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* *Paper in a book*: MALLEFET, J., P. VANHOUTTE & F. BAGUET (1992). Study of *Amphipholis squamata* luminescence. In: ALERA-LIACI & CANICATTI TI (eds), *Echinoderm Research*, L. Balkema, Rotterdam: 125-130.
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INVITED CONTRIBUTION

Fifty years after Alan M. Turing
An extraordinary theory of morphogenesis

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ABSTRACT. The publication of 'The Chemical Basis of Morphogenesis' by ALAN M. TURING in 1952 was a milestone for the development of mathematical biology and for many (biological) disciplines leaning on it. TURING provided an original solution to the problem of morphogenesis, by adapting a system of coupled differential equations to describe both chemical reaction and diffusion of morphogenetic substances in an initially homogeneous configuration. FOURIER'S analysis of the 'ring problem' in heat conduction, and the theory of spherical harmonics and their solution by (normalized) LEGENDRE'S associated functions form the mathematical backbone of TURING'S work on morphogenesis. TURING was up to more than providing a mathematical description of initial stages of embryonic development. Rather he was eager to unveil the mathematical foundations of living, biological organization. An investigation of the archival material of unpublished letters and manuscripts indicates that TURING was clearly determined to provide an argument for the generation of 'order-from-disorder'. Unfortunately, during his lifetime TURING remained unable to demonstrate the use of his model beyond the level of early embryonic stages. In the posthumously-published manuscripts several indications are found for further adaptation and improvement of TURING'S model to handle more accurately the reaction-diffusion processes in small organisms.

KEY WORDS : morphogenesis, reaction-diffusion theory, early embryonic development, spherical harmonics, normalized Legendre associated functions.

INTRODUCTION

In 1952 a paper was published that had a far-reaching impact not only for the application of the theory of reaction-diffusion mechanisms in biology, but also for developmental biology and embryology (TURING, 1952). Indirectly, genetics and the entire biological field were also affected. The author, however, was not a biologist but a mathematician. His 1952 paper was the only biological paper he published during his lifetime. ALAN MATHISON TURING (born 23 June 1912) unfortunately died under dramatic circumstances in June 1954, only two years after the publication of 'The Chemical Basis of Morphogenesis' (August 1952).

As early as 1953, the speculative value of TURING'S paper was recognised by J.W.S. PRINGLE (Department of Zoology, Cambridge) (1953), stating that TURING'S model of morphogenesis could 'provide a means of creating structure where no structure was initially present'. A system that is unstable with respect to its local concentrations of reacting molecules may be started on a course towards stability by a small event. However, if there is some initial heterogeneity due to factors other than the concentrations themselves, PRINGLE (1953) says this can provide 'the initial stimulus for morphogenesis if the heterogeneity has a component of its structure similar to the inherent tendency of the system'. The notion of an emerging self-explaining structure as well as the notion of the tendency of an unstable, homogeneous system towards a stable but heterogeneous system were both present in contemporary thought on morphogenesis. PRINGLE also refers to a personal communication of A.M. TURING with respect to the so-far unpublished work involving a model with non-linear differential equations for two morphogens.

The unpublished manuscripts and notes of ALAN TURING'S later research on 'The chemical theory of morphogenesis' have been collected at King's College Archive Centre (referred to as KCC). These were studied and pieced together by N.E. HOSKIN and B. RICHARDS and appeared – together with the 1952 paper – in the *Collected Works of A.M. TURING* (Volume *Morphogenesis* edited by P.T. SAUNDERS, 1992). In this posthumously published work, TURING's model indeed diverted from the linear case and also from geometrical constraints such as the ring of cells or the sphere. Moreover, an important conceptual role is reserved for the use of the mathematical theory of spherical harmonics and Legendre associated functions.

The extraordinary character of TURING'S work on morphogenesis links up with his outstanding achievements in fields such as mathematical logic, mechanical intelligence and pure mathematics. As a pinnacle in the twentieth century of mathematical and logical thought, he would rather work things out in a self-contained way than lean on others (see biography by SARA TURING, 1959, p. 119). As an example, in 1934 TURING proved the Central Limit Theorem independently of Lindeberg's proof of 1922 (HODGES,

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1983, p. 88). Also, TURING'S paper *'On Computable Numbers'* (1937) was independently provided but nevertheless antedated by Church's work in this field and published in 1936 (HODGES, 1983, p. 546). TURING was familiar with E. SCHRÖDINGER'S 1943 lecture *'What is Life?'*, deducing the crucial idea that genetic information must be stored at the molecular level (SCHRÖDINGER, 1944). But rather than follow up SCHRÖDINGER'S suggestion, TURING aimed at finding a parallel explanation of how a chemical soup of molecules could possibly give rise to a biological pattern, granted the transcription of genes into diffusible molecules (HODGES, 1983, p. 431).

However, TURING (1952) had some benefit of existing biological knowledge. The notion of diffusible molecules that affect embryological development and the discovery of the existence of chemical gradients that directed axis formation in the embryo, were especially elaborated by HÖRSTADIUS (1939, 1950, 1952, 1953). HÖRSTADIUS used eggs of the sea urchin *Paracentrotus lividus* (Echinodermata) as a model for the study of the development of an animal-vegetal gradient system in an initially spherical, symmetrical organism (for a review see BALINSKY, 1981). Moreover, since it became well known that also the cytoskeleton has a very important role in early embryogenesis, TURING'S conceptual basis of mere diffusible morphogenetic substances nowadays is considered too narrow to support the full range of embryological and developmental processes observed in biological species. The notion of 'positional information' (WOLPERT, 1969) – although more vague than TURING'S notion of morphogen (see HARRISON, 1987) – has regained popularity as a conceptual framework to embrace several biochemical mechanisms, acting in concert to direct morphogenesis (for a review see ALLAERTS & ROELANTS, 1993). Although mathematical contributions to the field of embryology have only occasionally been reported since the work of TURING (see e.g. GOODWIN & TRAINOR, 1980), mathematical modeling recently regained interest in the field of genome analysis (PERCUS, 2002).

We previously reported on the extraordinary position of TURING'S 1952 paper on morphogenesis with respect to the concept of positional information (ALLAERTS & ROELANTS, 1993), although we did not report in detail on the mathematical features of TURING'S work. In this study, we will focus on the mathematical core (section 1) and the origin and background of the 1952 paper (section 3). In section 2, TURING'S modifications of the reaction-diffusion model for small organisms are discussed, based on unpublished manuscripts and notes kept at King's College Archive Center and partly posthumously published in the *Collected Works of A.M. TURING* (SAUNDERS, 1992). Several examples have been found in the literature (biology as well as physics) to validate TURING'S theoretical findings. Finally, a few notes are added on TURING'S view on conscious living beings (section 4). An appendix is added for introduction into the mathematical techniques used by TURING to solve morphogenesis in a cylindrical (appendix a) and spherical configuration (appendix b). Also the mathematical fine-tuning of Turing's reaction-diffusion model as worked out by RICHARDS (see SAUNDERS, 1992), and the use of the technique of normalized Legendre associated functions to describe skeleton formation in radiolarian species are discussed (appendix c).

THE MATHEMATICAL CORE OF THE 1952 PAPER

ALAN TURING was not the first to use a mathematical model to describe complex dynamic systems in biology. Already VOLTERRA (1926), used a system of two coupled differential equations to describe the oscillatory behaviour of abundance numbers of prey and predator species. Later on, this model was referred to as the Lotka-Volterra system (MURRAY, 1989). But, in contrast to the Lotka-Volterra system, where an obvious relationship could be assumed between the prey species and the predator species feeding on it, the idea of morphogenetic substances that chemically reacted with each other, was an absolute *terra incognita.* The new feature of TURING'S work arose from the simultaneous consideration of diffusion as a factor influencing the concentrations in a region of space. In fact, TURING'S mathematical model of morphogenesis through chemical, diffusible substances shaped a new domain for mathematical modeling in biology, named reaction-diffusion theory (see also ALLAERTS & ROELANTS, 1993).

Unfortunately, TURING himself gave very few hints to explain the mathematical origins of his model. The central role of the cylindrical case in TURING'S 1952 paper, suggests some affinity with the central 'ring problem' in FOURIER'S (1822) analysis of heat transfer (see appendix a). This was probably general knowledge to the trained mathematician TURING. On the other hand, TURING did refer to the work of JEANS (1927) on *'Electricity and Magnetism'*, concerning the application of spherical harmonic functions to the problem of morphogenesis in a sphere.

The cylindrical case of morphogenesis

The central problem in TURING'S model of morphogenesis through diffusion of two (later: two or three) chemical substances is a classical application of reaction-diffusion theory. TURING'S approach starts from a radially symmetric (cylindrical) system, such as a ring of cells or a continuous ring of tissue. In the continuous, cylindrical case the equations for diffusion of the substances X and Y are:

$$\left.\begin{aligned}\frac{\partial X}{\partial t} &= a(X-h)+b(Y-k)+\frac{\mu'}{\rho^2}\frac{\partial^2 X}{\partial \theta^2}\\ \frac{\partial Y}{\partial t} &= c(X-h)+d(Y-k)+\frac{\upsilon'}{\rho^2}\frac{\partial^2 Y}{\partial \theta^2}\end{aligned}\right\} \quad (1)$$

In this set of equations, the cylindrical notation for the basic reaction-diffusion model is recognised, namely:

$$\frac{\partial C}{\partial t} = f(C) + D\frac{\partial^2 C}{\partial \theta^2} \qquad \text{(see also ALLAERTS, 1992)}$$

with C the vector of chemical concentrations, $f(C)$ the vector representing the chemical reactions of these chemical substances, D the diffusion matrix and $\partial^2 C/\partial \theta^2$ the second-order derivative along the polar angle co-ordinate θ. TURING'S set of equations (1) is a limiting case of the equations for reaction diffusion in a ring of cells, the ring having radius ρ. In the continuous case of the ring the diameter of the cells is incorporated into the notations used for describing the diffusibilities μ' and ν', which are relat-

ed to the cell-to-cell diffusion constants μ and ν of the substances X and Y respectively :

$$\mu = \mu'(\frac{N}{2\pi\rho})^2 \ , \ \nu = \nu'(\frac{N}{2\pi\rho})^2$$

It is important to note that TURING considers the diffusion constants μ , ν, μ' and ν' as constants, a fact that has important consequences for the biological process of morphogenesis (ALLAERTS & ROELANTS, 1993).

The general solution of (1) proposed by TURING, using Fourier transformation, is of the form (see appendix a) :

$$X = h + \sum_{s=-\infty}^{\infty} (A_s e^{p_s t} + B_s e^{p_s' t}).e^{is\theta} \quad \}(2)$$

$$Y = k + \sum_{s=-\infty}^{\infty} (C_s e^{p_s t} + D_s e^{p_s' t}).e^{is\theta}$$

where p_s, p_s' are the roots of the equation:

$$(p - a + \frac{\mu' s^2}{\rho^2})(p - d + \frac{\nu' s^2}{\rho^2}) = bc \quad (3)$$

The constants A_s, B_s, C_s and D_s are not independent, but are restricted to satisfy the set of equations:

$$A_s(p_s - a + \frac{\mu' s^2}{\rho^2}) = bC_s \quad \} \ (4)$$

$$B_s(p_s' - a + \frac{\mu' s^2}{\rho^2}) = bD_s$$

It is important to consider the nature of the solutions to the equation set (2). These equations represent Fourier series in an exponential notation, describing the deviations from the equilibrium concentrations *h, k* of the respective morphogenetic substances *X,Y* (Fig.1.a). Depending on the value of the roots *p, p'* of the characteristic equation (see appendix a), and on the value of the diffusion constants μ and ν and reaction rates *a, b, c, d,* the resulting geometrical representations of these equations are stationary or oscillatory waves. TURING (1952) is very much concerned with the physical or biological importance of these wave functions, which after a lapse of time may result in patterns on the ring (Fig. 1.b). The wave-lengths of these patterns depend on the circumference of the ring and the chemical data set (a,b,c,d,μ,ν) (TURING, 1952, p. 51; see also ALLAERTS & ROELANTS, 1993). In his unpublished work, TURING himself indicated that the restriction to a ring of cells was *"altogether an unnecessary one (...) for the conclusions for the ring of cells could be directly taken over by any arrangement of cells"* (SAUNDERS, 1992, p. 90). This remark, however, points to the main mathematical assumption of TURING's 1952 paper, namely that the reaction rates are linear functions of the concentrations, which is considered *"reasonably valid so long as only small variations of concentrations are concerned"* (SAUNDERS, 1992, p. 90). In section 2, the characteristics of a more refined reaction-diffusion model devoted to the case of small organisms will be discussed, starting from TURING'S posthumously published work (SAUNDERS, 1992).

The spherical case of morphogenesis

In 1954 ROBIN O. GANDY, university lecturer at Leicester (UK) and inheritor of A.M. TURING'S articles and unpublished manuscripts, expressed his concern about the preservation of TURING'S work, in particular his work on the cylindrical case of morphogenesis. This remark was found in a letter to M.H.A. NEWMAN (KCC: A/8), a professor of topology who played an important role as teacher and mentor of A. M. TURING in the pre-war period at Cambridge (HODGES, 1983, pp. 90-93). In 1951, TURING himself had already brought his work on spherical structures to the attention of the neurophysiologist J.Z. YOUNG (KCC: K/1, nr. 78), but TURING considered this case rather 'more difficult and doubtful' than the cylindrical case. Therefore, it is

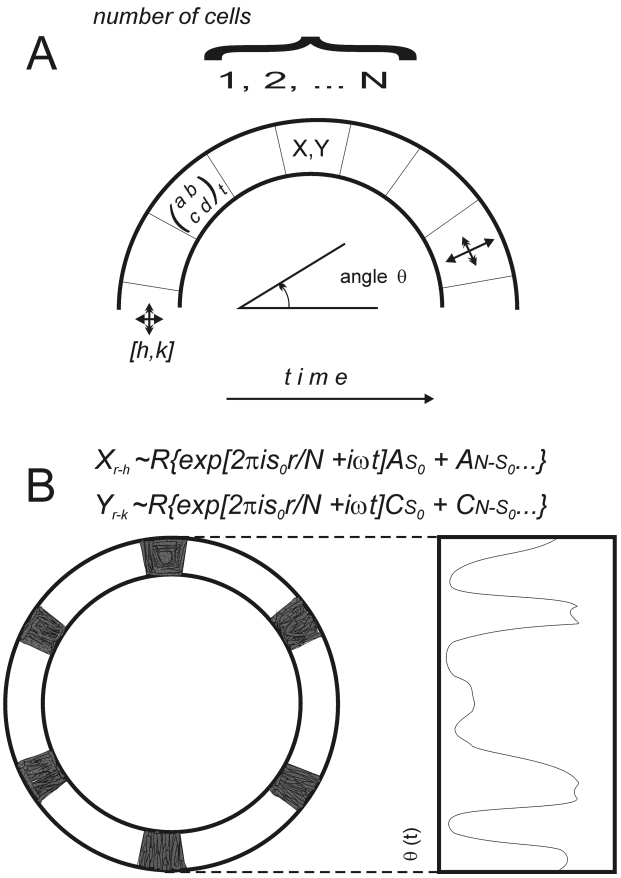

Fig. 1A. – Dimensions and parameters in TURING'S (1952) most simple model of morphogenesis, the reaction-diffusion system in a ring of cells (cylindrical case). *[h,k]* : equilibrium concentrations for morphogenetic substances *X,Y* ; $\begin{pmatrix} a & b \\ c & d \end{pmatrix}_t$: reaction rates at instant *t* ; double arrow: diffusibility; single arrow: deviation from equilibrium concentration after a lapse of time.

Fig. 1B. – Result of reaction-diffusion in a ring of cells after a lapse of time (according to TURING, 1952). The real parts *R* of the roots p_{S_0}, p'_{S_0} determine the wavelengths of the pattern resulting from equation (2) by the relation

$$X_{r-h} \approx \Re\left\{\exp\left[\frac{2\pi i S_0 r}{N} + i\varpi t\right] A_{S_0} + A_{N-S_0}...\right\},$$

the expression for the other morphogen *Y* being related through the relationship between the constants $A_S \sim C_S$,... Moreover, it can be easily shown that the roots p_{S_0} and p_{N-S_0} yield the same terms, using the relation :

$$\sin^2 \frac{\pi.(N - S_0)}{N} = \sin^2 \frac{\pi.S_0}{N}$$ (TURING 1952, p. 50).

somewhat surprising that GANDY did not mention TURING'S attempts to extend his model to spherical organisms, which in fact was already addressed to some degree in his 1952 paper. So, although in 1951 some doubt was expressed on the spherical extension of the model, TURING introduced the key mathematical features of this approach in 1952, as shown below (see also appendix b).

In the case of a hollow sphere of continuous tissue such as a blastula, the spherical notation for describing the diffusion of substances *X* and *Y* is needed. TURING uses the operator ∇^2 to indicate the superficial part of the Laplacian. ∇^2 is an abbreviation of the notation

$$\frac{1}{\rho^2}\frac{\partial^2 V}{\partial \phi^2}+\frac{1}{\rho^2 \sin^2\theta}\frac{\partial}{\partial\theta}(\sin\theta\frac{\partial V}{\partial\theta}) \ ,$$

where θ and ϕ are spherical polar co-ordinates on the surface of the sphere with radius ρ.

