, H. MATSUO, A.S. MANALO, H. ITO and M. MOCHIZUKI (1993) — Expression of epidermal growth factor and its receptor in the human ovary during follicular growth and regression. *Endocrinology*, **132** : 924-931.

MULHERON, G.W. and D.W. SCHOMBERG (1993) — The intraovarian transforming growth factor system. In : *The Ovary*. : edited by E.Y. Adashi and P.C.K. Leung. Raven Press Ltd., New York : 337-361.

ONAGBESAN, M., M. PEDDIE and I. WOOLVERIDGE (1993) — Demonstration of EGF/TGFα peptide in ovarian thecal cells of the domestic hen. *J. Endocrinol.*, Suppl. **137** : p. 175.

PEDDIE, M., M. ONAGBESAN and I. WOOLVERIDGE (1993) — The role of epidermal growth factor and other factors in the paracrine and autocrine control of ovarian follicular development in the domestic hen. In : *Avian Endocrinology*. : edited by P.J. Sharp. J. Endocrinol. Ltd., Bristol : 321-330.

PERRY-O'KEEFE, H., C.R. KINTNER, J. YISRAELI and D.A. MELTON (1990) — The use of *in situ* hybridisation to study the localisation of maternal mRNAs during *Xenopus* oogenesis. In : *In situ hybridisation : application to developmental biology and medicine*. : edited by N. Harris and D.G. Wilkinson. Cambridge University Press, Cambridge : 115-130.

PETITCLERC, E., P.E. POUBELLE and F. MARCEAU (1994) — Epidermal growth factor-induced rapid relaxation of the isolated rabbit mesenteric artery. *Eur. J. Pharmacol.*, **259** : 91-94.

PUCHTLER, H., F.S. WALDROP, S.N. MELOAN, M.S. TERRY and H.M. CONNER (1970) — Methacarn (methanol-Carnoy) fixation. Practical and theoretical considerations. *Histochemie*, **21** : 97-116.

ROY, S.K. and G.S. GREENWALD (1990) — Immunohistochemical localization of epidermal growth factor-like activity in the hamster ovary with a polyclonal antibody. *Endocrinology*, **126** : 1309-1317.

SHU, S., G. JU and L. FAN (1988) — The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci. Lett.*, **85** : 169-171.

VAN NASSAUW, L., F. HARRISSON and M. CALLEBAUT (1994) — Contractile activity of avian ovarian follicles. *A.R.T.A.*, **6** : 113-127.

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ALLATOSTATIC AND ALLATOTROPIC FACTORS IN THE BRAIN OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA*

by

DIRK VEELAERT[1], STEPHEN S. TOBE[2], C. G. YU[2],
LILIANE SCHOOFS[1] and ARNOLD DE LOOF[1]

[1]Zoological Institute, Katholieke Universiteit Leuven,
Naamsestraat 59, B-3000 Leuven ;
[2]Department of Zoology, University of Toronto, Ontario, Canada M5S 1A1

SUMMARY

A polyclonal antibody against allatostatin-5 (Dip-AST 2) of *Diploptera punctata* was used to demonstrate allatostatin-like material in the brain of *Schistocerca gregaria*. Immunoreactivity was found in cells of the pars lateralis and in axons running to the corpus allatum (CA). A methanolic brain extract of 7000 brains was fractionated by HPLC on a reversed phase C18 column. Using a sensitive juvenile hormone biosynthesis bioassay, we detected several fractions that influence juvenile hormone synthesis by the corpora allata of the cockroach *Diploptera punctata*. Four fractions have strong allatostatic activity and three fractions have allatotropic activity.

Key words : neuropeptide, corpora allata, juvenile hormone.

INTRODUCTION

The corpora allata (CA) of insects are the major sites of production of juvenile hormone (JH) which plays a central role in the control of metamorphosis and reproduction in most insects (TOBE and STAY, 1985). These small glands are connected to the brain by two pairs of nerves : the nervi corporis cardiaci I (NCA I), which originate in neurosecretory cells of the pars lateralis and the NCA II, which originate in cells of the suboesophageal ganglion (RADEMAKERS, 1977 ; KONINGS *et al.*, 1989) (Fig. 1). JH biosynthesis and release has been the subject of extensive research and two groups of signalling substances have been reported : the allatotropins (WILLIAMS,1961 ; GIRARDIE, 1967), responsible for stimulation of JH biosynthesis and the allatostatins (TOBE, 1980) responsible for inhibition. Recently, seven allatostatins were isolated form the cockroach, *Diploptera punctata* (WOODHEAD *et al.*, 1989, 1994 ; PRATT *et al.*, 1991), two from the cockroach *Periplaneta americana* (WEAVER *et al.*, 1994), five from the fly *Calliphora vomitoria*