The equations corresponding to set (1) in the cylindrical case, may be written as:

$$\frac{\partial X}{\partial t}=a(X-h)+b(Y-k)+\mu'\nabla^2 X \qquad \} \ (5)$$

$$\frac{\partial Y}{\partial t}=c(X-h)+d(Y-k)+\nu'\nabla^2 Y$$

To solve this set of differential equations, TURING (1952) refers to JEANS (1927), stating that *"(almost) any function on the surface of the sphere can be expanded in spherical surface harmonics"*. This according to TURING means that solutions of (5) are to be found which are expressions of the form:

$$\sum_{n=0}^{\infty}[\sum_{m=-n}^{n}A_n^m P_n^m(\cos\theta)e^{im\phi}] \qquad (6).$$

Something curious happened with the introduction of this notation. First, TURING'S (1952) reference to JEANS (1927) was erroneously cited as *'The Mathematical theory of elasticity and magnetism'*, whereas JEANS' textbook, edited from 1908 onwards, was on *'electricity and magnetism'*. Herein, indeed a chapter on methods for the solution of spherical problems was included, in which the theory on spherical harmonics takes a very prominent place. This theory attempts to provide a general solution of Laplace's equation $\nabla^2 V = 0$ (see Appendix b)*.

Then, Legendre's associated functions expressed as $A_n^m P_n^m(\cos\theta)$, are introduced by TURING (1952) without much ado as a solution to Laplace's equation. The upper indices *m* indicate that here the associated Legendre functions are used, which are linked to the Legendre functions $P_n(\cos\theta)$ through the relation:

$$P_n^m(\cos\theta)=(-1)^m \sin^m\theta.\frac{d^m P_n(\cos\theta)}{d(\cos\theta)^m}$$

(HOBSON, 1931, p. 90).

In appendix b, a more elaborate explanation is given of TURING'S use of Legendre's associated functions in the 1952 paper. The fact that TURING here also uses Legendre's associated functions of degree *m = -1*, but without referring to the notation $\overline{P_n^m}(\cos\theta)$ for the normalized Legendre associated functions (see appendix c), is in favour of the view that TURING was still in a process of refinement of his mathematical techniques (see extension of TURING'S work by RICHARDS in SAUNDERS, 1992).

TURING emphasizes that the expression in the square bracket of (6) is a 'surface harmonic of degree *n*', and that *"its nearest analogue in the ring theory is a Fourier component"* (TURING, 1952, p. 70). Moreover, an essential property of a spherical harmonic of degree *n* is when the operator ∇^2 is applied to it the effect is the same as multiplication by $-(n+1)/\rho^2$. Preferentially, manageably low values of the degree *n* (such as 1 or 2) are chosen (see appendix b).

The analogy with the ring theory, in which Fourier expansions are an important method (see appendix a), brings TURING (1952) to the following solution of (5):

$$X=h+\sum_{n=0}^{\infty}\sum_{m=-n}^{n}(A_n^m e^{iq_n t}+B_n^m e^{iq_n' t})P_n^m(\cos\theta)e^{im\phi} \qquad \} \ (7)$$

$$Y=k+\sum_{n=0}^{\infty}\sum_{m=-n}^{n}(C_n^m e^{iq_n t}+D_n^m e^{iq_n' t})P_n^m(\cos\theta)e^{im\phi}$$

where q_n and q'_n are the two roots of:

$$(q-a+\frac{\mu'}{\rho^2}n(n+1))(q-d+\frac{\nu'}{\rho^2}n(n+1))=bc$$

and,

$$A_n^m(q_n-a+\frac{\mu'}{\rho^2}n(n+1)=bC_n^m$$

$$B_n^m(q_n'-a+\frac{\mu'}{\rho^2}n(n+1)=cD_n^m$$

indicating that also here A_n^m, B_n^m, C_n^m and D_n^m are arbitrary but not independent constants, resulting from the solution of the differential equation set (see appendix a for analogy).

As in the cylindrical case, TURING suggests that one particular form of wave (and wavelength) predominates, so reducing (7) into:

$$X-h=e^{iqn_0 t}\sum_{m=-n_0}^{n_0}A_{n_0}^m P_{n_0}^m(\cos\theta)e^{im\phi} \qquad \} \ (8)$$

$$b(Y-k)=(q_{n_0}-a+\frac{\mu'}{\rho^2}n(n+1))(X-h)$$

This brings TURING to the extraordinary conclusion that the two morphogens diffusing on the sphere have proportional concentrations, and both of them are described by surface harmonics of the same degree n_0. This degree n_0 is chosen to maximize the greater of the roots q_{n_0}, q'_{n_0} (TURING, 1952, p. 70).

In Fig. 2, some examples are shown of applications found in biology and chemistry, that validate the use of spherical harmonics in processes describing early embryogenesis (GOODWIN & TRAINOR, 1980) or that provide evidence for sustained non-equilibrium chemical patterns, called 'Turing structures' later on (CASTETS et al., 1990).

* This theory was to a large extent worked out by the French mathematician A.M. LEGENDRE (1752-1833), contemporary of Sir P.S. DE LAPLACE (1749-1827) (for an historical review see E.W. HOBSON, 1931, pp. 16-17).

TURING himself suggested that the forms of 'various, nearly spherical structures', such as radiolarian skeletons (Fig. 2.c), were closely related to spherical harmonic patterns, an idea that has been elaborated further by B. RICHARDS (see SAUNDERS, 1992; appendix c). The best application of his theory, however, according to TURING (1952), seemed to be the gastrulation of the blastula. TURING referred to the early stage in the development of an embryo, characterized as a hollow spherical aggregation of cells which still are morphologically identical. As long as the size of the blastula is not more than the dimensionless diffusibility *(μ')*, the system is considered 'quite stable'. Near this point, however, TURING thinks the harmonics of degree 1 begin to develop, bringing the Legendre's associated functions (P_1^1) into play (TURING, 1952, p. 71; see appendix b). At his untimely death however, this idea remained unexplored.

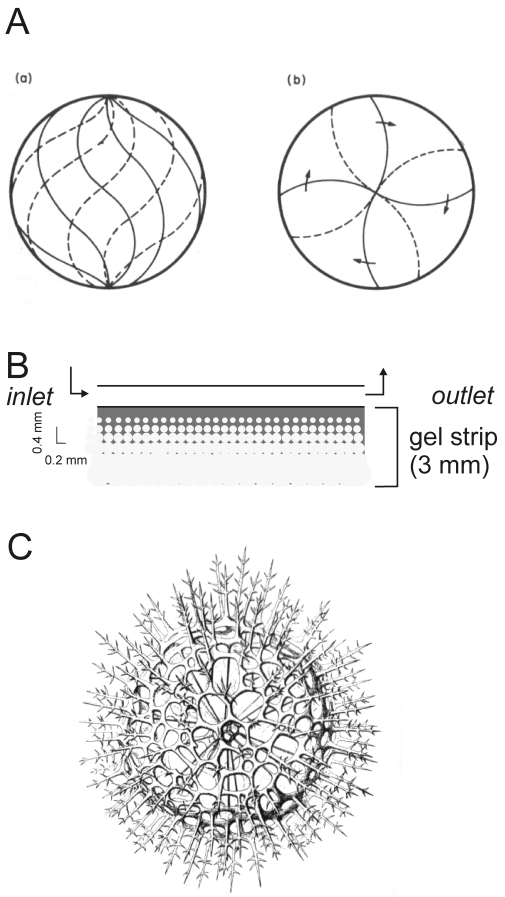

Fig. 2. – Applications of Turing structures in biological or chemical pattern formation:
A. – Spiral nodal lines derived from spherical harmonic functions have been used by GOODWIN & TRAINOR (1980) to describe the cleavage process in embryogenesis. Spiral nodal lines on the sphere from the side (left) and cleavage patterns up to third cleavage defined by spiral nodal lines seen from the animal pole (right) (after GOODWIN & TRAINOR, 1980, p. 766). Arrows show the movement of the blastomeres after cleavage.
B. – Evidence of sustained standing nonequilibrium chemical pattern in a single-phase open reactor suggested to be the first unambiguous evidence of a Turing structure (drawn after CASTETS *et al.*, 1990). The reactor is made of a chemically inert polyacrylamide gel, chemicals diffuse from the edges into the gel where the actual reactions take place.
C. – One of the examples, suggested by TURING (1952), of the spherical case of morphogenesis was the formation of the radiolarian skeleton (siliceous skeleton of *Trypanosphaera regina* after BARNES, 1974, p. 30). RICHARDS (see SAUNDERS, 1992) completed the mathematical solutions for the spherical case, making use of the normalized Legendre associated functions (see appendix c). The geometrical representations of these mathematical results revealed spheroid bodies with increasing numbers of fine radiant spines, when spherical harmonics of increasing degrees were introduced in the mathematical solution.

REFINEMENT OF THE MODEL FOR SMALL ORGANISMS

According to N.E. HOSKIN & B. RICHARDS (in SARA TURING, 1959, p. 137-144), at least two major modifications were adopted by TURING in his late, unpublished work on morphogenesis. These are: (1) the incorporation of quadratic terms in the differential equations in order to take account of a 'larger departure from a state of homogeneous equilibrium'; (2) the consideration that for small organisms the concentration function of the so-called growth-retarder or 'poison' substance (symbol V_j), were independent of position. The latter assumption requires that the organism is so small that the growth-retarder is uniformly diffused through it.

Today, it is a well-known fact that at least in the animal species studied so far, concentrations of morphogenetic substances with stimulatory effects and substances with inhibitory (or 'growth'-retarding) capacities do exist, and both occur in gradient-like or discrete distribution patterns. But this molecular biological knowledge obviously was not available in the early fifties, when TURING published his linear model for morphogenesis (1952) and the double helix strand model for DNA (WATSON & CRICK, 1953) was just discovered. For comparison, the homeobox-gene concept (and the idea of genes that regulate the patterned expression of morphogens) was proposed only in 1984 (see ALLAERTS, 1998 for references).The linear differential equations used in the 1952 paper were of the form $\frac{\partial X_i}{\partial t} = f_i(X_1,...,X_n) + \mu\nabla^2 X_i$ with *(i = 1,...,n)* for n different morphogens (9)and f_i the reaction function giving the rate of growth of X_i and $\mu\nabla^2 X_i$ the rate of diffusion of X_i (compare with equation 1 in section 1). In his 1952 paper, Turing considered the X_i's as variations from a homogeneous equilibrium, and, if the departures from equilibrium were only small, it would be permissible to linearize the f_i's and thus the differential equations. These conditions were assumed to be fulfilled in the 'initial' state of the morphogenetic system, where a homogeneous equilibrium state was present. Embryological studies afterwards have shown that the fertilized egg, although seemingly homogeneous at the macroscopic or even microscopic level, nevertheless has to be considered as a very dynamic and (in biochemical terms) far from equilibrium system.

In order to account for a larger departure from the initial, presumed homogeneous state, Turing introduced quadratic terms in the reaction functions. In the second part of the posthumously published manuscript *'Chemical Theory of Morphogenesis'* (SAUNDERS, 1992, pp. 88-

118), TURING gives the following general differential equation for the morphogen concentration function *U (t)* :

$$\frac{dU_j}{dt} = \left[\phi(-\nabla^2)U\right]_j GU_j^2 - HU_jV_j \qquad (10)$$

(SAUNDERS, 1992, p. 98),

where $\phi\,(-\nabla^2)$ denotes a function of the Laplacian of U_j, which has its maximum near the maximum wavelength, and V_j is the concentration function of the 'poison' substance. For small organisms, TURING considers two types of wavelengths of importance, namely the 'optimum' wavelength and the wavelength with zero root, i.e. the uniform distribution. According to TURING, the latter condition may be fulfilled in small and 'connected' organisms, where it is called a poison or growth-retarder (SAUNDERS, 1992, p. 98). *G* and *H* denote constants that are related to the use of spherical harmonics as solutions (see appendix b), and will appear to be solvable using the normalized Legendre associated functions and some reiteration procedure developed by RICHARDS (see SAUNDERS, 1992, p. 109; appendix c).

Now, since only solutions with the optimum wavelengths have a significant contribution, and due to the effective equilibrium of the growth-retarders, also $\partial V_j / \partial t = 0$, the following reduction of equation (10) is obtained for small organisms:

$$\frac{dU_j}{dt} = (P - HV).U_j + G\left[\Im U^2\right]_j \qquad (11)$$

(SAUNDERS, 1992, p. 98),

A special linear operator $\Im\,(U^2)$ is introduced with the property to remove from a function on a sphere all spherical harmonics except those of a particular degree (exhibiting a particular wavelength). Moreover, solutions must be equivalent under rotation of the sphere, and therefore also the squared harmonics are selected based on a specific degree (HOSKIN & RICHARDS, in S. TURING, 1959). HOSKIN & RICHARDS remarked that in most of TURING'S unpublished manuscripts it was very difficult to discover the results as far as worked out by TURING himself. A number of numerical computations were carried out on one of the first electronic computers at Manchester University (S. TURING, 1959, p. 139). Important in this respect is TURING'S use of spherical harmonics (already introduced in the 1952 paper, see section 1,b) and his use of linear operators applied to these spherical harmonics. Nevertheless, HOSKIN and especially RICHARDS provided a prolific extension of TURING'S suggestions and first steps into a more general theory of morphogenesis. Moreover, RICHARDS was able to demonstrate some numerical examples giving rise to organisms resembling the morphology of a radiolarian skeleton, as suggested by TURING (1952) (appendix c).

REMARKS ON THE ORIGIN OF 'THE CHEMICAL BASIS OF MORPHOGENESIS'

Among pre-war British mathematicians, two branches could be discerned. On the one hand, there were those such as G.H. HARDY, one of TURING'S teachers, who considered real mathematics (in contrast to trivial, applied mathematics) as not useful and doing 'no good' to society (HARDY, 1940). On the other hand, there were men such as A.M. TURING, who assured his followers that mathematics applied to biology and digital computing were as esthetic and indulging as pure mathematics could be. Owing to ANDREW HODGES' biography (1983) it is well accepted nowadays that ALAN TURING was a genius of his own kind. His first biographer, Alan's mother SARA TURING, already quoted the words of ROBIN O. GANDY, *"that the mark of his genius was that even in the most abstract realms of thought he always bore in mind completely concrete ideas and examples"* (S. TURING, 1959). On another occasion, GANDY wrote that TURING was *"unmethodical, or his methods were so individual"*, that his work was hard to follow (KCC: A/18). GANDY was a PhD student and friend of TURING, and soon became university lecturer at Leicester (UK). Reading the collected typescripts and manuscripts at King's College Archive Centre, one is in no doubt about the creative forces of TURING's personality, as he was endowed equally with sound mathematical discernment and a most subtle sense of humour. The question is, however, why and how the post-war mathematician switched over to the study of a typically biological subject such as the problem of morphogenesis of animals and plants?

ALAN TURING indeed was very concerned with the problem of finding a chemico-mechanical process that would explain the origin of changing symmetry patterns in a developing embryo. Referring to the collected letters of Alan to his mother (KCC, AMT/K-1), HODGES pointed out that Turing was familiar with E.T. BREWSTER'S book '*Natural Wonders every Child should know*' from his childhood on (HODGES, 1983, p. 11). In BREWSTER'S book an illustration was given of the process of blastula formation and gastrulation in the early embryo. The fundamental question exemplified in the phenomenon of gastrulation was: if the fertilized eggs were symmetrical and the chemical equations describing the molecular reactions in these structures were symmetrical, without knowledge of right or left, down or up, where did the decision to adopt a different symmetry came from? This phenomenon inspired MICHAEL POLANYI (1958) to claim that some 'immaterial' force must be at work. For TURING it meant that in some way information was created at this point of development (HODGES, 1983, p. 431). POLANYI, a chemist who became a Christian philosopher at Manchester, was an intellectual opponent of TURING – although on friendly personal terms (HODGES, 1997). TURING told GANDY that his new ideas were intended to 'defeat the argument from design'. From the onset, TURING'S approach to the problem of morphogenesis was closely tied with the problem of defining the driving force in embryonic axis formation (see also ALLAERTS & ROELANTS, 1993).

On the other hand, SCHRÖDINGER'S view (1944) of a molecular basis for genetic information was definitely insufficient to explain the formation of pattern. SCHRÖDINGER put forward his viewpoint of a genuine 'order-from-order' principle (SCHRÖDINGER, 1944, p. 81) far ahead of WATSON and CRICK'S double stranded helix model for the DNA molecule (WATSON & CRICK, 1953). Despite, and to some extent because of considerations of statistical physics (genes are too big to follow the expected inaccuracy of physical laws, expressed by the $\sqrt{n}$ rule)[1],

1 The $\sqrt{n}$ rule is an expression of the degree of inaccuracy to be expected in any physical law (SCHRÖDINGER, 1944). If *n* are the number of molecules in a given compartment, the relative error according to this rule will be 10 % if *n* = 100, but only 0.1 % if *n* = one million.

SCHRÖDINGER concluded that the molecular basis of the biological hereditary mechanism was not in contrast with statistical physics, and that quantum indeterminacy played no relevant biological role, except perhaps by *"enhancing their purely accidental character in such events as meiosis, natural and X-ray induced mutations"* (SCHRÖDINGER, 1944, p. 83).

Determined to provide an argument for the generation of 'order-from-disorder', TURING did not await the publication of WATSON and CRICK'S model either. Rather than following up SCHRÖDINGER'S suggestion, TURING sought an explanation of how a chemical soup of molecules in an embryo could possibly give rise to a biological pattern. In fact, TURING considered the effects of genes to belong to the class of effects that are not normally distributed, but that show a Poisson distribution instead (SAUNDERS, 1992, pp. 100-101): *"In some applications of the theory, it may be important to consider seriously the possibility that there may be only one or two molecules present, or even none..."* The statistical nature of diffusion and chemical reactions (the reactants being small, manageable molecules, not genes) was considered more satisfactory than the variations of reactions from cell to cell or irregularities of cell pattern (SAUNDERS, 1992, pp. 101-102).

In 1931 the foundations of mathematics were questioned by KURT GÖDEL'S argument showing that arithmetic must be incomplete and that assertions existed that could neither be proved nor disproved (see HODGES, 1983). The discovery of GÖDEL swept away two of the three demands of DAVID HILBERT'S proposed finite scheme of formal (mathematical) systems, namely the terms of consistency and completeness (HODGES, 1983, p. 92). GÖDEL'S work, however, "*left outstanding Hilbert's third question of decidability, the Entscheidungsproblem, namely the question of whether there exists a definite method which, at least in principle, can be applied to a given proposition to decide whether that proposition is provable"* (HODGES, 1997, p. 8). This question had survived GÖDEL'S analysis because *"its settlement required a precise and convincing definition of method"* (HODGES,1997). When TURING studied at Princeton, he was clearly disappointed at not being able to contact GÖDEL (who had left Princeton earlier)[2]. It was here that TURING'S contribution to pure mathematics came into play (TURING, 1936, 1937), but also that he began his conceptual contribution to the development of the computer (HODGES, 1983, 1997). Moreover, his endeavour branched off to extend the 'computable' to the realm of biological organisms.

With respect to the biological problems that became his new interests in the post-war period, TURING probably decided already in 1941 that the uncomputable, the unprovable and the undecidable were irrelevant to the problem of the mind (HODGES, 1997). Unfortunately, TURING remained unable to demonstrate the use of chemico-mechanical models beyond the level of early embryonic stages (in his 1952 paper) in order to describe the ongoing development up to a conscious (human) being, or to an application into the biology of cancer (S. TURING, 1959, p. 106). His published work on morphogenesis through a model described by coupled linear differential equations - for a system that was considered linear only when close to the origin - obviously was but a starting point for further investigation.

THE REALM OF CONSCIOUS LIVING BEINGS

In TURINGs correspondence with the neurophysiologist JOHN Z. YOUNG, he admits in a letter dated 8th February 1951 (KCC: K/1, Nr. 78), to be *"very far from the stage where I feel inclined to start asking anatomical questions"* (concerning the human brain). This would not occur, TURING said, until he had *"a fairly definite theory about how things were done"*. However, the organization of the brain seemed to be far more complicated than *"the polygonally symmetrical features of a starfish, flowers and leaf arrangements, or than the colour patterns on animals"*, although "*the formation of the brain structure should be one that could be achieved by the genetical embryological mechanism"* (TURING, KCC: K/1, Nr. 78). TURING is very confident that his work on reaction-diffusion theory will make clear *"what restrictions are really implicated"* (to the development of brain structure), and announces that he is interested in J.Z. YOUNG'S remarks on the stimulation under certain circumstances of neuron growth.

On another occasion, when comparing the activities of the human mind and the analytical properties of digital computers, TURING adopts a different stand on the phenomenon of human intelligence (TURING, 1950). Rather than examining the semantics of terms such as 'thinking' and 'machine', TURING argues that digital computatory algorithms and human intelligent activities can be completely matched or can be considered as perfect imitations of each other. That TURING considers the anatomical organization of the brain as of different order than the thinking performed by it, is indicated by the following premise of TURING'S approach: namely, putting forward the imitable properties of both systems *"has the advantage of drawing a fairly sharp line between the physical and intellectual capacities of a man"* (TURING, 1950, p. 434). Also *"there was little point in trying to make a 'thinking machine' more human by dressing it up in such artificial flesh (like a material which is indistinguishable from the human skin)"* (TURING, 1950, p. 434). According to Turing the realm of conscious human activities - being in no way performed by 'discrete state machines'-, cannot be apprehended without considering the 'educational aspect of learning'. For in contrast to the 'slow process of natural selection', the process of learning is much better to speed up the conditioning or teaching of intelligent human behaviour (TURING, 1950, p. 456). It is therefore not surprising that TURING showed hardly any interest in the anatomical characteristics of the brain (as he admits in the correspondence with J.Z. YOUNG), and nor did many of his followers in artificial intelligence and neural network theory.

Recently, attempts to describe the organization of the nervous system using an integration of neuro-anatomical and topological approaches were found in literature (Young et al., 1995; see also ALLAERTS, 1999). The integration of neuro-anatomical and topological approaches is well documented in the primate visual cortex, as shown by M.P. YOUNG and co-workers (YOUNG et al., 1995). Using

2 Later on, TURING provided a remarkable extension of GÖDEL'S theorem (see GANDY & YATES, 2001).

a topological approach, YOUNG succeeded in defining several topological characteristics of the visual system in the primate brain cortex, that correlated with known neuro-anatomical features. In other model systems of visual centers of the vertebrate brain, especially in the cat and the chicken, the role of learning and the biochemical processes underlying the adaptive capacities for learning of the visual system have been considerably well documented (see also ALLAERTS, 1999).

Contrary to the rather complicated visual system of the vertebrate brain – which indeed has served as a model system of the nervous system as a whole – , probably the best example in the nervous system for applying TURING'S reaction-diffusion theory is to be found in the regulation of axon growth. In agreement with TURING'S taste for manageably low numbers of key parameters in the model, the balance between neuron outgrowth stimulating and neuron outgrowth inhibiting factors in the regulation of axonal reconnection after spinal cord injury would be a suitable application field for TURING'S theory. In 1951, this was only a speculative idea, although TURING mentioned his interest on this point in his correspondence with J.Z. YOUNG (KCC: K/1, nr. 78).

CONCLUSIONS

The conclusion that TURING (1952) has worked out an extraordinary theory of morphogenesis is based on the following main arguments: (1) TURING worked out a complete mathematical model based on reaction-diffusion mechanisms in a very personal and self-contained way, no other template for such a model being available at his time; (2) the molecular, biological, and biochemical knowledge available to him was so scarce that his contribution to the conceptualization of the biological problem of morphogenesis and his pioneering role towards the development of theoretical biology can hardly be over-emphasized.

As pointed out by authors such as STEWART & GOLUBITSKY (1992) however, TURING'S model was in many ways imperfect and biologically inadequate. This was already recognised by TURING himself, who worked on a new theory with even greater mathematical complexity and incorporated several improvements on the original model. Unfortunately, this work was not finished at his death in 1954. We previously discussed a number of interpretations of TURING'S 1952 paper, such as the study of HARRISON (1987) (ALLAERTS & ROELANTS, 1993). Although HARRISON (1987) remarks that in TURING'S theory morphogens should be considered as diffusable cells (e.g. mesenchyme cells) rather than as chemical molecules, it can be doubted whether TURING would have agreed with this interpretation. Indeed, in his unpublished material he gave an even more restrictive meaning to the word morphogen, *"viz. chemical substance, the variation of whose concentration is described by a variable in the mathematical theory"* (KCC: AMT/C/26/5). One may regret the fact that, so far, TURING'S theory was at best exemplified in the formation of radiolarian skeletons, and not for instance in the gastrulation of the blastula, an event that better reflects the common sense of chemical processes influencing embryonic development. TURING must have been aware of this difference when stating that *"gastrulation was the most important application of his theory"* (TURING, 1952, p. 71), also because in this process the result of morphogenesis was a breakdown of the spherical symmetry to a lower degree (ALLAERTS & ROELANTS, 1993; ALLAERTS, 1999).

The importance of TURING'S mathematical theory of morphogenesis may very well extend beyond the latter questions related to embryonic development (such as the problem of symmetry breakdown), and provide genuine mathematical tools for remote biological questions. TURING'S 1952 paper was entitled a *'chemical **basis** for morphogenesis'*, whereas in the collected notes and manuscripts the title *'chemical **theory** of morphogenesis'* occurs (SAUNDERS, 1992). Although it can be doubted whether this title was chosen by TURING – the title on the manuscript shows a different handwriting, probably that of R.O. GANDY –, it is obvious that TURING in his last years mostly confined his morphogenetic studies to the domain of growth in plants, and especially the problem of phyllotaxis. However, in the same notes applications of morphogenetic theory in other domains, such as an application of this theory to the pathogenesis of cancer or to the spread of epidemics, are mentioned as well (SAUNDERS, 1992, p. 100). Anno 2002, it is needless to say that new mathematical approaches in these areas might be very valuable.

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APPENDIX

a) Use of Fourier's method for the cylindrical case of morphogenesis

An important mathematical technique used by TURING is the expansion of a function of angle θ into a Fourier series. In both the 1952 paper and in the collected unpublished works (SAUNDERS, 1992), TURING often uses a combination of a Fourier series (in one variable) and a Fourier integral (in the other variable) (SAUNDERS, 1992, p. 74).

TURING is rarely explicit regarding the biographical sources of his mathematical methods. In the case of Fourier's method, this is probably common mathematical knowledge. For most biological readers, however, this may not be easy to grasp, so some explanation of the techniques used by TURING (1952) is given below.

The problem of conduction or diffusion in a ring, in which the dependent variable depends only on one co-ordinate and the time t, is a classical example of FOURIER'S 'ring problem'. According to CARSLAW & JAEGER (1959), it was the first problem to which FOURIER applied his mathematical theory on series of periodical functions, and for which the results of his mathematical investigations were compared with the facts of experiment (FOURIER, 1822).

The expansion of a function X of angle θ into a Fourier series is of the form:

$$X(\theta) = \sum_{s=-\infty}^{\infty} G_s e^{is\theta} \qquad (1)$$

$X(\theta)$ being values of X at $t = 0$

provided that the first derivative of X is continuous. To denote its angular nature, θ is also commonly expressed in the form $\frac{2\pi}{\varpi} z$.

In the case of true periodical functions it is more convenient to expand to the goniometrical functions *sin* and *cos* because of the real periods, than to expand to exponential functions.

Using the Taylor expansions of the complex exponential function and the goniometrical functions, the following important relation directly follows:

$$e^{is\theta} = \cos s\theta + i \sin s\theta \qquad (2)$$

Consequently, taking the real parts of the Fourier expansion, a Fourier series is obtained of the form:

$$f(x) = \sum_{s=-\infty}^{\infty} (b_s \cos s\theta + c_s \sin s\theta) \qquad (3)$$

Substituting the co-ordinates x_r, y_r (denoting a small but linear deviation of the equilibrium concentrations for $X = h$ and $Y = k$ in the r-th cell) by the new co-ordinates $\xi_0, \ldots \xi_r, \ldots \xi_{N-1}$ and $\eta_0, \ldots \eta_r, \ldots \eta_{N-1}$, according to

$$x_r = \sum_{s=0}^{N-1} \exp\left[\frac{2\pi irs}{N}\right].\xi_s \qquad (4)$$

$$y_r = \sum_{s=0}^{N-1} \exp\left[\frac{2\pi irs}{N}\right].\eta_s$$

and using the relation derived from equation (2) between the complex exponential functions:

$$e^{2is\theta} + e^{-2is\theta} = 2 - 4\sin^2 s\theta \qquad (5)$$

TURING (1952) obtains the following set of linear differential equations in ξ_s, η_s with constant co-efficients :

$$\frac{d\xi_s}{dt} = (a - 4\mu \sin^2 \frac{\pi s}{N})\xi_s + b\eta_s \qquad (6)$$

$$\frac{d\eta_s}{dt} = c\xi_s + (d - 4\nu \sin^2 \frac{\pi s}{N})\eta_s$$

In simplified notation this set is of the form:

$$\frac{dx_1}{dt} = a_{11}x_1 + a_{12}x_2 \qquad (6^*)$$

$$\frac{dx_2}{dt} = a_{21}x_1 + a_{22}x_2$$

It is important to note that the co-efficients $a_{11},..a_{22}$ are constants, depending on the diffusibilities μ, ν and chemical reaction rates (see section 1.a. of main text), so diffusion constants are considered constant throughout the morphogenetic system (ALLAERTS & ROELANTS, 1993). The solutions of set (6*) are of the form $x_1 = \alpha_1 . e^{kt}$, $x_2 = \alpha_2 . e^{kt}$, where α_1, α_2 and k are chosen so that the exponential functions fulfill the set of linear equations:

$$k\alpha_1.e^{kt} = (a_{11}\alpha_1 + a_{12}\alpha_2).e^{kt} \quad \text{(PISKOUNOV, 1980, p. 121)}$$

$$k\alpha_2.e^{kt} = (a_{21}\alpha_1 + a_{22}\alpha_2).e^{kt}$$

The latter set has to be resolved with respect to α_1, α_2, yielding:

$$(a_{11} - k)\alpha_1 + a_{12}\alpha_2 = 0$$

$$a_{21}\alpha_1 + (a_{22} - k)\alpha_2 = 0$$

If k is chosen so that the determinant of the set of equations is different from zero, the only solution is the trivial solution where $\alpha_1 = \alpha_2 = 0$. However, if the determinant $\Delta(k)$ is zero, non trivial solutions are obtained from solving the so-called 'characteristic equation' of the system (see PISKOUNOV, 1980, p. 122):

$$\Delta(k) = \begin{vmatrix} a_{11} - k & a_{12} \\ a_{21} & a_{22} - k \end{vmatrix} = 0$$

or $(a_{11} - k)(a_{22} - k) - a_{12}a_{21} = 0$ (7).

The characteristic equation given by TURING (1952, p. 48) for the set of linear differential equations (6) is as follows:

$$(p - a + 4\mu \sin^2 \frac{\pi s}{N})(p - d + 4\nu \sin^2 \frac{\pi s}{N}) - bc = 0 \qquad (8)$$

of which p_s and p'_s are called the roots of the characteristic equation in p. In the case that p_s and p'_s are two distinct roots (either real or complex), the solution of set (6) is of the form:

$$\xi_s = A_s.e^{p_s t} + B_s.e^{p'_s t} \qquad (9)$$

$$\eta_s = C_s.e^{p_s t} + D_s.e^{p'_s t}$$

where TURING (1952, p. 48) states that A_s,, B_s,, C_s and D_s are arbitrary but not independent co-efficients, which are restricted to satisfy :

$$A_s(p_s - a + 4\mu \sin^2 \frac{\pi s}{N}) = bC_s \qquad (10)$$

$$B_s(p'_s - a + 4\mu \sin^2 \frac{\pi s}{N}) = bD_s$$

This follows the matrix notation for multiplication of the matrix of set (6) with the column-matrix of the solutions of the characteristic equation (8). The interdependence of A_s,, B_s,, C_s and D_s therefore directly follows the mathematical procedure for solving the set of linear differential equations (see also PISKOUNOV, 1980, Vol. 2, pp. 593-598). This mathematical result is interpreted by TURING (1952) in such a way that also important biological conclusions regarding the diffusion of morphogens are inferred from it (see main text, section 1).

By substituting (9) back into (4) and replacing the variables x_r, y_r (departures from equilibrium) by X_r, Y_r (the actual concentrations), the expression similar to (2) in section 1 (main text) is obtained for reaction and diffusion in a ring of cells. For the continuous ring of tissue, the limiting case is considered where the Fourier series is summed from $-\infty$ to $+\infty$.

b) Use of Legendre's Associated Functions and spherical harmonics

In the appendix to Part II, entitled *'Chemical Theory of Morphogenesis'* (based on TURING'S drafts used by N. HOSKIN and B. RICHARDS for the *'Morphogen Theory of Phyllotaxis'*, SAUNDERS, *ibidem*, p. 117), the use of normalised Legendre associated functions is introduced (see also appendix c). In the latter manuscript, which was actually worked out (on a suggestion by TURING) by RICHARDS, one of TURING'S students, reference is given to the work of E.W. HOBSON (1931). This is a textbook on spherical and ellipsoidal harmonics, written for trained mathematicians. The Legendre's associated functions also occur in TURING'S (1952) paragraph 12, devoted to *'Chemical waves on spheres. Gastrulation'*, where they appear as solutions to the harmonic functions on the sphere. We here present some important features of the theory of spherical harmonics and LA-functions, in order to elucidate the biological inferences, that, according to TURING (1952) can be obtained from these mathematical techniques. For the mathematical deductions, a detailed survey is given in HOBSON (1931).