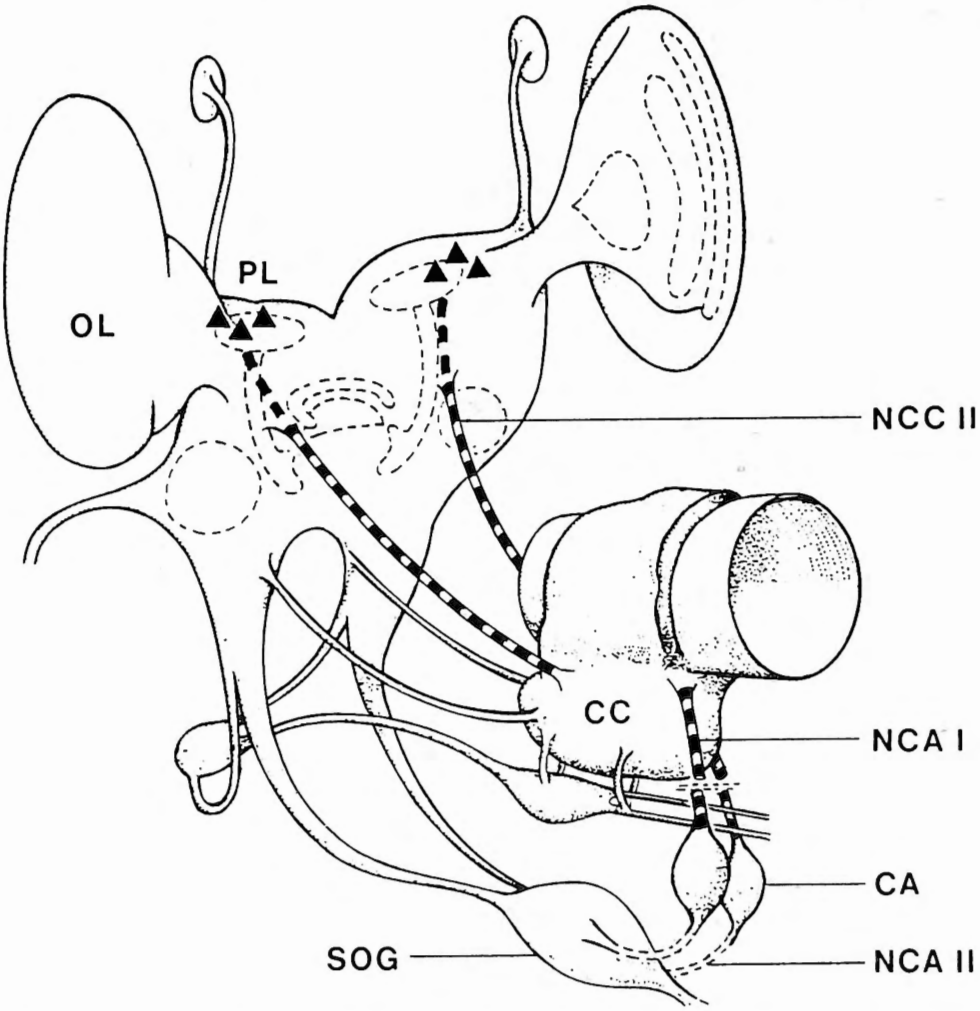

Fig. 1. — Diagram of corpora allata (CA) (KONINGS *et al.*, 1989) of *Schistocerca gregaria* with their connections to the brain via the nervi corporis cardiaci II (NCC II) and the nervi corporis allati I (NCA I) and to the suboesophageal ganglion (SOG) via the nervi corporis allati II (NCA II). Allatostatin immunoreactive cell bodies projecting to CA : closed triangles. PL, pars lateralis ; CC, corpus cardiacum ; OL, optic lobe.

(DUVE *et al.*, 1993) and one from the tobacco hornworm *Manduca sexta* (KRAMER *et al.*, 1991).

In locusts the presence of allatotropic factors (FERENZ and DIEHL, 1983 ; FERENZ 1984 ; GADOT and APPLEBAUM,1985 ; REMBOLD *et al.*, 1986 ; COUILLAUD and GIRARDIE 1990 ; LEHMBERG *et al.*, 1992) and allatostatic factors (VEELAERT *et al.*, 1995) has been reported but none have yet been purified. In this paper we

describe the presence of allatostatic and allatotropic factors in an extract of 7000 brains of *Schistocerca gregaria* and their partial purification by HPLC on a reversed phase C18 column.

MATERIAL AND METHODS

Animals and immunocytochemistry

Schistocerca gregaria (Forskal) was raised according to ASHBY (1972). Brains from adults were dissected in locust saline (NaCl 168 mM, KCl 6.4 mM, $NaHPO_4$ 1.2 mM, $NaHCO_3$, 3.0 mM, $MgCl_2$ 3.6 mM, $CaCl_2$ 2.1 mM). Immunohistochemical staining was accomplished by means of the peroxidase-anti-peroxidase method as described by VANDESANDE and DIERICKX (1976). Tissue preparation and specificity was controlled as described by VEELAERT *et al.* (1995).

Tissue extraction and HPLC purification

7000 brains of *Schistocerca gregaria* were extracted in methanol/water/acetic acid (90:9:1) and concentrated on C18 reversed phase Sep-pak cartridges (Waters Associates, Milford, MA). The Sep-pak cartridges were subsequently eluted with 50 % acetonitrile containing 0.1 % TFA and with 80 % acetonitrile containing 0.1 % TFA. The 50 % eluate was chromatographed on a Delta-Pak C18 column (250 × 10 mm) (Waters Associates, Milford, MA) on a Gilson HPLC system. HPLC conditions were as follows : solvent A, 0.1 % TFA in water ; Solvent B, 50 % acetonitrile in 0.1 % aqueous TFA. Initial conditions : 100 % A, then linear gradient to 100 % B over 180 min ; flow rate 6 ml/min ; detector : 5 absorption units full scale (AUFS) set at 214 nm. Peaks were collected manually every 4 minutes.

Juvenile hormone biosynthesis assay

Samples of 11 brain equivalents were dried in a Speedvac concentrator and assayed on the CA of *Diploptera punctata* (Eschscholtz) according to TOBE and CLARKE (1985). They were dissolved in methionine-free medium 199 containing Ficoll (20 mg/ml) and L-[methyl]-^{14}C-methionine (2.1GBq/mmol ; Amersham). Following 3-h incubations, samples were extracted and quantified as described by TOBE and CLARKE (1985). Eight glands were tested per group. Inhibition was determined by comparison with control glands, incubated at the same time (parallel incubations), to which no extract had been added. This established the control rates of JH release, and rates of release by treated glands were then expressed as a percentage of the control values.

RESULTS AND DISCUSSION

Allatostatin immunoreactivity is found in three cells of the pars lateralis (Fig. 2) with axons projecting to the CA. These cells belong to a group of cells at the rostro-ventral part of the pars lateralis whose axons contribute to the NCC II and the NCA I (KONINGS *et al.*, 1989).

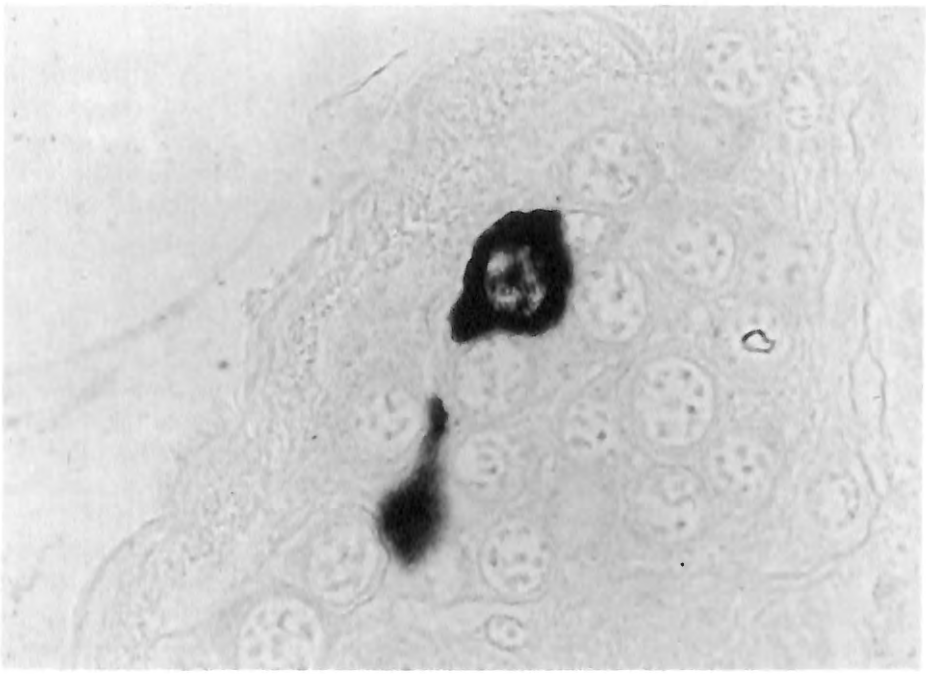

Fig. 2. — Allatostatin immunoreactivity in two cells in the pars lateralis of the brain of *Schistocerca gregaria*, 800 ×.

The inhibition of juvenile hormone biosynthesis by the fractions separated by HPLC is shown in Fig. 3. Fractions 12 till 23 show inhibition. Strong inhibition was found in fractions 15, 17, 18, 20 (more than 70 %). We found stimulation of juvenile hormone biosynthesis in fraction 7, 8 and 37 (more than 30 %).