Starting from Laplace's equation in polar co-ordinates r, θ, ϕ:

$$\frac{\partial}{\partial r}(r^2 \frac{\partial V}{\partial r}) + \frac{1}{\sin\theta}\frac{\partial}{\partial \theta}(\sin\theta \frac{\partial V}{\partial \theta}) + \frac{1}{\sin^2\theta}\frac{\partial^2 V}{\partial \phi^2} = 0 \quad (11),$$

and substituting $V = R\,\Theta\,\Phi$, where R, Θ, Φ are functions of r, θ, ϕ, respectively, it can be shown that some terms of Laplace's equation can be separated in only one variable. This implies that the equation can only be satisfied if these are constant terms, resulting in the general form of the solution for $\Phi = C \cos m\phi + D \sin m\theta$. Again, substituting $\cos\theta = \mu$ and $\Theta = u$, equation (11) is transformed into:

$$\frac{d}{d\mu}\left\{(1-\mu^2)\frac{du}{d\mu}\right\} + \left\{n(n+1) - \frac{m^2}{1-\mu^2}\right\}u = 0 \quad (12),$$

where n is derived from the solution of the first term of Laplace's equation :

$R = Ar^n + Br^{-n-1}$.

In the particular case where $m = 0$, equation (12) becomes:

$$\frac{d}{d\mu}\left\{(1-\mu^2)\frac{du}{d\mu}\right\} + n(n+1)u = 0 \quad (13),$$

which is known as Legendre's equation (HOBSON, 1931, pp. 9-10). The complete solution of Legendre's equation (13), where n denotes a positive integer, is of the form:

$$u = AP_n(\mu) + BQ_n(\mu) \quad (14),$$

where $P_n(\mu)$ is called Legendre's polynomial or function of the n-th degree, $Q_n(\mu)$ is the Legendre's function of the second kind and n-th degree (which has both real and complex values), and A and B denote arbitrary constants (HOBSON, *ibidem*, p. 13). As explained below, the most important term is $P_n(\mu)$, which is an algebraic function of $\mu = \cos\theta$ of degree n, and is given by:

$$P_n(\mu) = \frac{1.3.5...(2n-1)}{1.2.3...n}\left\{\mu^n - \frac{n(n-1)}{2(2n-1)}\mu^{n-2} + \frac{n(n-1)(n-2)(n-3)}{2.4.(2n-1)(2n-3)}\mu^{n-4} - ...\right\} \quad (15)$$

The first values of the polynomial $P_n(\mu)$ are as follows:
$P_0(\mu) = 1$, $P_1(\mu) = \mu$, $P_2(\mu) = \frac{1}{2}(3\mu^2 - 1)$,
$P_3(\mu) = \frac{1}{2}(5\mu^3 - 3\mu)$, and so on, and :

$P_n(0) = 0$ for all n.

RODRIGUEZ (*see* HOBSON, 1931, p. 18) gave a more convenient expression for the calculation of Legendre's function in terms of $(\mu^2 - 1)$, namely:

$$P_n(\mu) = \frac{1}{2^n.n!}(\frac{\partial}{\partial\mu})^n(\mu^2-1)^n \quad (16)$$

Moreover, making use of Laplace's definite integral expression for $P_n(\mu)$ (HOBSON, p. 25):

$$P_n(\mu) = \frac{1}{\pi}\int_0^{\pi}(\mu \pm \sqrt{\mu^2-1}\cos\phi)^n\,d\phi \quad (17)$$

the following recursion formula is obtained between three consecutive Legendre's functions:

$$nP_n - (2n-1)\mu P_{n-1} + (n-1)P_{n-2} = 0 \quad (18).$$

For a proper understanding of TURING'S use of the above equations, the question of the geometrical relevance of these algebraic relations is not without importance. It can be shown that the expression $P_n(\cos\theta) = 0$ defines a system of nodal lines (see also Fig. 2A) on the sphere, perpendicular to the axis and symmetrical to the diametral plane $\theta = \pi/2$ (HOBSON, 1931, p. 19). Therefore, $P_n(\mu)$ is called a 'zonal' harmonic, dividing the spherical surface into zones[3].

The above properties of Legendre's polynomial $P_n(\mu)$ require some introduction into the theory of spherical harmonics. This theory dates back to the work of W. THOMSON (LORD KELVIN) in England and A. CLEBSCH in Germany (HOBSON, 1931, p. 119). According to JEANS (1927, p. 208), any solution of Laplace's equation (11) is called a spherical harmonic. The most important class of harmonics consists of rational integral functions of three independent variables, e.g. the polar co-ordinates r, θ, ϕ. For the physical interpretation of these harmonics, it is important to note that the value of any finite single-valued function of position on a spherical surface can be expressed as a series of rational integral harmonics, each of the form $r^n P_n$, provided the function has only a finite number of discontinuities and of maxima and minima on the surface (JEANS, 1927, p. 211). This rule is probably the one referred to by TURING (1952, p. 70), when stating that *"any function on the sphere, or at least any that is likely to arise in a physical problem, can be expanded in spherical surface harmonics"*. JEANS (1927) wrote his textbook for students in physics and engineering, interested in the application of advanced mathematical techniques to electricity and magnetism.

So far, only the Legendre's functions of the first kind $P_n(\mu)$ are considered. This is due to the fact that, if n = integer, one of the two Legendre polynomials in equation (14) is finite, the other is infinite. When dealing with complete spheres, it is impossible for the Legendre's function of the second kind $Q_n(\mu)$ to become finite. However, in cases where the infinities of the Q_n harmonic can be excluded, for instance by excluding certain parts of the sphere, it may be necessary to take both P_n and Q_n into account (JEANS, 1927, p. 237).

Another simplification so far was that only the solutions of Laplace's equation (12) have been considered where $m = 0$, giving rise to Legendre's equation (13). When considering the general solution of the form $\Phi = C \cos m\phi + D \sin m\phi$, with m = integer, then so-called 'tesseral' harmonics constitute the solution to Laplace's equation. These tesseral harmonics are expressed in terms of so-called Legendre's associated functions (shortly: LA-functions), with symbols $P_n^m(\mu)$, $P_n^m(\mu)$. The general solution of equation (14) now becomes:

$$u = AP_n^m(\mu) + BQ_n^m(\mu)$$

$$\text{with } P_n^m(\mu) = \frac{1}{2^n.n!}(1-\mu^2)^{m/2}.\frac{\partial^{m+n}}{\partial\mu^{m+n}}(\mu^2-1)^n \quad (19),$$

in which RODRIGUEZ' expression (16) for Legendre's functions in terms of $(\mu^2 - 1)$ is recognized. An alternative, shorter notation is given by:

$$P_n^m(\mu) = \sin^m\theta\,\frac{\partial^m P_n(\mu)}{\partial\mu^m} \quad (20) \text{ (JEANS, 1927, p. 239).}$$

Equation (19) vanishes if $m + n > 2n$, i.e. if $m > n$, so also here important simplifications can be obtained. Moreover, since $Q_n^m(\mu)$ cannot be a rational integral function of $\sin\theta$ and $\cos\theta$ it is concluded that from the solution of Laplace's equation only the part with $P_n^m(\mu)$ gives rise to rational integral harmonics (JEANS, 1927, p. 239). Therefore, the solution of Laplace's equation is of the form:

$$P_n^m(\mu)(C_m \cos m\phi + D_m \sin m\phi) \quad (21)$$

which is the equivalent - TURING uses exponential rather than goniometrical terms (see appendix a) - of the expression found in

3 The idea of determining harmonics by the position of the poles was suggested by C.F. GAUSS, but was first developed by J.C. MAXWELL, although the definiteness of this method was contested by others (see HOBSON, 1931, p. 132).

TURING'S solution for morphogenesis in the spherical case (TURING, 1950, p. 70). According to JEANS (1927, p. 239), there are *(2n+1)* tesseral harmonics of degree *n*, namely:

$$P_n(\mu)\ ,\ cos\ \phi\ \ P_n^1(\mu)\ ,\ sin\ \phi\ \ P_n^1(\mu)\ ,\ ...,$$

$$\cos n\phi\ \ P_n^n(\mu),\ sin\ n\phi\ \ P_n^n(\mu).$$

For manageably low values of *n*, these are also the examples of LA-functions used by TURING (1952, p. 71)**. For instance, for $n = 1$ only three tesseral harmonics are possible:
$cos\ \theta = \mu$ (since $P_1^0(\mu)$ is always a solution), $sin\ \theta\ cos\ \phi$ and $sin\ \theta\ sin\ \phi$ (JEANS, 1927, p. 239).

For higher values of *n*, LA-functions can be calculated making use of expression (20) and the recursion formula for Legendre polynomials (18).

Concluding of this second part of the appendix, it is clear that TURING (1952) gave very little introduction to the mathematical methods used for solving the spherical case of reaction-diffusion theory (these notions were only briefly mentioned on the upper half of p. 70). Further on, mathematical techniques are used mostly in analogy with the cylindrical case (see appendix a). This also holds for the interdependence of the constants A_n^m, B_n^m, C_n^m and D_n^m, which follows the matrix formulation for solving the set of differential equations, analogous to the cylindrical case, where LA-functions constitute the co-efficients of the set of differential equations.

Finally, it is not without importance that JEANS (1927, p. 229-230) devoted a paragraph to 'nearly' spherical surfaces. JEANS shows that nearly spherical surfaces can be treated in the same way as spherical surfaces, for the squares of the harmonics describing the small deviations can be neglected. Interestingly, also TURING (1952, p. 71) regards the forms of various 'nearly' spherical structures as closely related to the latter spherical harmonic patterns (see main text).

c) Use of normalized Legendre associated functions

The normalized Legendre associated functions (shortly: normalized LA-functions) are introduced in B. RICHARDS extension of TURING'S posthumously published manuscripts (SAUNDERS, 1992, Part III, pp. 107-118), with a number of references to HOBSON (1931). The rationale is to provide a more exact solution for the morphogenetic equations in the spherical case. As shown at the end of appendix b (**), TURING (1952) also used LA-functions of degree $m = -1$, which refer to the notion of normalized LA-functions. RICHARDS makes extensive use of them in order to describe the reaction-diffusion process in small organisms (see section 2 of main text).

From the differential equation describing the reaction-diffusion process in small organisms, the solution obtained for the concentration function $U(\theta, \phi, t)$ is of the form:

$$U(\theta,\phi,t) = \sum_{m=-n}^{m=n} S_m(t).\overline{P_n^m}(\cos\theta).e^{im\phi} \qquad (22),$$

where U being real and $\overline{P_n^m}(\cos\theta)$ being the normalized LA functions, which are defined by :

$$\overline{P_n^m}(\cos\theta) = A_n^m P_n^m(\cos\theta) \qquad (23),$$

where $P_n^m(\cos\theta)$ represents the usual LA-function, with the condition that $P_n^m(\cos\theta) = P_n^{-m}(\cos\theta)$ and

$$A_n^m = \sqrt{\frac{(2n+1)(n-m)!}{(n+m)!}} \qquad \text{(SAUNDERS, 1992, p. 117).}$$

This property, referring to Hobson (1931, p. 162), is inferred from the theory of conjugate systems of harmonics. A conjugate system of harmonics of degree *n* is defined as a system of *(2n+1)* harmonics, such that for any pair of them the product of these harmonics equals zero, or:

$$\frac{1}{4\pi}\iint \overline{P_n^r}(\cos\theta).\overline{P_n^s}(\cos\theta)dS = \begin{cases} 1 & \text{for } r = s \\ 0 & r \neq s \end{cases} \qquad (24)$$

(SAUNDERS, 1992, p. 108).

The biological relevance of these conjugate systems is obvious, for it enables an important simplification in the number of harmonics describing concentration or potential functions on the sphere. When applied to the differential equation for reaction-diffusion in small organisms (see section 2), it follows that the function $\Phi(\nabla^2)$, which depends on the concentration function U, now can be replaced by a constant I, or:

$$\Phi(\nabla^2)U = IU$$

Accordingly, the general differential equation describing the changes of morphogen concentrations in time is given by:

$$\frac{dU}{dt} = IU + GU^2 - HUV \qquad (25)$$

(SAUNDERS, 1992, p. 108).

According to RICHARDS (see SAUNDERS, 1992, p. 108-111) equation (25) can now be used to derive the unknown solutions $S_m(t)$ in expression (22), following the recursion formula:

$$S_m = \sum_{i=-n}^{i=n}\sum_{j=-n}^{j=n} S_i S_j L_n^{i,j,-m} \qquad (26),$$

where the auxilliary functions $L_n^{p,q,r}$ and $E_n^{p,q,r}$ are defined as follows:

$$L_n^{p,q,r} = \frac{1}{4\pi}\int_0^{2\pi}\int_{-1}^{1} \overline{P_n^p}(\cos\theta)\overline{P_n^q}(\cos\theta)\overline{P_n^r}(\cos\theta).e^{i(p+q+r)\phi}d\cos\theta.d\phi$$

and

$$E_n^{p,q,r} = \frac{1}{2}\int_{-1}^{1} \overline{P_n^p}(\cos\theta)\overline{P_n^q}(\cos\theta)\overline{P_n^r}(\cos\theta).d\cos\theta$$

An important simplification results from application of the following conditions:

$$L_n^{p,q,r} = \begin{cases} E_n^{p,q,r} = E_n^{/p/,/q/,/r/} \\ 0 \end{cases} \quad \text{for} \quad \begin{cases} p+q+r = 0 \\ p+q+r \neq 0 \end{cases}$$

(SAUNDERS, 1992, p. 117).

Numerical examples of these calculations using the recursion formula (26) have been provided by RICHARDS (see SAUNDERS, 1992, pp. 110-114). According to RICHARDS, it is important to remember that the solutions represent deviations from the sphere; a correct balance between the oscillations of the concentration function U and the radius of the initial sphere can be obtained when looking at a suitable biological species (SAUNDERS, 1992, p.111). Examples of such suitable biological species are found in the marine organisms of the class Radiolaria (see Fig. 2.c). These unicellular organisms are surrounded by a skeleton, generally composed of silica, which forms sharp spines that radiate from the outer shell of the skeleton. RICHARDS' calculations for solutions of degree $n = 4$ reveal spheroid bodies with spines at each pole and four around the equator, among others. More complex geometrical forms are obtained by using solutions of higher degree.

** TURING (1952, p. 71) also uses LA-functions with negative integer degree (-1). This results from the use of normalized LA-functions, which notions are explained in appendix c.

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Diet of the Barn Owl (*Tyto alba*) and Little Owl (*Athene noctua*) in wetlands of northeastern Greece

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ABSTRACT. The diets of the Barn Owl (*Tyto alba*) and Little Owl (*Athene noctua*) were studied through pellet analysis in four northeastern Greek wetlands. Results from the months February-September showed that in all areas, the most important prey for the Barn Owl were small mammals (mainly *Mus* spp., *Microtus rossiaemeridionalis*, *Crocidura* spp. and *Apodemus* spp.) The mammalian prey composition showed winter-summer and among-areas differences. Except in the Evros Delta, where small mammals were dominant by both numbers and biomass, the most numerous prey of the Little Owl were insects (mainly Orthoptera and Coleoptera), but small mammals (mainly *M. rossiaemeridionalis* and *Mus* spp.) dominated the diet by biomass. Significant seasonal differences in prey composition occurred in the Evros Delta, while the summer diets were also different among wetlands. Barn Owl median prey biomass (MPB) was significantly higher than Little Owl MPB in summer, whereas prey diversity and evenness values were higher in the latter species as a consequence of considerable amounts of insects in its diet. In both owl species, MPB differed significantly among the wetlands in the year of study, while seasonal differences occurred occasionally in some of the wetlands. The total prey overlap (Pianka's symmetric equation) of the two owl species in the summer (range 27%-53%) was lower than the mammalian prey overlap (60%-97%) and both were greater than those found by other authors in most parts of the Mediterranean.

KEY WORDS : Barn Owl *Tyto alba*, Little Owl *Athene noctua*, diet, wetland, Greece.

INTRODUCTION

The Barn Owl (*Tyto alba* (Scopoli, 1769)) and the smaller-sized Little Owl (*Athene noctua* (Scopoli, 1769)) are sympatric in most of continental Europe and partly around the Mediterranean (Cramp, 1985), belonging to common trophic guilds (that is groups of coexisting species with more or less dietary overlap, Herrera & Hiraldo, 1976). The food habits and prey relationships of the Barn Owl have been much more widely studied but considerable geographical variation has been observed in prey composition of both species (Bunn et al., 1982 ; Mikkola, 1983 ; Cramp, 1985 ; Taylor, 1994). The diet of both owl species is different from that of the central European populations (Herrera & Hiraldo, 1976). Across the Mediterranean, which is characterized by drier and warmer climate than continental Europe, most studies on both species have been carried out in inland or island areas while information is lacking from coastal wetlands. Such areas, widely occurring in northeastern Greece, have been greatly transformed into cultivations but still include natural habitat of great conservation importance (MEHPW[1]). Nowadays, despite delineation and managed wetland projects, adequate habitat conservation is still lacking.