Our results show that brain extracts of *Schistocerca gregaria* contain allatotropic and allatostatic factors and that it is possible to separate them by HPLC. Nothing is known about the nature of the allatotropic factors, but according to their retention times they must be very hydrophilic. Recently, VEELAERT *et al.* (1995) demonstrated that *Schistocerca gregaria* contains allatostatin (Dip-AST-2 ; DONLY *et al.*, 1993) -like material and that brain extracts of *Schistocerca gregaria* also inhibit juvenile hormone biosynthesis by the CA of *Locusta migratoria* and *Diploptera punctata*.

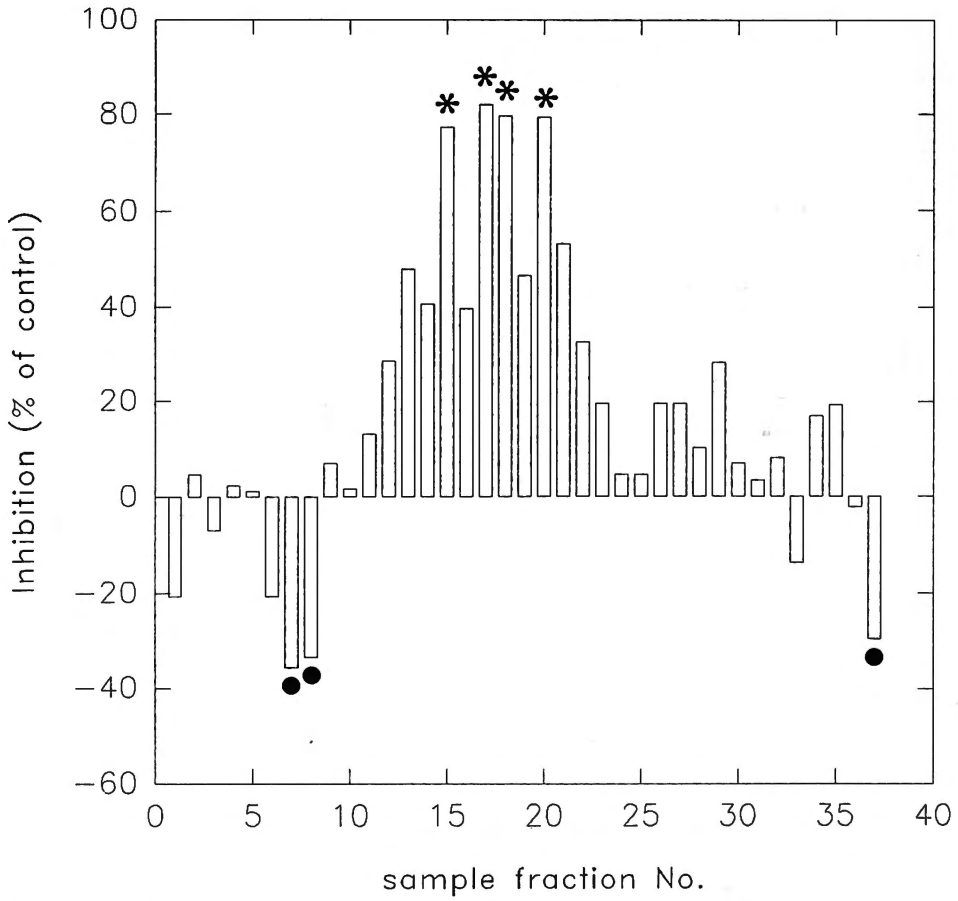

Fig. 3. — Inhibitory effect of 11 brain equivalents of *Schistocerca gregaria* on JH biosynthesis by 2 CA of virgin *Diploptera punctata* cockroaches (n = 8). *, allatostatic; ●, allatotropic.

Hence, *Schistocerca gregaria* contains an allatostatin-like peptide that might be involved in regulating the production of JH by the CA. Screening the HPLC fractions with the juvenile hormone biosynthesis assay in combination with an ELISA or RIA for allatostatin-like material is probably the best way to purify peptides affecting the CA. Progress is being made in the final purification of the biologically active peptides.

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REFERENCES

ASHBY, G. (1972) — Locusts. In : *The UFAW handbook on the care and management of laboratory animals.* Edited by UFAW, Churchill Livingstone, Edinburgh, London : 582-587.

COUILLAUD, F. and A. GIRARDIE (1990) — Disconnected corpora allata provide a novel assay for allatostimulating factors. *J. Exp. Zool.*, **253** : 330-333.

DONLY, B. C., Q. DING, S. S. TOBE and W. G. BENDENA (1993) — Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata. Proc. Natl. Acad. Sci. USA*, **90** : 8807-8811.

DUVE, H., A.H. JOHNSON, A.G. SCOTT, C.G. YU, K.J. YAGI, S.S. TOBE and A. THORPE (1993) — Callatostatins : neuropeptides from the blowfly *Calliphora vomitoria* with sequence homology to cockroach allatostatins. *Proc. Natl. Acad. Sci. USA*, **90**(6) : 2456-2460.

FERENZ, H.J. (1984) — Isolation of an allatotropic factor in *Locusta migratoria* and its effect on corpus allatum activity in vitro. In *: Biosynthesis, metabolism and mode of action of invertebrate hormones* (Ed. by Hoffman J. and Porchet M.) Springer, Berlin : 2-96.

FERENZ, H.J. and I. DIEHL (1983) — Stimulation of juvenile hormone biosynthesis in vitro by locust allatotropin. *Z. Naturforsch.*, **38**C : 856-858.

GADOT, M. and S.W. APPLEBAUM (1985) — Rapid in vitro activation of corpora allata by extracted locust brain allatotropic factor. *Arch. Insect Biochem. Physiol.*, **2** : 117-129.

GIRARDIE, A. (1967) — La pars intercérébralis chez *Locusta migratoria* et son role dans le développement. Thèse de doctorat, Strassbourg : 124 pp.

KONINGS, P.N.M., H.G.B. VULLINGS, O.J.M. KOK, J.H.B. DIEDEREN and W.F. JANSEN (1989) — The innervation of the corpus cardiacum of *Locusta migratoria* : a neuroanatomical study with the use of lucifer yellow. *Cell Tissue Res.*, **258** : 301-308.

KRAMER, S. J., A. TOSCHI, C. A. MILLER, H. KATAOKA, G. B. QUISTAD, J. P. LI, R. L. CARNEY and D. A. SCHOOLEY (1991) — Idenitification of an allatostatin from the tobacco hornworm *Manduca sexta. Proc. Natl. Acad. Sci. USA*, **88** : 9458-9462.

LEHMBERG, E., H.J. FERENZ and S.W. APPLEBAUM (1992) — Maturation and responsiveness to extracts of corpora allata from male *Locusta migratoria* containing allatotropic factors. *Z. Naturforsch.*, **47**(5-6) : 449-452.

PRATT, G.E., D.E. FARNSWORTH, K.F. FOK, N.R. SIEGEL, A.L. MCCORMACK, J. SHABANOWITZ, D.F. HUNT and R. FEYEREISEN (1991) — Identity of a second type of allatostatin from the cockroach brains : an octadecapeptide amide with a tyrosine-rich address sequence. *Proc. Natl. Acad. Sci. USA*, **88** : 2412-2416.