The aims of this study were : a) to describe and compare the diets of the Barn Owl and the Little Owl in coastal wetlands of northeastern Greece b) to compare the owls' diet composition between winter and summer in each wetland and among wetlands studied and, c) to compare the results of this study with others carried out in Greece and in other Mediterranean countries. The study of the diets of these and other owl species is being continued in Greece, thus the results of this study have a preliminary character.

MATERIAL AND METHODS

Pellets of Barn Owl and Little Owl were collected on successive dates from February to August 1987, in known roosts. The study areas were in four wetlands in northeastern Greece, (described briefly below). The distances between them ranged from 15 to 92 km.

The Evros Delta (40º84' N, 26º07' E), is the easternmost Greek wetland. This area includes a high diversity of habitats, such as extensive saltmarshes and salty grounds, lagoons, sand dunes and sandy islands, reed beds, tamarisk and riverine forest, temporary and permanent freshwater marshes and extensive cultivation areas (Babalonas, 1979 ; Britton & Hafner, 1979 : unpublished report).

Porto Lagos (40º01' N, 25º08' E), is a small village situated within a wide wetland complex including the shallow, polluted, brackish Lake Vistonis on the north, surrounded by reed beds and forest remnants. Extensive coastal lagoons fringed marginally with saltmarshes, sandy beaches and livestock grazing fields extend to the southwest.

Lafres (39º05' N, 25º00' E) comprises a complex of two coastal lagoons close to each other named "Lafri" and

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1 MEHPW: Ministry of Environment, Housing and Public Works. Project for delineation of Ramsar Convention wetlands. Wetland: Evros Delta (1985; 1986a; 1986b), Athens (in Greek).

"Lafrouda" surrounded by rocky cliffs with *Quercion ilicis* vegetation (MEHPW, 1986b). Extensive grasslands, saltmarshes, sandy beaches and cultivation areas occur mainly to the north of the lagoons.

Lake Mitrikou (hereafter Mitrikou) (40º99' N, 25º32' E) is a shallow freshwater lake extending over 2.3 km^2, surrounded by extensive reed beds and cultivation areas, situated in the vicinity of a coastal wetland complex (MEHPW, 1986a).

Samples from February to March were combined in a category hereafter called "winter", whereas samples collected from April to August comprised a category that will be called "summer".

The analysis of food from pellets may involve biases, especially in the case of the Little Owl, which captures invertebrates, such as annelid worms, that are difficult to recover from pellets. Nevertheless, analysis of pellets is still the most suitable method of studying the diet of owls. Pellets were analysed using reference books (Mammals : Lawrence & Brown, 1973 ; Chaline, 1974. Birds : Brown et al. (1987). Reptiles : Arnold & Burton (1980). Insects : Chinery (1981). Mean weight of each prey was taken from the literature (Reptiles : Helmer & Scholte (1985 : unpublished report). Birds : Perrins (1987). Mammals : Macdonald & Barret (1993)). Due to uncertainties in the taxonomic position of mice *Mus* sp. in our area and the impossibility of reliably distinguishing mice *Apodemus* sp. by cranial characters alone (Vohralik & Sofianidou, 1992), we did not separate them by species. The diets of each owl species were analysed separately for the winter and summer periods (where samples were available) in terms of numerical and biomass proportions, and were compared between winter and summer and between species within common periods. The trophic diversity (NB) was estimated on a prey class level by using the antilog of the Shannon-Wiener index (Shanon & Weaver, 1963), while in order to standardize the trophic diversity for comparison within and among the areas we calculated evenness index (Marti, 1987). The diet overlaps (based on prey classes) between species in each wetland were estimated by Pianka's symmetric equation (Pianka, 1973 ; Alatalo, 1981 ; Marti, 1987). These indices have been widely used in similar studies and were suitable for comparing our results with other studies.

In comparing the diet of Barn Owls among wetlands, due to the importance of mammals in their diet, we presented data on only mammalian prey to the species level, whereas other prey types were presented to a class level. Numerical proportions were compared by the χ^2 test. While for statistical reasons median prey weights were estimated and compared (by Mann-Whitney U-test or Kruskal-Wallis test), average prey weights were also estimated for comparisons with the literature. Cluster analysis was also performed to investigate whether dietary compositions were clumped by geographical area or by owl species.

RESULTS

Barn Owl

The diet of the Barn Owl in all studied areas consisted mainly of mammals, although birds, amphibians and arthropods were also included in lower proportions (Table 1). Of

TABLE 1

Diet of the Barn Owl in wetlands of northeastern Greece. W: winter; S: summer.

	Evros Delta				P. Lagos				Lafres				Mitrikou			
	% Nr		% Biomass		% Nr		% Biomass		% Nr		% Biomass		% Nr		% Biomass	
Prey	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**
CRUSTACEA	-	-	-	-	-	-	-	-	-	0.2	-	0.1	-	-	-	-
CHILOPODA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
INSECTA	-	-	-	-	2.3	0.6	0.2	0.1	1.7	0.2	0.1	0.0	0.6	1.2	0.0	0.2
AMPHIBIA	-	-	-	-	-	-	-	-	0.3	0.5	0.7	0.8	-	-	-	-
AVES	-	-	-	-	5.8	4.2	14.7	9.9	2.8	2.2	9.0	4.1	12.9	11.1	39.6	43.4
MAMMALIA	100	100	100	100	92.0	95.3	85.1	90.0	95.1	96.8	90.2	95.0	86.5	87.7	60.4	56.3
Neomys spp.	0.0	2.0	0.0	1.5	-	-	-	-	-	-	-	-	-	-	-	-
Suncus etruscus (Savi, 1822)	0.9	0.0	0.1	0.0	0.6	2.0	0.1	0.3	1.4	1.0	0.2	0.1	3.2	12.3	0.4	1.6
Crocidura suaveolens (Pallas, 1811)	28.2	31.3	12.3	13.8	19.3	16.2	7.9	6.6	21.5	14.1	10.0	5.5	18.7	8.6	7.4	3.4
Crocidura leucodon (Hermann, 1780)	1.4	0.0	0.8	0.0	1.9	1.7	1.1	0.9	8.7	6.7	5.4	3.5	21.9	25.9	11.5	13.6
Crocidura spp.	1.4	-	0.7	-					1.0	0.7	0.6	0.3	-	-	-	-
Soricidae unidentified	0.0	1.0	0.0	0.4	-	-	-	-	-	-	-	-	-	-	-	-
Pipistrellus spp.	-	-	-	-	0.3	0.3	0.1	0.1	-	-	-	-	-	-	-	-
Microtus rossiaemeridionalis Ognev, 1924	37.7	22.2	54.9	32.7	20.3	22.1	27.6	29.9	22.2	42.3	34.4	55.5	8.4	8.6	11.0	11.4
Arvicola terrestris (Linnaeus, 1758)	-	-	-	-	0.3	-	1.3	-	-	-	-	-	-	-	-	-
Rattus rattus (Linnaeus, 1758)	-	-	-	-	1.0	1.1	3.9	4.5	-	-	-	-	-	-	-	-
Apodemus spp.	7.3	22.2	10.6	32.7	7.4	11.2	10.1	15.2	3.5	9.4	5.4	12.3	5.8	3.7	7.6	4.9
Micromys minutus (Pallas, 1771)	-	-	-	-	0.6	1.4	0.2	0.5	-	0.2	-	0.1	-	-	-	-
Mus spp.	22.7	21.2	19.9	18.8	40.2	38.8	32.9	31.6	36.1	22.0	33.5	17.3	27.7	27.2	21.8	21.4
Muridae unidentified	-	-	-	-	-	-	-	-	0.7	0.2	0.8	0.2	0.6	1.2	0.6	1.2
Rodentia unidentified	0.5	0.0	0.7	0.0	-	-	-	-	-	-	-	-	-	-	-	-
Number of prey items	220	99			311	358			288	404			155	81		

1916 prey items of the Barn Owl in the four wetlands studied (both seasons, 1987), 95% were mammals, 4% birds and 1% amphibians and arthropods. The mammalian prey, including at least 11 species, consisted mainly of *Mus* spp. (31% numerically), *Microtus rossiaemeridionalis* (26%), *Crocidura* spp. (26%) and *Apodemus* spp. (8%) but there were differences between seasons depending on the species and area. These species also were the most important in terms of biomass in both seasons. The total proportions of mammalian prey both by numbers and biomass were similar in each wetland between winter and summer. Birds, being a much less important diet constituent, varied more in proportions among areas but differences between winter and summer were not considerable. The evenness values and median prey weights (except in Lafres) were similar between winter and summer (Table 2). The median prey biomasses (with an overall average of 14.4 g), (Table 2), differed significantly among the four wetlands due to differences in the ranges of individual prey weights (winter : $\chi^2 = 20.081$, df = 3, P = 0.0002 ; summer : $\chi^2 = 32.874$, df = 3, P < 0.001, Kruskal-Wallis χ^2 tests).

TABLE 2

Prey size parameters and prey diversity indices of the Barn Owl and Little Owl in northeastern Greek wetlands. Statistics between adjacent median prey weight values were performed using Mann-Whitney U tests.

Sample	N	Median (g)	Average (g)	Range (g)	Significance*	Diversity	Evenness
Tyto alba							
Evros Delta, winter	220	12.0	13.7	2-20	n. s.	0.00	0.00
Evros Delta, summer	99	12.0	13.6	6-20		0.00	0.00
Porto Lagos, winter	311	12.0	14.9	1-80	n. s.	1.39	0.46
Porto Lagos, summer	358	12.0	15.2	2-100		1.23	0.43
Lafres, winter	288	12.0	12.9	0.5-100	P < 0.0001	1.27	0.39
Lafres, summer	404	20.0	15.1	2-30		1.19	0.36
Mitrikou, winter	155	12.0	15.7	0.5-100	n. s.	0.42	0.59
Mitrikou, summer	81	12.0	15.4	2-70		0.41	0.55
Athene noctua							
Evros Delta, winter	428	20.0	14.3	0.2-70	P < 0.0001	1.89	0.58
Evros Delta, summer	361	12.0	10.3	0.1-70		2.48	0.83
Porto Lagos, summer	105	2.0	4.8	0.1-25		2.20	0.55
Lafres, summer	118	0.5	6.9	0.1-25		2.91	0.72

Little Owl

In the study areas, the Little Owl preyed mainly on small mammals and insects, but also, to a lesser extent, on other invertebrates, birds, reptiles and fish (Table 3). In total insects were the most numerous prey, making up 52% of 1012 prey items, followed by mammals (41%, at least eight species) being the most important biomass, with *Microtus* and *Mus* sp. predominating (17% and 11% respectively). The remaining prey types (birds, reptiles, fish and various invertebrates) made up 7% of the total by number. In the Evros Delta, insect proportions increased in the summer whereas mammal proportions dropped. This resulted in a significantly greater median prey weight and a lower evenness in winter (Table 2). Median prey weight was significantly different in the summer among the three wetlands, being considerably highest in the Evros Delta (Kruskal-Wallis $\chi^2 = 22.781$, df = 2, P < 0.0001) reflecting differences in the use of prey types. Diversity indices had relatively high values (Table 2) reflecting the considerable diversity in prey use by the Little Owl.

TABLE 3

Diet of the Little Owl in wetlands of northeastern Greece. W: winter, S: summer of 1987.

	Evros Delta				Porto Lagos		Lafres	
	% Nr		% Biomass		% Nr	% Biomass	% Nr	% Biomass
Prey	**W**	**S**	**W**	**S**	**S**		**S**	
MOLLUSCA	-	0.6	-	0.2	-	-	0.8	0.1
CRUSTACEA	-	0.3	-	0.1	-	-	-	-
ARACHNIDA	-	-	-	-	-	-	0.8	0.1
DIPLOPODA	-	-	-	-	1.9	0.2	2.5	0.2
ANNELIDA	0.2	0.0	-	-	-	-	-	-

TABLE 3

Diet of the Little Owl in wetlands of northeastern Greece. W: winter, S: summer of 1987.

	Evros Delta				Porto Lagos		Lafres	
	% Nr		% Biomass		% Nr	% Biomass	% Nr	% Biomass
Prey	**W**	**S**	**W**	**S**	**S**		**S**	
INSECTA	17.3	44.6	0.8	3.9	75.5	23.1	53.4	4.4
Orthoptera	2.1	9.4	0.3	1.8	46.7	20.5	0.8	0.2
Dermaptera	0.5	3.9	-	0.1	2.9	0.1	9.3	0.6
Coleoptera	14.7	31.3	0.4	2.0	24.9	2.5	41.5	3.6
Hymenoptera	3.7	3.6	-	-	1.0	-	1.7	0.0
PISCES	-	0.6	-	0.3	-	-	-	-
REPTILIA	1.2	1.9	1.1	5.7	-	-	0.8	0.5
AVES	1.9	1.9	3.9	7.0	5.8	26.5	5.1	16.1
MAMMALIA	79.2	49.9	94.3	82.7	17.3	50.2	36.4	78.6
Neomys anomalus Cabrera, 1907	-	-	-	-	-	-	0.8	1.2
Crocidura leucodon (Hermann, 1780)	0.2	0.3	0.1	0.2	-	-	0.8	1.0
Crocidura suaveolens (Pallas, 1811)	2.8	1.9	1.2	1.1	2.9	3.6	1.7	1.4
Crocidura spp.	-	0.6	-	0.4	-	-	-	-
Suncus etruscus (Savi, 1822)	0.2	-	-	-	-	-	-	-
Pipistrellus pipistrellus (Schreber, 1774)	-	0.6	-	0.3	-	-	-	-
Microtus rossiaemeridionalis Ognev, 1924	42.1	25.5	59.2	49.7	4.8	19.9	3.4	9.6
Apodemus spp.	3.5	4.4	4.9	8.7	-	-	12.7	35.9
Mus spp.	21.5	11.4	18.1	13.3	6.7	16.7	16.1	27.2
Muridae indetermined	0.9	0.6	1.0	0.8	1.9	6.0	-	-
Rodentia indetermined	3.7	2.8	5.3	5.4	1.0	4.0	0.8	2.4
Mammalia indetermined	4.2	1.9	4.4	2.8	-	-	-	
Number of prey items	428	361			105		118	

In all areas mammals were more numerous in the diet of the Barn Owl and insects in the diet of the Little Owl. Total prey overlap in the summer ranged from 27% to 53%, while the overlap in mammal prey was much higher ranging from 60% to 97%. Both prey diversity and evenness were considerably higher in the diet of the Little Owl than in the diet of the Barn Owl (Table 2). In the cluster analysis of biomass dietary proportions for all samples and areas, most prey samples of each owl species were clumped together despite the fact that they originated from different areas.

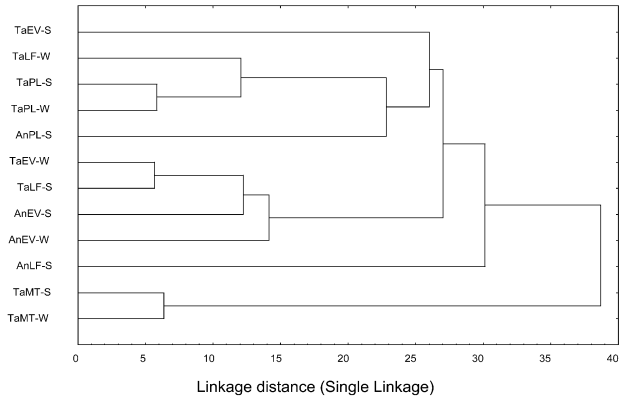

Fig. 1.– Dendrogram showing linkage distance of dietary similarity of Barn Owl and Little Owl in northeastern Greek wetlands. Ta: *Tyto alba.* An: *Athene noctua*. EV: Evros Delta. MT: Lake Mitrikou. PL: Porto Lagos. LF: Lafres. S: summer. W: winter.

DISCUSSION

Seasonal variation in the owls' diets

From our samples we could conclude that there were differences in the composition of mammalian prey of the Barn Owl between summer and winter in most areas. Seasonal dietary differences have also been found in some other studies (e.g. Campbell et. al., 1987 ; Taylor, 1994) but have not been clear in others (Smith et. al., 1972 ; Parker, 1988). Such differences have been attributed to seasonal fluctuation in abundance and behavioural changes of mammalian prey (Webster, 1973 ; Brown, 1981 ; Goszczynski, 1981 ; Taylor, 1994). Bunn et al. (1982) attributed seasonal variations in the predation of shrews to differences between habitat types in the different areas.

Regarding the Little Owl, a seasonal change in prey diversity and evenness indices (as in the Evros Delta), denotes an opportunistic exploitation of food resources. Other studies have also revealed seasonal changes in the Little Owl's diet (Cramp, 1985 ; Zerunian et al., 1982 ; Mikkola, 1983). The highest prey diversity at Lafres suggests that prey was taken from a more diverse spectrum of habitats, probably because the relative transformation of this area by man is low. Certainly, more information on the

seasonal behaviour of small mammals in our study areas would throw more light on the variation of the owls' diet.