RADEMAKERS, L.H.P.M. (1977) — Identification of a secretomotor center in the brain of *Locusta migratoria*, controlling secretory activity of the adipokinetic hormone producing cells of the corpus cardiacum. *Cell Tissue Res.*, **184** : 381-395.

REMBOLD, H., B. SCHLAGINTWEIT and G.M. ULRICH (1986) — Activation of juvenile hormone synthesis in vitro by a corpus cardiacum factor from *Locusta migratoria. J. Insect Physiol.*, **32** : 91-94.

TOBE, S.S. (1980) — Regulation of the corpora allata in adult female insects. In : *Insect biology in the future : VBW 80*. Edited by Locke M and Smith DS. Academic Press, New York : 45-367.

TOBE, S.S. and N. CLARKE (1985) — The effect of L-methionine concentration on juvenile hormone biosynthesis by corpora allata of the cockroach *D. punctata. Insect Biochem.*, **15** : 175-179.

TOBE, S.S. and B. STAY (1985) — Structure and regulation of the corpus allatum. *Adv. Insect Physiol.*, **18** : 305-432.

VANDESANDE, F. and K. DIERICKX (1976) — Immunocytochemical demonstration of separate vasotocinergic and mesotocinergic neurons in the amphibian magnocellular neurosecretory system. *Cell Tissue Res.* **179** : 289-296.

VEELAERT, D., L. SCHOOFS, S.S. TOBE, C.G. YU, H.G.B. VULLINGS, F. COUILLAUD and A. DE LOOF (1995) — Immunological evidence for an allatostatin-like neuropeptide in the central nervous system of *Schistocerca gregaria, Locusta migratoria* and *Neobellieria bullata. Cell Tissue Res.*, **279** : 601-611.

WEAVER, R.J., Z.A. FREEMAN, M.G. PICKERING and J.P. EDWARDS (1994) — Identification of two allatostatins from the CNS of the cockroach *Periplaneta americana* : novel members of a family of neuropeptide inhibitors of insect juvenile hormone biosynthesis. *Comp. Biochem. Physiol.*, **107**C : 119-127.

WILLIAMS, C.M. (1961) — The juvenile hormone. II. Its role in the endocrine control for molting, pupation and adult development in the cecropia silkworm. *Biol. Bull.* (Woods Hole), **121** : 572-585.

WOODHEAD, A.P., B. STAY, S. L. SEIDEL, M.A. KHAN and S.S. TOBE (1989) — Primary structure of four allatostatins : neuropeptide inhibitors of juvenile hormone synthesis. *Proc. Natl. Acad. Sci. USA*, **86** : 5997-6001.

WOODHEAD, A.P., M. A. KHAN, B. STAY and S.S. TOBE (1994) — Two new allatostatins from brains of *Diploptera punctata. Insect Biochem. Mol. Biol.*, **24** : 257-263.

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GALLBLADDER CONTRACTIONS IN CHICKENS, GUINEA PIGS AND MICE FOLLOWING TREATMENT WITH SIMMONDSIN OR CHOLECYSTOKININ

by

SABIEN VERMAUT (1), PAUL BUSSELEN (2), STUART SPENCER (3), MARNIX COKELAERE (2), GERDA FLO (2) and EDDY DECUYPERE (1)
(1) Laboratory for Physiology and Immunology of Domestic Animals, Kardinaal Mercierlaan 92, B-3001 Heverlee (Belgium)
(2) Disciplinary Research Center, E. Sabbelaan 53, B-8500 Kortrijk (Belgium)
(3) AgResearch, Ruakura Agricultural Centre, East Street, Private Bag 3123, Hamilton, New Zealand

SUMMARY

The jojoba plant (*Simmondsia chinensis*) is a native oilseed shrub of the Sonoran desert. Feeding jojoba meal — a byproduct of the oil extraction — results in reduced food intake in rats, chickens, ewes and rodents. This inhibitory effect is due to the presence of a glycoside, simmondsin. Its satiating effect is either by a stimulation of the secretion of cholecystokinin (CCK) as a satiating factor or by acting as a CCK-agonist itself. In this study, the effect of CCK and simmondsin on gallbladder contraction *in vivo* and *in vitro* was tested using mice, guinea pigs and chickens as experimental models.

In vivo, total gallbladder contraction was observed after i.p. injection of a high dose of (mammalian) CCK-8 in mice, while no contraction at all was observed in chickens. *In vitro* however, the isolated gallbladders from both mice and chickens contracted, but the treshold for gallbladder contraction induced by CCK-8 was higher in chickens than in mice. Moreover, the contraction pattern was continuously pulsatile and oscillating in chickens while a single, large contraction was observed in mice. Simmondsin had no direct contractile effect on gallbladder strips in mouse, chicken or guinea pig. This suggests that it probably has its satiating effect by stimulating release of endogenous CCK. It was further demonstrated that simmondsin stimulated duodenal CCK secretion in guinea pig since gallbladder contractions *in vitro* were intensified, when the latter was flooded with duodenal secretions collected after *in vitro* treatment with simmondsin. Moreover, in this *in vitro* system a CCK-A antagonist (devapezide) neutralised the effect of duodenal CCK, obtained after simmondsin stimulation.

Keywords : gallbladder, CCK, simmondsin, chicken, mice, guinea pig.

INTRODUCTION

The jojoba plant (*Simmondsia chinensis*) is a native oilseed shrub of the Sonoran desert (BOOTH *et al.*, 1974). Feeding jojoba meal — a byproduct of the oil extraction — results in reduced food intake in rats (BOOTH *et al.*, 1974 ; COKELAERE *et al.*, 1992), chickens (NGOU NGOUPAYOU *et al.*, 1982 ; ARNOUTS *et al.*, 1993), ewes (MANOS *et al.*, 1986) and rodents (SHERBROOKE *et al.*, 1976). This inhibitory effect is due to the presence of a 2-cyanomethylene-3-hydroxy-4,5-dimethoxycyclohexyl - B-D-glycoside, called simmondsin (ELLIGER *et al.*, 1973, 1974a, 1974b ; BOOTH *et al.*, 1974). It appears that simmondsin exerts its satiating effect by a stimulation of the cholecystokinin (CCK) system, since its effect is inhibited by the simultaneous administration of devapezide, a peripheral CCK-receptor antagonist (COKELAERE *et al.*, in press). It is, however, not clear if simmondsin induces satiation by stimulating release of CCK, or by acting directly as a CCK-agonist. In order to discriminate between these two possibilities, the effect of simmondsin has been compared with the effects of CCK on gallbladder contractions *in vivo* and *in vitro*.

MATERIAL AND METHOD

Drugs used

Hexane-extracted jojoba meal was re-extracted with acetone in a Soxhlet extractor for 8 h. After crystallization, the mixture of simmondsin and its analogues, diluted in water, was put on a Sephadex column for preparative chromatography. The simmondsin fraction was purified by TLC (silica gel TLC plates Merck no. 5553 ; solvent, ethylacetate/ethanol 70 :30 with 10 % H_2SO_4) monitored by IR spectrophotometry (VAN BOVEN *et al.*, 1993).