Interspecific prey variation

In each area, differences in the diet of the two owl species were found a) in the general prey composition, containing more invertebrates in the Little Owl (as in BUNN et al., 1982 ; CAPIZZI & LUISELLI, 1995). b) in the relative composition of mammalian prey and c) in the median prey weight, being lower in the Little Owl (as a consequence of invertebrate predation). Differences in hunting techniques and different morphology and digestive efficiency of the two owl species may partly account for a different representation of prey remains in pellets (BUNN et al., 1982). The different average prey weight seems to be the result of different energetic demands of each species, with the larger species (the Barn Owl) generally taking larger prey (MARTI, 1974). The cluster analysis, which revealed a considerable dietary clumping by owl species, i.e. dietary differentiation of the two species in the study area, possibly suggests a degree of prey selection by both species. This is also supported by the low total prey overlap of the two species. However, the suggestion of selection of a kind of prey is speculative unless supported by experimental evidence. In Spain, the owls' dietary overlap was even lower (5%, HERRERA & HIRALDO, 1976 ; DELIBES et al., 1984) and in parts of Europe these owls do not co-exist in the same areas, therefore they exist in different trophic guilds (JAKSIC, 1988). In contrast, there was a considerable mammalian prey overlap found both in this study and other Mediterranean studies (94%, GOTTA & PIGOZZI, 1997) suggesting that the situation of the trophic guilds in the Mediterranean countries is more complex than originally believed and needs further investigation.

Geographical variation in prey use

In our area the differences between areas in the composition of main mammalian prey in the diet of both owls probably reflect geographical particularities in prey composition. Geographical variation in prey use by the Barn Owl has been attributed to various factors that influence the prey, such as habitat (including human-induced changes), geographical location, altitude, rainfall and temperature (HERRERA, 1974a ; 1974b ; DUENAS & PERIS, 1985 ; MARTI, 1988 ; TORRE et al., 1996 ; YOM-TOV & WOOL, 1997). Dietary variation of the Little Owl in different areas was reported by ZERUNIAN et al. (1982) and attributed to different habitat use.

TABLE 4

Importance of small mammals in the diet of barn owls across the Mediterranean Region

	Diet content (% by number)		Total number of mammal species	Most frequent mammal prey by number		Most important mammal prey by biomass		No. of species forming 80% of diet by		Prey weight (g)		
	Small mammals	Rodents		Species	% of diet	Species	% of diet	Number	Biomass	Average	Range	References
1. Greece												
This study	94.8	66.7	11	*Mus spp.*	30.9	*Mus* spp.[a] #	30.4	4	5	14	1-70	this study, 1987
Euboea island	92.8	83.6	8	*Mus spp.*	32.9	*Rattus norvegicus*	60.1	4	3	24	2-60	AKRIOTIS, unpubl. data
Korfu island	90.2	74.5	15	*Apodemus sylvaticus*	50.6	*Apodemus sylvaticus*	52.2	4	4	19	1-60	BÖHR, 1962
Krete island	96.5	87.6	7	*Mus musculus*	72.1	*Mus musculus*	57.0	2	3	15	2-60	CHEYLAN, 1976
Attica (Dafni)	67.6	53.5	6	*Apodemus mystacinus*	29.6	*Apodemus mystacinus*	76.5	***	2	15	2-40	CHEYLAN, 1977
Attica (Hymettus)	84.9	81.7	4	*Mus domesticus*	47.1	*Mus domesticus*	38.5	3	****	21	6-100	TSOUNIS & DIMITROPOULOS, 1992
2. Elsewhere												
Spain *[b]	96.8	79.2	10	*Mus spp.*	44.4	*Mus* spp.	37.3	4	4	17	4-30	BRUNET-LECOMPTE & DELIBES, 1984
	87.1	68.8	12	*Mus musculus*	44.9	*Mus musculus*	37.0	4	4	20	10-30	HERRERA, 1974a
France (Provence)	99.5	84.8	8	*Mus musculus*	54.1	*Mus musculus*	41.8	2	3	16	1-30	CHEYLAN, 1976
Italy *	96.1	77.4	12	*Apodemus spp.*	33.7	*Apodemus spp.*	49.0	5	3	18	5-80	LOVARI et al., 1976
Italy *	97.6	80.6	13	*Microtus savii*	38.6	*Microtus savii*	43.2	7	4	20	2.5-105	CAPIZZI & LUISELLI, 1996
Slovenia	99.0	32.2	18	*Neomys anomalus*	20.9	*Neomys anomalus*	19.0	8	9	14	1-70	TOME, 1992
Palestine *	77.7	65.0	11	*Microtus socialis*	46.1	*Microtus socialis*	-	3	-	-	-	DOR, 1947
Israel	99.0	97.6	5	*Meriones spp.*	49.8	*Meriones* spp.	49.8	2	2	48	20-120	YOM-TOV & WOOL, 1997
Israel	96.2	92.4	7	*Gerbillus dasyurus*	59.5	*Gerbillus dasyurus*	-	2	-	-	-	REKASI & HOVEL, 1997
Egypt	45.3	50.0	5	*Mus musculus*	42.8	*Rattus rattus*	26.8	**[c]	**[d]	35[e]	9-201	GOODMAN, 1986
Morocco *	99.7	88.7	4	*Mus musculus*	69.7	*Mus musculus*	-	4	-	-	-	SAINT-GIRRONS & THOUY, 1978

a.# Microtus rossiaemeridionalis contributed similarly to biomass, 30.3%.
b.* Compiled by TAYLOR, 1994
c.** Five mammal prey species comprised only 52.6% of the diet by number and c. 50% by biomass.
d.*** Six mammal prey species comprised only 67.6% of the diet by number.
e.**** Four mammal prey species comprised only 75.8% of the diet by biomass.

TABLE 5

Comparison of the main prey categories of Little Owls in Mediterranean countries.

Prey	Spain	Spain	Italy	Italy	Egypt	Euboea isl. Greece	Astypalea isl. Greece	NE Greece
INVERTEBRATES	95.9	96.2	95.7	88.2	14.3	97.6	99.4	38.3
PISCES	-	-	-	-	-	-	-	0.2
AMPHIBIA	0.2	1.6	-	-	-	-	-	-
REPTILIA	0.5	0.6	0.5	-	12.5	0.1	0.3	1.3
AVES	0.4	0.3	1.1	0.5	16.1	0.3	0.1	2.9
MAMMALIA	2.8	1.3	2.7	11.3	57.1	2.4	0.2	57.3
Insectivora	-	-	0.3	1	1.8	0.4	0.2	3.1
Chiroptera	-	-	-	-	-	-	-	0.2
Rodentia	2.5	1.1	1.2	10.3	55.4	1.6	-	51.6
Other	-	0.2	1.2	-	-	-	-	2.5
Total number of prey items	5018	631	3405	1636	56	1763	1068	1012
Prey Diversity (NB)	1.23	1.27	1.15	1.46	3.19	1.13	1.06	2.36
Eveness (N)	0.39	0.33	0.33	0.57	0.71	0.31	0.33	0.86
References	Herrera & Hiraldo, 1976	Jaksic & Marti, 1981	Zerunian et al., 1982	Capizzi & Luizelli, 1996	Goodman, 1988	Akriotis, unpubl. data	Angelici et al., 1997	This study, 1987

In northeastern Greece, habitat differences between study areas are considerable (MEHPW, 1985 ; 1986a ; 1986b), probably accounting for differences in the composition of the small mammals in the diet of both owls. The dominance of mice in the diet of both owls was possibly due to this prey's greater abundance in the study area as a previous trapping study suggested : 42% of a total of 473 small mammals trapped in northeastern Greece were mice (Vohralik & Sofianidou, 1992). *Microtus* may actually be abundant, because it also makes up a considerable part of the diet of other raptors in Evros (Alivizatos & Goutner, 1997). Thus the highly opportunistic Little Owl, being typically insectivorous in other parts of Europe (see further), exploited this abundant mammalian prey source.

Comparisons with other studies

Mice were the most frequent mammalian prey of the Barn Owl's diet in most Mediterranean areas. The mean proportion of small mammals in this species' diet in our study area (94.8%), fell within the range of the respective proportions in other parts of Greece (84.9% to 96.5%) (Table 4). In northeastern Greece, the total number of mammalian prey species fell within the range of that in other Mediterranean areas (4-18), but the estimated average prey weight was 14.2 g (2.9 g to 15.4 g), being among the lowest in the Mediterranean, the rest of Europe (12.8 g to 25.0 g) and the New World populations (Taylor, 1994). The information in Table 4 suggests that Barn Owls, mainly small mammal predators across the Mediterranean countries, seem to exploit locally available and/or abundant prey, probably according to the local situations, without a particular pattern being apparent in regard to geographical location.

Little Owls preyed upon a wide prey spectrum varying among northeastern Greek study areas. In four Italian neighbouring areas such prey variability resulted from habitat differences (Zerunian et al., 1982). The diet of Chilean, American and Spanish *Athene* populations presented discrepancies that reflected differential availability and not selection of prey (Jaksic & Marti, 1981 ; Jaksic, 1988). Thus, habitat and opportunistic feeding behaviour of Little Owls seems to affect the composition of their diet locally.

In most Mediterranean countries Little Owls prey mainly upon invertebrates (Table 5). It has been suggested that the relative proportions of invertebrates in the diet during the breeding season increase gradually from mid-Europe to the Mediterranean (Herrera & Hiraldo, 1976 ; Mikkola, 1983). However, in northeastern Greece and Egypt (also in Sicily, Lo Verde & Massa, 1988), there were considerably higher proportions of mammals ; a fact that may have considerable consequences in the biology of these populations of the Little Owl and is worthy of further studies.

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The localization and expression of HNK-1-immunoreactive molecules in chicken embryos

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ABSTRACT. HNK-1 (Leu-7 antigen) is a unique carbohydrate moiety bound to the subsets of a number of cells and extracellular matrix glycolipids and glycoproteins, acting as an adhesive. Using immunohistochemistry and Western immunoblot method, we observed the localization and expression of the HNK-1 epitope in chicken embryos at different developmental stages. The results of immunohistochemistry show that HNK-1-immunoreactivity (HNK-1-IR) is distributed extensively in different tissues, and the intensity of immunoreaction varies with the development stages in chicken embryos. In the nervous system, the HNK-1-IR decreases gradually in brain but remains almost stable in spinal cord with embryo development, and it is strong in the neural tube and the neural crest at stage 11. At later stages, all forming ganglia are labeled. The special conductive tissues of the heart are also recognized by the HNK-1 antibody. In the alimentary tube, HNK-1-IR mainly appears in the Auerbach plexus, the Meissner plexus, and the mucosal membrane. Endothelial cells, pit cells and/or fat-storing cells may contribute to the HNK-1-IR of the liver. The results of Western blot show that there are at least three kinds of HNK-1-reactive molecules expressed in different tissues of different stages, and the kinds of these molecules remain relatively identical with developmental stages. Our results from chicken embryos demonstrate that HNK-1 epitope is expressed widely in the entire embryonic period, and the distribution of HNK-1-positive molecules shows histological specificity.

KEY WORDS : HNK-1-immunoreactivity, development, epitope, chicken embryo, immunohistochemistry, Western immunoblot.

INTRODUCTION

The monoclonal IgM (immunoglobulin M) antibody HNK-1, which recognizes a carbohydrate epitope, was first generated to Human Natural Killer cells (Abo & Balch, 1981). In 1983, Schuller-Petrovic, et al. found that the nervous system shared HNK-1 antigenic determinants with lymphocytes. Thereafter, HNK-1 antibody became a valuable tool in studying the developing nervous system and other systems (Holley & Yu, 1987 ; Nordlander, 1989 ; Metcalfe et al., 1990).

The HNK-1 epitope is a 3'-sulfated gulcuronyl-substituted-lactoseries oligosaccharide bound to certain glycolipids (Ilyas et al., 1984), proteoglycans (Margelis et al., 1987) and subsets of a number of cells and extracellular matrix adhesion molecules. The glycolipid (Chou et al., 1986) and glycoprotein (Voshol et al., 1996) both have the sulfate-3-GlcAβ1→3Gal1→4GlcNAc domain at the non-reducing end. There are a number of cell adhesion molecules in the nervous system representing the HNK-1-immunoreactivity (HNK-1-IR), including neural cell adhesion molecules (N-CAMs), L1 (Kielhauer et al., 1985), myelin-associated glycoprotein (MAG) (McGarry et al., 1983), P0 (Bollensen & Schachner, 1987), peripheral myelin protein 22 (PMP22) (Snipes et al., 1993), J1 (Kruse et al., 1985), ependymins (Lakos et al., 1994), sulfoglucuronyl glycolipids (SGGL) (Chou et al., 1987), etc. The multiple expression of the HNK-1 epitope is involved in cell-cell or cell-extracellular matrix recognition during various choreographed stages of cell proliferation, migration, differentiation and maturation. Moreover, these molecules play an important role in learning and memory (Schmidt et al., 1995 ; Pradel et al., 1999). Most of the HNK-1-reactive molecules are expressed only in embryos or in very early postnatal stages, and restricted to a particular stage in adults.

The HNK-1-IR is distributed on the surface of migrating neural crest cells and their derivatives during development (Vincent et al., 1983 ; Bronner-Fraser et al., 1986 ; Sadashiani et al., 1990)). This indicates that the HNK-1 epitope engages in the organization of nervous system directly or indirectly by the cell adhesion molecules in vertebrates. For HNK-1, there are many reports about the migratory routes of the neural crest cells and the factors that influence migration in the embryos (Bronner-Fraser, 1986 ; Hirata et al., 1997). Although some authors also studied embryos, they devoted their attention to the cell level or some specific tissue (Metcalfe et al., 1990 ; Sakai, et al., 1994 ; Blom, et al., 1999). Many molecules have HNK-1-IR during development, but little is known about the expression of HNK-1 epitope throughout the entire embryonic development. Although the location of the HNK-1 can be ascertained by immunohistochemistry, this method cannot distinguish which kind of HNK-1-positive molecule is expressed. Moreover, it is not clear whether only one class or different classes of these molecules function at a certain stage. Here we studied HNK-1 immunoreaction using immunohistochemistry combined with Western blot technology in the different stages of chicken embryos. These results provide some evidence for understanding the roles of HNK-1 during embryonic development.

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MATERIAL AND METHODS

Animals and tissue preparation

Fertile White Leghorn chicken eggs were obtained from commercial sources and incubated in an auto-incubator (Grumbach, made in Germany) at 37.5˚C-37.8˚C and 60% relative humidity. The embryos were used after different incubation times, divided into five experimental groups : 39 hours, 3-4, 7.5, 11.5 and 15 days. According to the morphological stage series of HAMBURGER & HAMILTON (1951), the above groups were named as stages 11, 20, 32, 37-38 and 41, respectively. Three to five embryos were used in each group.

The embryos were fixed for 4-24hr in cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The preparations were then washed, dehydrated in ascending graded alcohols, cleared in xylene, and embedded in paraffin. Transverse or longitudinal 8-10μm paraffin sections were made and every sixth section collected on a slide with gelatin-chrome alum. Each slide was deparaffinized through xylene and descending graded alcohols, and then rinsed with distilled water.

Immunohistochemical staining

After pretreatment with hydrogen peroxide (H_2O_2), the above slides were incubated in normal goat blocking serum and then in a 1 :10 dilution of mouse HNK-1 IgM antibody overnight at 4˚C. Following thorough rinsing, biotin-labeled goat anti-mouse IgM (Sigma, 1 :200) was applied, followed by ABC (avidin-biotin complex ; Vector products) solution at room temperature. Finally, the sections were reacted with a mixture of diaminobenzidine (DAB, Sigma) and H_2O_2 until the reaction product was clearly visible. Sections were rinsed in water, dehydrated in ascending ethanols, cleared in xylene, mounted in Canada balsam and coverslipped. Some sections were lightly counterstained with hematoxylin and eosin.

For control, the primary antibody was omitted, and replaced by normal goat serum.

Western immunoblot

Based on our immunohistochemistry data in this experiment, we chose three stages (20, 32 and 37-38) for Western blot. Fourteen embryos were used from each stage. The sample protein was extracted from the whole embryos in stage 20, and from the brain and the trunk of embryos in the other stages, with 5 volumes of 0.05mol/L Tris-HCl buffer, pH7.5, containing 10mM EDTA, 1% sucrose, 2% Triton X-100, 100mM NaCl and 1mM PMSF. Homogenates were centrifuged for 1hr at 10,000g at 4˚C, and supernates collected and stored at -70˚C. Protein determinations were carried out according to LOWRY et al. (1951) using bovine serum albumin (BSA) as standard. Protein samples were heated for 5min at 100˚C in sample buffer containing mercaptoethanol and sodium dodecyl sulfate, and electrophoresed (BIO-RAD) on 7.5% resolving gel in the discontinuous buffer system of LAEMMLI (1970). Approximately 100 μg of total protein was loaded in each lane. For immunoblot, proteins were transferred onto nitrocellulose membrane in 25mmol/L Tris, 192mmol/L glycine and 20% methanol, pH8.3. After non-specific protein binding was blocked with 10% goat serum, the nitrocellulose membrane was incubated successively with HNK-1 antibody (1 :20), biotin-goat anti-mouse IgM, and ABC kit. Finally, colour development was achieved with H_2O_2 and DAB. The marker bands were obtained from the same gel and stained with Coomassie Brilliant Blue R-250.