Sulphated CCK octapeptide (CCK-8) was purchased from Sigma (St. Louis, USA) and devapezide (MK-329), a specific CCK-A-receptor antagonist, was donated by Merck Sharp and Dohme Research Laboratories. All solutions were prepared daily prior to injection. Before each injection they were diluted with physiological saline (0.9 % NaCl) to the required concentration. For the *in vitro* work, solutions were added to Krebs phosphosaline buffer (KPS ; pH 7.4, 37° C).

Experiments

In vitro

Strips of gallbladder of mice (BALB\C), guinea pig or chicken (Hisex) were mounted in a superfusion bath and connected to an isometric force transducer. After ca. 60 min of perfusion with KPS, the preparations became spontaneously active and sensitive to low doses of CCK-8. The effects of increasing concentrations of CCK-8 (1 nM CCK-8 up to 100 nM CCK-8) and 10 mM simmondsin were therefore tested after ca. 80 min of superfusion.

To investigate the possible secretion of endogenous CCK, chicken duodenum was isolated, turned inside out and perfused with KPS (serosal solution) in a re-circulatory fashion. This re-iterative way was chosen to concentrate any product released by the gut. The outside (mucosa) was also continuously bathed in KPS, but separated from the inside circulation. After 30 minutes of perfusion the serosal perfusate was collected (solution A) and replaced with a new KPS, to which 10 mM simmondsin was added. Following a further 30 minutes perfusion the perfusate was collected (solution B).

The serosal solutions were then tested for their contracting activity on chicken gallbladder. Similar perfusates were collected after incubation with guinea pig duodenum and were tested on guinea pig gallbladder.

After 30 minutes the serosal solution B of the guinea pig was tested again in the presence of the specific CCK receptor antagonist devazepide. Devapezide was dissolved in DMSO (10 mg/ml) and then diluted in KPS in a concentration of 1 μg/ml. For control the same amount of DMSO without devazepide was tested.

In vivo

Standard gallbladder contraction assays were performed following the method described by MAKOVEC *et al.* (1987). Experiments were performed on 30 mice (CD1, 25 weeks, both sexes), weighing approximately 30 g. They received a standard diet and were fasted overnight (17 hours) before the experiments started. Water was freely available throughout. Five mice were injected intraperitoneally (i.p.) with 1.25 μg CCK-8/kg body weight (BW) in 80 μl saline. Five were injected i.p. with 1 g simmondsin/kg BW in 80 μl saline. Five were injected i.p. with 800 μg/kg BW MK-329, soluted in glycerol and PEG 400, diluted in 100 μl saline, followed 15 minutes later by an i.p. injection of 1.25 μg CCK-8/kg BW in 80 μl saline. For control i.p. injections, the same concentration of glycerol and PEG 400 diluted in 100 μl saline, followed by an injection of 80 μl saline, was tested in 5 mice. Fifteen minutes after injection, the mice were killed and their gallbladder was removed and weighed.

A similar procedure was repeated with 64 male chickens (Hisex, 3 weeks old), weighing approximately 160 g. Sixteen animals were injected i.p. with 6.25 μg CCK-8/kg BW in 500 μl saline, whereas 16 birds were injected (i.p.) with 1 g simmondsin/kg BW in 500 μl saline. Sixteen control animals received 500 μl saline only. The last 16 chickens received non treatment but were fed after the same starvation period. In each treatment gallbladders were removed at 15 or 30 minutes after the injection ($n = 8$ for each group).

The data were analyzed using analysis of variance followed by Duncan's multiple range test using the SAS program (SAS, 1985).

RESULTS AND DISCUSSION

Below 100 nM, CCK had no effect in eliciting *in vitro* gallbladder contraction in chicken (data not shown), whereas in mice (Fig. 1) and guinea pig (data not shown), doses as low as 1 nM were effective. DIMALINE *et al.* (1990) reported,

however, that the treshold dose of CCK for *in vitro* chicken or guinea pig gallbladder contraction was 0.1 nM. Moreover, in our study, the contraction pattern in chicken gallbladder was continuously pulsatile and oscillating, while a single, large contraction was observed in mice (Figs 1-2-3). This oscillating contraction pattern in chicken gallbladder contraction wasn't observed by DIMALINE *et al.* (1990). The sensitivity of the technique for measuring the contractions can be different, because the tissue bath techniques used by these authors were not reported.

In our *in vitro* studies there were initially no contractions in the gallbladder of mice, guinea pig and chicken before 60 minutes, after which spontaneous contractions started to appear. This might suggest that there is a basal CCK secretion by the gallbladder itself. It would appear that in the fresh tissue preparation *in vitro*, there is insufficient CCK to produce an effect, but after one hour of incubation enough endogenous CCK may have been produced to start contractions. Such a possibility is further supported by the finding that the CCK antagonist, devapezide, eliminates the spontaneous contractions. We know of no previous suggestions that the gallbladder itself may produce CCK.

Simmondsin, at 10 mM *in vitro*, had no direct contractile effect on gallbladder strips in mouse, chicken or guinea pig (Figs 1-2). This observation indicates that simmondsin does not act as a CCK agonist on mammalian or chicken gallbladder receptors. In guinea pig, however, a substance found in duodenal perfusates after stimulation with simmondsin (solution B), did stimulate guinea pig gallbladder contraction. These data suggest that simmondsin induces CCK release from guinea pig duodenum.

Gallbladder contractions could be seen prior to any application of simmondsin to the gut (Fig. 4). Simmondsin treatment however (Solution B), intensified these contractions, an action which could be inhibited by the peripheral CCK-A-receptor antagonist, devapezide (Figs 4-5). Devapezide also eliminated all spontaneous contractions in the absence of solution B, supporting the possibility of a CCK-liberation by the gallbladder itself.

In contrast, in the chicken experiment, solution B did not show any contractile effect on chicken gallbladder strips (Fig. 3). However it is not possible to determine whether this is because the chicken tissue had not secreted CCK, or whether the secretion was insufficient to stimulate gallbladder contraction.

The mammalian hormones gastrin and CCK share a common biologically active COOH-terminal pentapeptide sequence and have overlapping spectra of biological activities. However, there are important functional differences between the two peptides. CCK is a potent stimulant of gallbladder contraction and pancreatic enzyme secretion, while gastrin is largely ineffective in these systems, but it is a potent stimulation of gastric acid secretion (WALSH *et al.*, 1987). CCK-8 like molecules have previously been identified in avian brain and intestine (DOCKRAY, 1979), and a molecule identical to mammalian CCK-8 has been isolated from chicken brain (FAN *et al.*, 1987). The presence of an intestinal peptide that is both chemically and biologically similar to complete sequence mammalian CCK (1-33) in birds has not yet been proven.

DIMALINE *et al* (1990) have suggested that the factors which determine specificity of action of CCK and gastrin are different in birds and mammals. They have isolated chicken intestinal peptides which showed biological gastrin-like actions, but which were chemically more like CCK. This chicken CCK-like peptide was less potent than CCK-8 in causing contraction of both avian and mammalian gallbladder *in vitro*. A proline residue, immediately adjacent to the sulphated tyrosine in this chicken CCK-like peptide, may produce a steric effect that lowers its activity on gallbladder contraction (DIMALINE *et al.*, 1990). In our study, this steric effect could also explain the difference in effect between mammals and birds after stimulation with solution B.

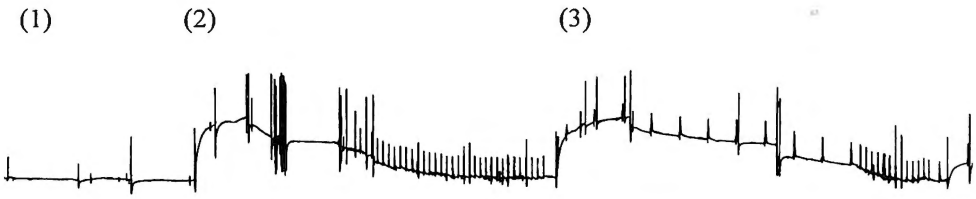

Fig. 1. — Simmondsin and CCK-8 were administered to mouse gallbladder with intermittent washes with KPS, in the following order : (1) 10 mM simmondsin, (2) 1 nM CCK-8/ml, (3) 1 nM CCK-8/ml.

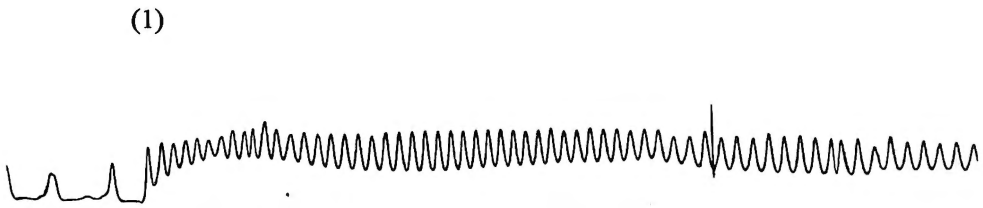

Fig. 2. — CCK-8 was administered to chicken gallbladder in a concentration of 100 nM CCK-8/ml (1).

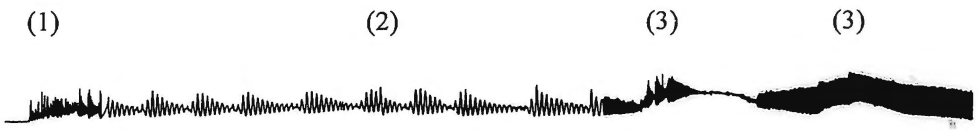

Fig. 3. — (1) Spontaneous contraction of chicken gallbladder begins. Chicken duodenal perfusate — after stimulation with 10 mM simmondsin - (solution B) (2) and 100 nM CCK-8/ml (3) were administered on chicken gallbladder tissue with intervening washes with KPS, in the following order : (2), (3), (3).

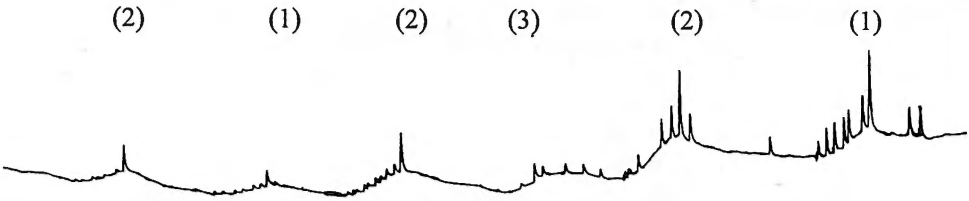

Fig. 4. — Guinea pig duodenal perfusate (1) before stimulation with simmondsin (solution A), or (2) after stimulation with 10 mM simmondsin (solution B) and (3) KPS were administered to guinea pig gallbladder tissue in the following order : (2), (1), (2), (3), (2), (1).

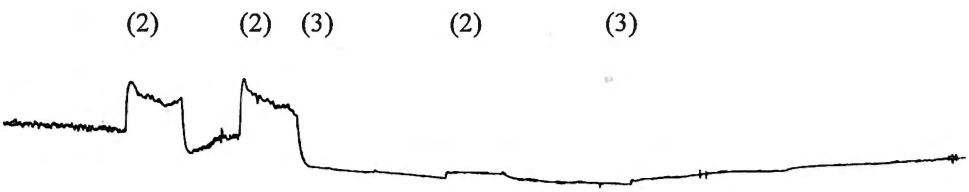

Fig. 5. — (2) Duodenal perfusate fluid after stimulation with 10 mM simmondsin (solution B) and (3) 1 µg/ml CCK-A antagonist were administered in the following order : (2), (2), (3), (2), (3).

In the *in vivo* studies, total gallbladder contraction was apparent by 15 minutes after i.p. injection of a high dose of (mammalian) CCK-8 (1 µg CCK-8/kg BW) in mice, as reported by others (MAKOVEC *et al.*, 1987). In contrast, no loss of gallbladder weight was observed in chickens, even at a higher dose of 5 nmol CCK-8/kg BW (Tables 1-2). The higher dose needed for *in vitro* gallbladder contraction both *in vitro* and *in vivo*, and the different pattern of contraction seen in chicken gallbladder, may explain why *in vivo* CCK did not empty gallbladders in chickens, whereas it did in mice.

From the *in vitro* studies it was already anticipated that an *in vivo* i.p. injection of a high dose of simmondsin (1 g/kg BW) would not elicite gallbladder contraction in chickens. It has not been proven yet that *in vivo*, simmondsin stimulates the liberation of CCK in mammals at a dose, effective to establish gallbladder contractions. However, it seems that endogenous CCK can stimulate gallbladder contractions in mice *in vivo*, because re-feeding the mice after a period of starvation caused a total gallbladder emptying (Table 1). The lack of effect of simmondsin in these studies may have been a result of looking at gallbladder weights using the standard 15 min test ; a longer period might have allowed time for simmondsin-stimulated, endogenous CCK production and gallbladder contraction to be seen. In birds, the presence and activity of chicken CCK is still not clear as gallbladders were not emptied after re-feeding . These results confirm the conclusions of MARTINEZ *et al.* (1993) that in birds the receptors mediating CCK-effects are different from those of mammals. The role of endogenous CCK in satiety in chickens has also been

questioned (COVOSA and FORBES, 1994) because an i.p. injection of devapezide could not block the reduction in feed intake after an i.p. injection of CCK-8.