RESULTS

The localization of HNK-1-immunoreaction

At stage 11, HNK-1-positive neural crest cells were detected at the medial and dorso-lateral sides of somites, from where these cells initiated their migration to the ventral trunk. Moreover, HNK-1-immunoreactive cells were ubiquitously distributed along the rostro-caudal axis at the neural tube level (Fig. 1A), and the HNK-1-reactive fibres were distributed on the wall of the neural tube (Fig. 1B).

At stage 20, labelled cells or materials were scattered in the migratory pathway of the crest cells (Fig. 1C). All ganglia derived from the neural crest, including the ganglia of cranial nerves, spinal ganglia and sympathetic ganglia, were labeled, and were as compact as crest cells (Fig. 1B). Also, HNK-1-IR was found in the conductive tissue of the heart (Fig. 1D) and dotted around the notochord (Fig. 1C).

At stage32, the HNK-1-IR was the same as that in stage 20 in the conductive tissues of the heart and in the ganglia.

Compared with the brain, the intensity of HNK-1-IR in the spinal cord was relatively stable in different stages. The spinal cord has three morphological parts : the substantia alba, the substantia grisea and the central canal (Fig. 2A). The HNK-1-IR was stronger in the substantia alba than in the substantia grisea (Fig. 2B). At stage 20 HNK-1-IR was around the notochord, but concentrated on a narrow area of the ventral notochord at stage 32 (Fig. 2B). The antibody also stained fibres innervating limbs (Fig. 2C) and the vicinity of blood vessels (Fig. 2F). In the alimentary tube, the HNK-1-IR was distributed in the Auerbach plexus (myenteric plexus), the Meissner plexus (plexus submucosus) (Fig. 2D) and the mucus membrane, and was also scattered in the liver (Fig. 2E).

At stage 37-38, the intensity of HNK-1-IR in the brain was weaker than at early stages, but in the trunk it was similar to that at stage 32 (Fig. 2F). No HNK-1-IR was observed in the brain at stage 41.

We also investigated localization of the HNK-1 epitope in the brains of chickens at postnatal days 1 and 3, and of adult white-rumped munia (*Lonchura striata swingoei*). No reactive cells or fibres were observed in these cerebrums.

In controls, where only normal goat serum was used, no specific staining was observed.

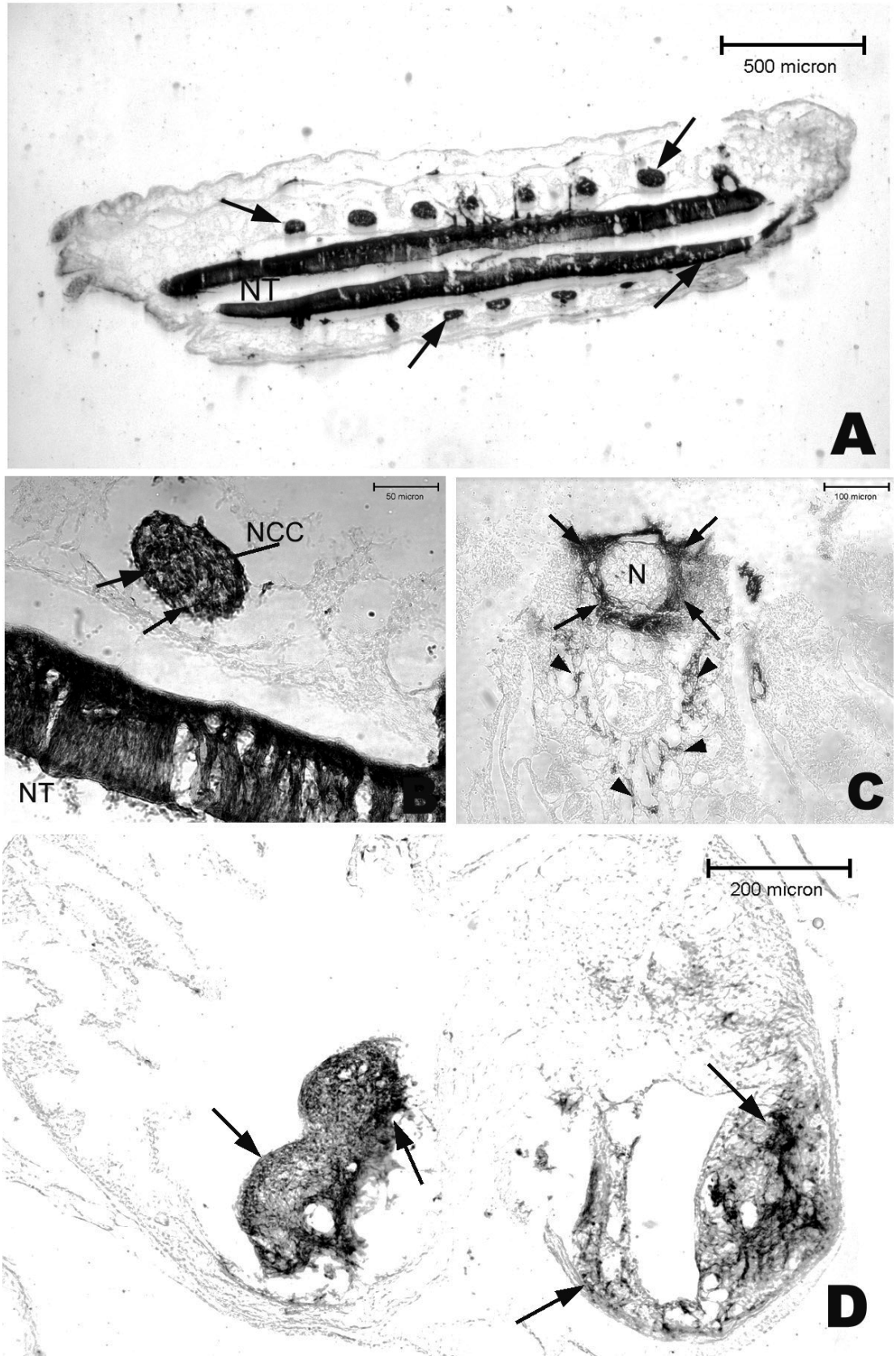

Fig. 1. – A. Whole mount of the chicken embryo at stage11. The arrows indicate where HNK-1 antibody labels the neural tube and neural crest. – B. High-power micrograph of a whole mount of a chicken embryo at stage 11. Arrows show two HNK-1-positive cells out of many. – C. Transverse section through the notochord at stage20. Large arrows indicate the surrounding of the notochord and small arrows indicate the ventral migratory pathway of the neural crest cells. – D. Longitudinal section of the heart at stage20. Arrows show HNK-1-positive conduction system of the heart. (NT: neural tube; NCC: neural crest cell; N: notochord).

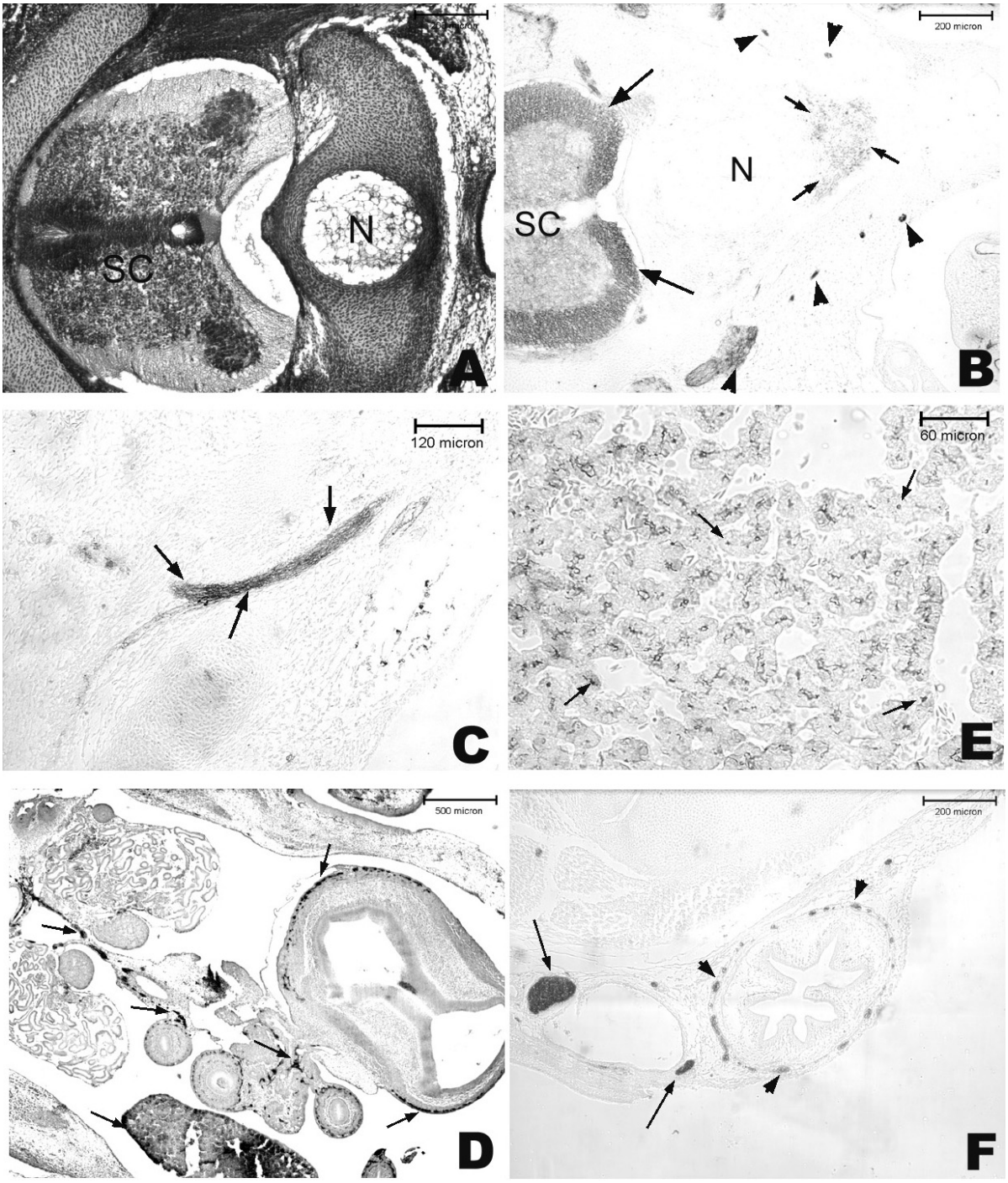

Fig. 2. – A. Transverse section of chicken embryo through spinal cord at stage32, stained with hematoxylin and eosin . It shows three parts of the neural tube: the substantia alba, the substantia grisea and the central canal. – B. Transverse section through the spinal cord at stage32. HNK-1 antibodies label the spinal cord (large arrows), the ventral side of the notochord (middle arrows) and the ventral migratory pathway of the neural crest cells (short arrows) . – C. Transverse section of the trunk of chicken embryo at stage32. HNK-1 antibodies label the nerve of the limb (arrows). – D. Transverse section of the trunk of chicken embryo at stage32 (hematoxylin-counterstained). Arrows show HNK-1-positive local nervous system in the mucosa of the alimentary tube. – E. Section through the liver of chicken embryo at stage32 (hematoxylin-counterstained). Arrows show some of the many HNK-1-positive products. – F. Transverse section of the trunk of chicken embryo at stage37-38. HNK-1 antibody labels the gut (short arrows) and the vicinity of the blood vessel (long arrows). (N: notochord; SC: spinal cord)

Western immunoblots

The results showed that there were more than three kinds of immunoreactive molecules at all the stages we investigated (Fig. 3). Moreover, from stage 20 to stage 37-38, the size of molecules bearing the HNK-1 epitope remained the same. The immunoblot showed the appearance of labeled protein bands at 40, 80 and approximately 110-200kDa. The most prominent band had a molecule size of approximately 80kDa. Close to this band, there was anoth-

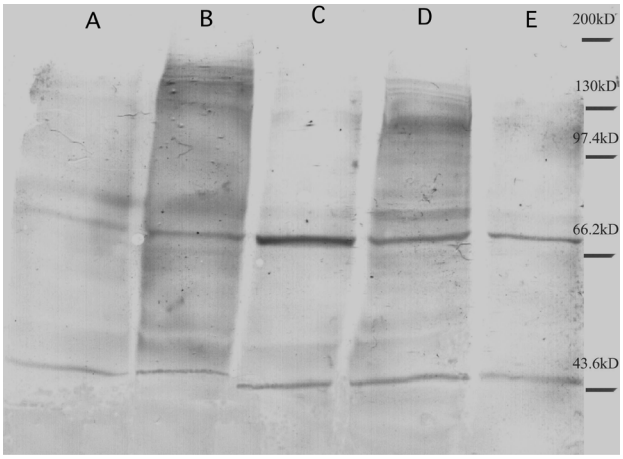

Fig. 3. – Western blot analysis of total proteins of the embryos
Lane A is blot obtained from whole embryos of stage 20; Lanes B and C are blots obtained from stage32 (B, from brain tissues; C, from trunk tissues); Lanes D and E are blots obtained from stage37-38 (D, from brain tissues; E, from trunk tissues); The five marker bands on the right were obtained from the same gel and stained with Coomassie Brilliant Blue R-250, corresponding to 200kD, 130kD, 97.4kD, 66.2kD and 43.6kD respectively from top to bottom.

er band stained very lightly in lanes A, B and D. In lanes C and E, the sites of this band were hard to see.

DISCUSSION

The spatial and temporal specificity of the HNK-1 epitope expression

Our results showed that HNK-1-IR in the brain decreased gradually with embryo development, and disappeared in postnatal and adult brains. This implies that the molecules with the HNK-1 epitope may play an important role in early development of the chicken brain. A similar tendency of HNK-1-IR was found in the 3-4 weeks postnatal mouse cerebrum (Schwarting et al., 1987). However, in the adult rat and mouse cortex, HNK-1-IR was expressed selectively on GABA-engic (GABA : γ-aminobutyric acid) neurons containing the calcium-binding parvalbumin (Kosaka et al., 1990 ; Ren et al., 1994). These observations indicate that the HNK-1 epitope engages in the organization of central nervous system, with species differences between rodents and birds during brain development.

During the development of the neural tube in chicken embryos, we found that HNK-1-IR was stronger in the substantia alba than in the substantia grisea at stages 11, 20, and 32, and stronger in the ventral substantia alba than in the dorsal side at stage 32. The substantia alba mainly consisted of myelinated fibres, and the substantia grisea consisted of non-myelinated fibres. This indicate that the myelin-forming Schwann cells mainly secrete HNK-1 adhesive molecules such as MAG, P0 and PMP22 etc. Martin et al. (1988, 1992) reported that HNK-1-positive molecules were preferentially expressed on motor neurons and motor nervous fibres in the ventral root of the spinal cord in mice, especially on the Schwann cells. By contrast, HNK-1-IR was represented both in ventral and dorsal roots in the lamprey (Hirata et al., 1997). In addition, we found that HNK-1-IR occurred in the limb nerves at later development. These investigations indicate that the HNK-1 epitope participates in the formation of myelin and the differentiation of motor neurons in spinal cord.

In conjunction with previous investigations (Bronner-Fraser, 1986 ; Loring & Erickson, 1987 ; Kuratani, 1991), our results revealed that migration of trunk neural crest cells in chicken embryos is also along ventral pathways. The same migration pattern was also identified in zebrafish (*Brachydanio rerio*) embryos (Raible et al., 1992) and in swordtail (*Xiphophorus helleri*) embryos (Hirata et al., 1997). In chicken and in *Xenopus* species (Sadaghiani & Vielkind, 1990), neural crest cells are present in the dorsal and lateral sides of the neural tube. They also migrate further to the ventral side of the notochord, and even to the area surrounding the dorsal aorta, where the sympathetic nervous system is formed. In the lamprey (*Lampetra reissneri*) on the other hand, Hirata et al. (1997) found no HNK-1-positive cells that migrate ventrally beyond the notochord at axial levels. These findings demonstrate that the migratory pathway of neural crest cells is relatively conservative in fish, birds and mammals.

In the heart of chicken embryos, Luider et al. (1993) and our results showed that HNK-1-IR is present in the septum of atriorum and ventriculorum where the special conductive tissues of the heart are located. The conductive tissues of the heart are not derived from neural crest cells, but these tissues displayed the HNK-1-IR in embryos. This phenomenon was also observed in rats (Nakagawa et al., 1993 ; Sakai et al., 1994 ; Wenink et al., 2000). Chuck et al. (1997) found that HNK-1-IR was more intense in His-Punkinje system than in normal myocardium in avian embryos. This shows that HNK-1-positive proteins implicated in cell interactions play an important role in the formation and maintenance of function of the cardiac conduction system. In human embryos with abnormal atrial automaticity, HNK-1-IR was found to be expressed temporarily around the left and right atrial pacemaker (Blom et al., 1999). This indicates that HNK-1 plays an important role in the development of conductive tissues of the heart.