TABLE 1

Mouse gallbladder weights 15 min. after i.p. injection of saline, CCK-8, simmondsin, PEG 400 + glycerol and saline, MK-329 + saline or after feed intake. Values are means ± standard errors (SE). Means with no common superscript differ significantly ($p < 0,05$) by Duncan's multiple range test

Treatment	*Gallbladder weight (mg) (mean ± SE) (n = 5)*
saline (ip)	25,4 ± 6,8[a]
CCK-8 (ip)	3,5 ± 0,7[b]
saline (ip)	25,4 ± 6,8[a]
simmondsin (ip)	40 ± 7,1[a]
PEG : glycerol (ip) + saline (ip)	58,7 ± 6,7[a]
devapezide (MK-329) (ip) + CCK-8 (ip)	30,8 ± 1,5[a]
after feed intake	6,7 ± 4,1[b]

TABLE 2

Chicken gallbladder weights 15 min or 30 min after i.p. injection of saline, CCK-8, simmondsin or after feed intake. Values are means ± standard errors (SE). Means with no common superscript differ significantly ($p < 0,05$) Duncan's multiple range test

Treatment	*Gallbladder weight after 15 min (mg) (mean ± SE) (n = 5)*	*Gallbladder weight after 30 min (mg) (mean ± SE) (n = 5)*
saline (ip)	96.8 ± 17.8[a]	107.4 ± 43.8[a]
CCK-8 (ip)	94.0 ± 11,5[a]	112.0 ± 10,2[a]
simmondsin (ip)	112.5 ± 8.6[a]	120.9 ± 9.9[a]
after feed intake	100.2 ± 15.4[a]	108.3 ± 9.3[a]

CONCLUSIONS

In conclusion, these *in vitro* studies show that.simmondsin does not directly stimulate CCK-receptors in mouse, chicken or guinea pig gallbladder. Our results suggest that simmondsin induces release of a CCK-like substance from guinea pig duodenum, but we could find no evidence that simmondsin could induce CCK production in chickens. *In vivo*, it could not be shown that simmondsin induced

gallbladder contraction in either mice or chickens ; it is even doubtful whether exogenous or endogenous CCK is able to stimulate chicken gallbladder contractions.

REFERENCES

ARNOUTS, S., J. BUYSE, M.M. COKELAERE and E. DECUYPERE (1993) — Jojoba meal (*Simmondsia chinensis*) in the diet of broiler breeder pullets : physiological and endocrinological effects. *Poultry Sci.*, **72** : 1714-1721.

BOOTH, A.N., C.A. ELLIGER and A.C. JR. WAISS (1974) — Isolation of a toxic factor from jojoba meal. *Life Sci.*, **15** : 1115-1120.

COKELAERE, M.M., H.D. DANGREAU, S. ARNOUTS, E.R. KÜHN and E. DECUYPERE (1992) — The influence of pure simmondsin on food intake in rats. *J. Agric. Food Chem.*, **40** : 1839-1842.

COKELAERE, M.M., P. DAENENS, G. FLO, E. KUHN, M. VAN BOVEN, S. VERMAUT, J. BUYSE and E. DECUYPERE — Simmondsin : physiological effects and working mechanisms in rats. *Proc. IX Intern. Conf. on jojoba and its uses*, Catamarca, Argentinia ; 25-29 September 1994, Ed. L.H. Princen ; A.O.C.S. 1995 (in press).

COVASA, M. and J.M. FORBES (1994) — Effects of the CCK receptor antagonist MK-329 on food intake in broiler chickens. *Pharmacol. Biochem. Behav.*, **48**(2) : 479-486.

DIMALINE, R. and C.M. LEE (1990) — Chicken gastrin : a member of the gastrin/CCK family with novel structure-activity relationships. *Am. J. Physiol.*, **259** : 882-G888.

DOCKRAY, G.J. (1979) — Cholecystokinin-like peptides in avian brain and gut. *Experientia*, **35** : 628-630.

ELLIGER, C.A., A.C. JR. WAISS and R.E. LUNDIN (1973) — Simmondsin, an unusual 2-cyanomethylenecyclohexyl glycoside from *simmondsia californica*. *J. Chem. Soc., Perkin Trans.*, **19** : 2209-2212.

ELLIGER C.A., A.C. JR. WAISS and R.E. LUNDIN (1974a) — Structure and stereochemistry of simmondsin. *J. Org. Chem.*, **39** : 2930-2931.

ELLIGER, C.A., A.C. JR. WAISS and R.E. LUNDIN (1974b) — Cyanomethylenecyclohexyl glycosides from *simmondsia californica*. *Phytochemistry*, **13** : 2319-2320.

FAN, Z.W., J. ENG, M. MIEDEL, J.D. HULMES, T.-C. E. PAN and R.S. YALOW (1987) — Cholecystokinin octapeptides purified from chinchilla and chicken brains. *Br. Res. Bull.*, **18** : 757-760.

MAKOVEC, F., M. BANI, R. CEREDA, R. CHISTA, M.A. PACINI, L. REVEL and L.C. ROVATI (1987) — Antispasmodic activity on the gallbladder of the mouse of CR 1409 (Lorglumide) a potent antagonist of peripheral CCK. *Pharmacol. Res. Communic.*, **19** : N° 1 : 41-48.

MANOS, C.G.JR., P.J. SCHRYNEMEECKERS, D.E. HOGUE, J.N. TELFORD, G.S. STOEWSAND, D.H. BEERMAN, J.G. BABISH ; J.T. BLUE ; B.S. SHANE and D.J. LISK (1986) — Toxicologic studies with lambs fed jojoba meal supplemented rations. *J. Agric. Chem.*, **34** : 801-805.

MARTINEZ V., M. JIMÉNEZ, GONALONS, P. VERGARA (1993) — Effects of cholecystokinin and gastrin on gastroduodenal motility and coordination in chickens. *Life Sci.*, **52** : 191-198.

NGOU NGOUPAYOU J.D.N., P.M. MAIORINO and B.L. REID (1982) — Jojoba meal in poultry diets. *Poult. Sci.*, **61** : 1692-1696.

SAS INSTITUTE (1985) SAS/STAT. — *Guide for Personal Computers. Version 6.* SAS Institute Inc., Cary, NC.

SHERBROOKE W.C. (1976) — Differential acceptance of toxic jojoba seed (*Simmondsia chinensis*) by four sonoran desert heteromyid rodents. *Ecology*, **57** : 596-602.

VAN BOVEN, M., N. BLATON, M.M. COKELAERE and P. DAENENS (1993) — Isolation, purification and stereochemistry of simmondsin. *J. Agric. Food Chem.*, **41** : 1605-1607.

WALSH, J.H. (1987) — Gastrointestinal hormones. *Physiology of the Gastrointestinal Tract.* L.R. Johnson (Ed.). New York, Raven : 181-252.

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BOOK REVIEWS

La phorésie chez les acariens. Aspects adaptatifs et évolutifs, par Françoise ATHIAS-BINCHE, Éditions du Castillet, Perpignan, 1994, 180 pages, 65 illustrations.

Les acariens sont des arachnides rarement plus épais qu'un cheveu, qui étonnent à plus d'un titre. Ainsi, tous les modes de phorésie (la phorésie est un processus par lequel un animal se fixe à un autre pour émigrer d'un habitat) connus dans le règne animal sont pratiqués par les acariens. En outre, le développement de la phorésie s'est souvent accompagné d'adaptations extraordinaires tant chez les acariens que chez les vecteurs. Un des exemples les plus frappants d'adaptation extrême chez le phorétique est celui des acariens astigmates du groupe des hypodéridés qui, après s'être introduits sous la peau de leur vecteur, se transforment en petits sacs inertes. De même, l'existence de poches abdominales ou « acarinarium » chez certaines abeilles constitue, sans doute, l'exemple d'adaptation le plus spectaculaire chez les vecteurs.

Au fil des 180 pages de son ouvrage, le Dr. Athias-Binche nous fait découvrir les diverses facettes de la phorésie chez les acariens et met l'accent sur plusieurs aspects intéressants du phénomène tels que la spécificité de la phorésie, son évolution vers le parasitisme, les diverses stratégies de dispersion, les effets sur le vecteur, etc.

Ce livre, abondamment illustré, possède un caractère didactique évident pour les acarologues débutants. De plus, la synthèse qu'il propose quant à nos connaissances concernant la phorésie chez les acariens paraît être une première. Cet ouvrage n'existe malheureusement qu'en version française, avec toutefois un résumé en anglais. Notons que son existence comble, dans une certaine mesure, l'importante lacune en ouvrages de synthèse existant dans la littérature acarologique francophone.

Toute médaille ayant son revers, nous ne manquerons pas de relever quelques défauts, et plus particulièrement la présence de certaines généralisations abusives, difficilement acceptables par un spécialiste. Ces approximations sont toutefois excusables au vu de la multiplicité et de la complexité des phénomènes associés à la phorésie. Signalons également la trop forte tendance qu'a l'auteur de disserter sur des sujets intéressants mais fort éloignés du thème initial.

Nous suggérons d'inclure, dans une prochaine édition du livre, un tableau reprenant l'ensemble des genres existant chez les acariens et l'importance de la phorésie chez chacun d'eux (nombre d'espèces, type de phorésie, etc.). Le lecteur aura ainsi d'emblée une vue plus synthétique de l'importance et du rôle de la phorésie chez les acariens.

Malgré ces remarques, « La phorésie chez les acariens » reste un livre intéressant par l'importance des données et des exemples qu'il renferme. Nous ne pouvons donc que conseiller sa lecture pour peu qu'elle se réalise de manière critique, c'est-à-dire en tenant compte des remarques indiquées ici.

Laurent VAN ASSELT
et Georges WAUTHY,
Département d'Entomologie,
I.R.Sc.N.B.

Les Hyménoptères Sphecidae d'Europe occidentale, par J. BITSCH et J. LECLERCQ, Faune de France, Tome 79.

Les zoologistes européens, en particulier nos collègues belges, seront heureux de constater que la Fédération française des Sociétés de Sciences Naturelles (qui siège au Muséum, rue Cuvier à Paris) poursuit courageusement la publication des volumes de la Faune de France, ouvrage de référence tellement apprécié et utilisé par les naturalistes. Cette collection, combien utile, vient de s'enrichir d'un nouveau volume, consacré à un groupe d'insectes particulièrement intéressant, ne serait-ce que sous l'angle de l'éthologie, mais fort difficile d'accès jusqu'ici pour les non spécialistes, en raison du nombre élevé d'espèces et des difficultés que présentait leur identification. Rien que pour la France, en effet, on compte quelque 400 espèces, réparties en 70 genres !

Bien que ne différant pas essentiellement des autres volumes de la collection dans son principe et sa présentation, le volume consacré aux Sphécides me paraît mériter une mention particulière, en raison du soin minutieux avec lequel toutes les données morphologiques, taxonomiques, chorologiques et nomenclaturales ont été vérifiées, exploitées et synthétisées. Il est vrai que la réalisation de cet ouvrage **up to date** a demandé la collaboration de deux grands spécialistes, tous deux professeurs émérites de deux grandes universités : Jacques Bitsch, de l'Université Paul Sabatier de Toulouse, et Jean Leclercq, de la Faculté Agronomique de Gembloux.

Relevons dès l'abord la qualité de l'illustration : le présent ouvrage (le volume 1, de 320 pages, qui ne concerne que la sous-famille des *Crabroninae,* soit 96 espèces dans la seule faune française) est riche de 59 planches de dessins au trait, particulièrement soignés et précis, et de 98 cartes de distribution.

Les clés d'identification, il faut le souligner, sont rédigées de manière claire et synthétique mais sans employer des abréviations, qui, sous prétexte de gagner de la place, rendent parfois la lecture de ces clés fort pénible. Ces clés d'identification ne conduisent pas seulement aux espèces observées en France, mais concernent toutes les espèces européennes, ce qui augmente considérablement l'intérêt de ces clés et, par conséquent, la portée de l'ouvrage. Les espèces non observées sur le territoire français sont indiquées entre parenthèses dans les clés, mais sont néanmoins mentionnées et traitées comme les autres dans le corps du texte, avec un peu moins de détails. Cela justifie amplement ce titre de l'ouvrage qui annonce « Sphecidae d'Europe occidentale ».

Pour chaque espèce (surtout pour celles de la faune française, évidemment), les auteurs fournissent une documentation particulièrement fouillée. La liste des synonymies est complétée par les références des travaux, de quelque nature que ce soit, traitant de l'espèce en question. Après la description minutieuse, mais aussi concise que possible, de chacun des deux sexes, on trouve un paragraphe (souvent très étoffé) sur l'éthologie et l'écologie de l'espèce (ou du moins ce qu'on en connaît actuellement) : le milieu habité, les modes de nidification, les proies, les fleurs butinées, etc.

Enfin, un dernier paragraphe est consacré à la distribution biogéographique : répartition à l'échelle mondiale, puis distribution en France et en Corse.

Ce dernier commentaire biogéographique se réfère, pour chaque espèce, à une carte d'occupation de la France par départements : chaque département où l'espèce a été observée est couvert d'une trame (lignée, quadrillée ou uniformément noircie) suivant le nombre d'observations (1, 2 à 4 ou plus de 4) publiées ou vérifiées sur du matériel de collection. Cette façon de procéder peut dérouter le lecteur, féru de biogéographie, qui s'attendrait plutôt à trouver des cartes en coordonnées UTM, comme celles de la série des « Atlas provisoires des Insectes de Belgique et des régions limitrophes » inaugurée et patronnée dès 1969 par Jean Leclercq en personne. L'explication réside dans le fait que la présentation de cartes détaillées en coordonnées UTM aurait nécessairement dépassé les limites de l'ouvrage, tant sur le fond (en déplaçant peut-être exagérément son intérêt vers la biogéographie, alors qu'il reste tant à faire à ce niveau) que sur la forme (chaque carte exigeant une page entière pour être lue aisément). Cette documentation biogéographique ne sera d'ailleurs pas perdue, puisqu'elle est publiée par J. Leclercq en collaboration avec Y. Barbier (d'ailleurs réalisateur des cartes de l'ouvrage par un programme informatique de l'Université de Mons) dans le n° 27 des « Notes fauniques de Gembloux ». La cartographie de la Faune de France des Sphécides est, sous sa forme « départementale », suffisamment imagée pour illustrer l'analyse biogéographique que proposent les auteurs, et devrait stimuler l'intérêt des utilisateurs de l'ouvrage, et tout spécialement les naturalistes de terrain.

Rarement, un ouvrage de systématique a été réalisé avec autant de soin et de souci de couvrir de manière exhaustive et critique toute l'information concernant une famille d'insectes aussi diversifiée. C'est une précieuse contribution à l'inventaire de la richesse faunistique de l'Europe occidentale (on dira aujourd'hui : « de sa biodiversité ») et un modèle à suivre.

Ch. JEUNIAUX
Université de Liège

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