The enteric nervous system, including the Auerbach plexus and the Meissner plexus etc., is derived from neural crest cells. As well as in the enteric nervous system of the alimentary tube, we also found HNK-1-IR in the mucus membrane and liver of chicken embryos. A similar phenomenon was observed in the vicinity of the hepatic sinusoid in rats (Peinad, 2000). The question is, which functions of these HNK-1-positive cells were active in the alimentary tube during development. It is known that many kinds of APUD (Amine Precursor Uptaking and Decarboxylating) cells are scattered and mingled with the endothelial cells of the alimentary tube and in the digestive glands, regulating digestion and metabolism. Margolish et al. (1987) found that some APUD cells were chromaffin cells, which expressed some HNK-1-positive glycoproteins and glycolipids. The HNK-1-positive cells in the alimentary tube of chicken embryos may, therefore, be APUD cells. In liver tissues there are four kinds of cells : endothelial, Kupffer, fat-storing and pit. Some endothelial cells can secrete an active substance with the HNK-1 epitope, such as chondroitin sulfate, and the fat-storing

cells are descendants of the neural crest cell. The pit cell is a large granular lymphocyte, and it possesses the activity of natural killer cells. It is possible that the HNK-1-IR in the liver of chicken embryos may be attributed to some secretory endothelial cells, fat-storing cells and/or pit cells.

The above indicates that HNK-1 can engage in organization of the nervous system, including the central and peripheral systems. It can influence the development of the conductive tissues of the heart, and the development of the system to regulate digestion and metabolism.

The multiple expressions of the HNK-1 epitope

Our results of Western immunoblot show that there are at least three kinds of HNK-1-reactive molecules participating in development of chicken embryos at each stage. The blots show a slight difference of HNK-1-positive molecular expression between brain tissues and trunk tissues. For example, 110-200kD bands could be detected clearly from the brain tissues, but were only faintly visible from the trunk tissues. Many more kinds of HNK-1-positive molecules are expressed in the brain than in the trunk at the same stage. It is very interesting to find that the same epitope was expressed on many kinds of molecules. This result is similar to that from avian dorsal root ganglia (DRG) with HNK-1 antibody (Tucker et al., 1984). In 11-day-old avian embryos, three major bands of 130, 200 and 300kD were detected from DRG, while a major diffuse band spreading from 300 to 200kD and two other components at 180 and 130kD were observed in the cerebrum. Moreover, some other antibodies, such as the monoclonal antibodies VC1.1 (Naegele & Barnstable, 1991), L3 (Kücherer et al., 1987), zn-12 (Metcalfe, et al., 1990) could similarly react with several molecules. These antibodies probably recognized one lapping, or identical carbohydrate epitopes. Generally, the molecules detected by these antibodies had weights ranging from 20 to 300kD, and our result tallies with this point. However, we do not yet understand the mechanism and the significance of overlapping expression from the different HNK-1 molecules, nor how these molecules interact with each other. It is likely that these molecules can be expressed at a precise time and in specific cells during the development of the nervous system. Future studies will need to determine and separate the different kinds of HNK-1-reactive molecules with specific antibodies, to determine which kinds of HNK-1-reactive molecule play different roles at different stages.

Our results revealed that the HNK-1 epitope is distributed quite widely in embryogenesis. Interestingly, we found that HNK-1-positive cells are accumulated not only in the neural crest at stage 11, but also in the ganglia at later stages. Thus, we infer that the molecules with HNK-1-IR could advance cell-cell adhesion or cell-matrix adhesion so as to contribute to cell accumulation and ganglion formation. These findings help us to understand the roles of HNK-1-positive molecules during development in the chicken embryo.

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An update on the inland cladoceran and copepod fauna of Belgium, with a note on the importance of temporary waters

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ABSTRACT. A field survey of mostly small and shallow inland water bodies in Flanders yielded three cladoceran and two copepod species new to the fauna of Belgium. These new records involve two *Moina* species (*M. micrura, M. weismanni*), *Simocephalus serrulatus*, the calanoid copepod *Mixodiaptomus kupelwieseri* and the cyclopoid copepod *Metacyclops minutus*. Special attention was paid to the fauna of temporary pools and wheel tracks. In the pools sampled in spring, the zooplankton was dominated by *Daphnia obtusa, Eucyclops serrulatus* and/or *Diacyclops bicuspidatus*. The crustacean fauna of the pools sampled in summer was dominated by *Moina macrocopa* and *Metacyclops gracilis*, whereas *Metacyclops minutus* was dominant in the pools sampled in autumn. Even though species diversity was generally found to be low in the wheel tracks and pools sampled, the taxa were found to be rather typical.

KEY WORDS : Cladocera, Copepoda, new records, temporary waters, vernal pools, Belgium.

INTRODUCTION

Temporary waters bodies are common on all continents, but biological and ecological research on these habitats lags behind the studies on permanent lentic and lotic waters (e.g. Schwartz & Jenkins, 2000). This lack of attention probably stems from the small size of many of these habitats, the fact that they contain no fish, and their transient nature. As such, these habitats have been considered of limited economic value. From an ecological point of view, however, these habitats have considerable added value, because they contain taxa that are absent from more permanent water bodies (Eder & Hödl, 1996 ; Maier et al., 1998). Although biodiversity in these habitats is often not spectacularly high, many taxa inhabiting temporary water bodies have special adaptations to the ephemeral nature of the habitat and can be considered ecological specialists (Brendonck, 1996 ; Williams, 2000). Since temporary waters often lack fish, they also function as a refuge for taxa that are not able to coexist with fish because of their large body size (Kerfoot & Lynch, 1987). The best representatives of this strategy are the large branchiopods, that, in the course of evolution, were excluded from many habitats by smaller-sized zooplankton species that were less vulnerable to fish predation (Kerfoot & Lynch, 1987), but managed to survive in all kinds of, mostly temporary, water bodies that lack fish. Large branchiopods can thus be considered "flagship" species of small temporary waters (Eder & Hödl, 1996). Temporary ponds are threatened all over the world, not only because of deteriorating water quality, but also because their number is decreasing dramatically, largely due to human impact such as draining, agriculture, and urban development (Brown, 1998). Temporary waters are under threat all over Europe, but their status is more dramatic in Western than in Central Europe (e.g. Mura, 1993). In areas such as Flanders (Belgium), most temporary pools and ponds have disappeared, and the large branchiopods have disappeared for about a century (Brendonck, 1989). Of the two records of large branchiopods in Belgium in the last decades (*Leptestheria dahalacensis* (Rüppel, 1837), Brendonck et al., 1989 ; *Chirocephalus diaphanus* (Prévost, 1803), Loneux (Walravens, 1998), the conchostracan *Leptestheria dahalacensis* recorded from the region of Brussels no doubt was accidentally introduced.

There is virtually no knowledge on the biota in the remaining temporary ponds in Flanders, such that a comparison with ponds in less impacted areas is impossible. The purpose of the present paper is to present the results of a survey of a limited number of temporary pools in Flanders, in an effort to gain insight on whether these water bodies contain typical crustacean zooplankton taxa, even though the large branchiopods have disappeared. In addition, we report on the occurrence of *Moina* species in Flanders. *Moina* species are often an important component of the zooplankton in temporary waters (Goulden, 1968), but virtually no data have been reported on the occurrence of *Moina* species in Flanders. We have observed *Moina* species in both temporary and more permanent water bodies. Finally, we also report on an additional new record for the cladoceran fauna of Belgium.

MATERIAL AND METHODS

The field surveys consisted of two parts. First, we conducted a survey of the microcrustacean taxon composition

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in a set of 30 temporary pools and wheel tracks. We sampled ten temporary waters in spring 2000 (March-April), 13 in summer 2000 (July) and seven in autumn 2000 (November) (Table 1). All samples were taken with a plankton net of 85 µm mesh size. The samples were immediately preserved with 4% formaldehyde.

In addition to this survey of the zooplankton composition of a limited set of temporary waters, we also carried out several field surveys directed at the documentation of the occurrence of representatives of *Moina* in Flanders. Between 1988 and 2000, numerous water bodies were sampled and screened for the presence of *Moina* species.

Finally, we also report another new record for the Belgian cladoceran fauna. This species was observed during field surveys of the pond complex of De Maten (Cottenie et al., 2001 ; Cottenie & De Meester, in press). The pond complex consists of 35 shallow and interconnected ponds ranging in size from <1 ha to approx. 10 ha. In summer (July) 1996, 14 of these ponds were intensively sampled with a dip net with mesh size 200 µm and analysed in detail for their taxon composition in microcrustacean zooplankton, screening a minimum of 300 individuals. As the earlier work on these samples focused on patterns of similarities in species composition among ponds (Cottenie et al., 2001) and on patterns of species diversity (Cottenie & De Meester, in press) without providing a species list, we here report on the new records.

RESULTS

New records

Moina micrura Kurz, 1874

Material : several egg-bearing parthenogenetic and ephippial females and two males.

Locality : pond near Lake Donkmeer (Overmere, Eastern Flanders ; August 1988)

M. micrura was originally described from Central Europe, and considered as a highly variable, cosmopolitan species (Goulden, 1968). In Europe, this species has a circummediterranean-pontic distribution, with the northernmost occurrence at Hamburg (Flössner, 2000). It is also known from The Netherlands (Notenboom-Ram, 1981). Its occurrence in Belgium was anticipated by Dumont (1989a). The species can be easily differentiated from the two *Moina* species that have previously been reported from Belgium (*M. brachiata* and *M. macrocopa* : Dumont, 1989a) by its small size, large eye, deep supra-ocular depression, and the structure of its postabdomen and claw.

Moina weismanni Ishikawa, 1896

Material : seven parthenogenetic females, two ephippial females and two males.

Locality : dead arm of the River Schelde near Melle (Eastern Flanders ; August 1988).

M. weismanni was earlier known from the Far East (Goulden, 1968). Margaritora et al. (1987) have recorded this species for the first time in Europe in Italy. Hudec (1990) reported the occurrence of this species from Czechoslovakia, Hungary and Yugoslavia, and Petkovski (1991) reported the species from SW Yugoslavia (Macedonia), proving that the species inhabited the Balkan Peninsula too. The present record is the first from Western Europe. *M. weismanni* is very similar to *M. micrura* : the parthenogenetic females of the two species are very difficult to separate. The sexual females can, however, easily be recognized, because of the unique surface ornamentation of the ephippium of *M. weismanni* (Fig. 1). The central and dorsal part of the ephippium of *M. weismanni* is characterized by raised knobs, which can be easily seen in lateral view. In addition to the ephippium, the female´s distinctive features are the short and thick antennules and the 15-21 fine, long spicules of the pecten on the postabdominal claw (Hudec, 1990). In the Central European *M. micrura*, the antennules are long and spindle-like, and the pecten has 10-15 short, thick horns that are nearly triangular-shaped. The distinctive characters of *M. weismanni* males are the long antennules that are thick near the base and show four short, thick hooks at the tip. *M. micrura* males have long and thin antennules, with three long and thin hooks on the tip (Hudec, 1990). The ventro-anterior part of the carapace has fine, long hairs in *M. weismanni*, while it is entirely naked in *M. micrura*.

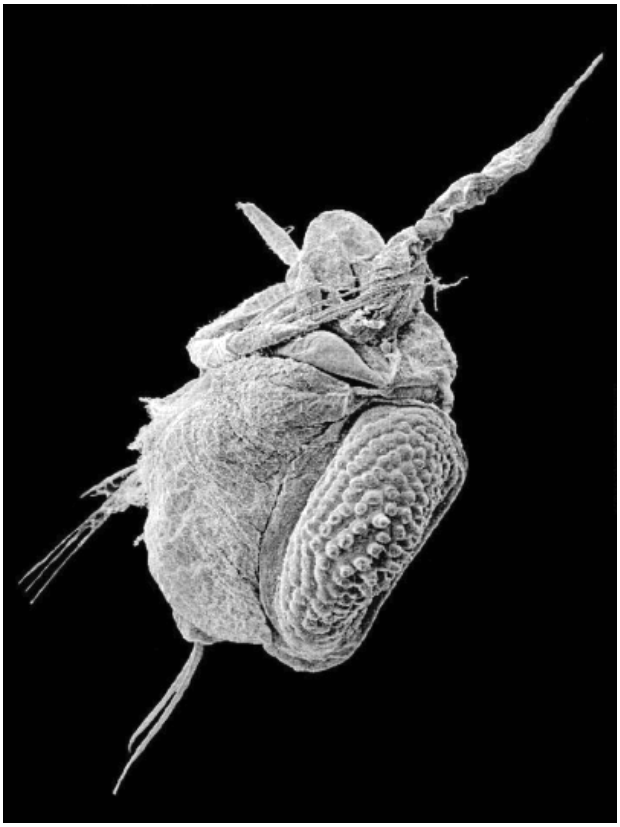

Fig. 1. – *Moina weismanni*, female showing ornamentation of ephippia.

Simocephalus serrulatus (Koch, 1841)

Material : 62 parthenogenetic females.

Locality : five different ponds in the pond complex of De Maten (July 1996).

Simocephalus serrulatus has a world-wide distribution, but has never been recorded from Belgium. It has been found all over Europe, except the Iberian peninsula, Ire-

land and the Arctic region (Flössner, 2000) and was anticipated to occur in Belgium in the review by Dumont (1989a). This species is very characteristic, and easy to recognize thanks to the large prominence of the dorso-posterior valve separated from the rest of the valves by deep embayment. Further characteristic features are the denticles on the frons and on the ventral, posterior and on about one third of the dorsal margins of the valves. It seems to be quite widespread in the pond complex of De Maten, although it proved much less abundant than the two other *Simocephalus* species (*S. vetulus* and *S. expinosus*), which were both represented by several hundreds of individuals in the same samples.

Mixodiaptomus kupelwieseri (Brehm, 1907)

Material : several females and males.

Locality : a vernal pool in Bourgoyen Nature Reserve (Gent, Eastern Flanders ; March 2000).

Mixodiaptomus kupelwieseri was the only calanoid copepod in our samples from temporary pools, and was abundant in the one pond in which it was found. This record greatly extends the known distribution area, since previously it was known from Central and Southern Europe (Austria, Moravia, Hungary, Italy, Greece) and from the Camargue (Dussart, 1967 ; Einsle, 1993). It is a typical inhabitant of temporary waters. It is found in autumn and winter in Southern Europe (Dussart, 1967) and in spring in Central Europe (Brtek, 1954).

Dumont (1989b) listed seven calanoids from Belgium, four of them belonging to Diaptomidae. *Mixodiaptomus kupelwieseri*, the fifth diaptomid species, but the only member of the genus *Mixodiaptomus* in Belgium, can be easily differentiated from the other diaptomids, particularly based on the structure of the fifth legs of the female and male (Einsle, 1993).

Metacyclops minutus (Claus, 1863)

Material : several females and males.

Locality : seven temporary pools located near Leuven, Erps-Kwerps, Nederokkerzele and Kampenhout (November 2000).

M. minutus is a typical species of temporary pools (Maier, 1992a), its occurrence in our material was limited to temporary pools sampled in autumn. With the exception of at one site it occurred in very low numbers. However, in six of the seven pools it was the only species found.

This is the third species of the genus *Metacyclops* found in Belgium. Based on the antennule containing 11 segments it can be separated from *M. problematicus* (which is only known from one locality). *Metacyclops gracilis* has two apical spines on the endopod of the 4th leg, while there is only one seta on the endopod of the 4th leg of *M. minutus* and a further distinctive character is the absence of the inner seta on the basis of the 1st leg in the latter species. It is cosmopolitan (Einsle, 1993), distributed all over Europe, though mostly rare; in Germany it is listed in the red data book (Maier et al., 1998).

The occurrence of Moina in Flanders

Altogether four species of *Moina* are presently known from Belgium. In addition to the two new species reported to occur in Belgium (see above, *M. micrura* and *M. weismanni*), *M. brachiata* and *M. macrocopa* have already been reported (Dumont, 1989a). During our survey, we did not detect *M. brachiata*, while *M. macrocopa* was found in several wheel tracks near Willebringen and Tielrode in July 2000.

Community composition of crustacean zooplankton in temporary pools and wheel tracks

Table 1 lists the taxa observed in a total of 30 temporary pools and wheel tracks. In total, seven cladocerans and nine copepods were recorded. In spring, only three cladoceran species were observed, all of which were widespread and common. The dominance of *Daphnia obtusa* as the only daphnid in all but one of the pools sampled in spring is striking. Copepods were prominent members of the zooplankton in the pools sampled in spring, with a total of three cyclopoid species and one calanoid species. The calanoid *Mixodiaptomus kupelwieseri* has been discussed above. There was only little overlap in the occurrence of the different cyclopoid copepods, which tended all to be very common. In summer, six cladocerans and six copepods were observed. The two larger temporary pools sampled (Haasrode) were dominated by *Simocephalus* species, *Daphnia curvirostris* (one pond) and three cyclopoid copepods (*Macrocyclops albidus, Eucyclops serrulatus* and *Acanthocyclops robustus*). *Moina macrocopa* reached very high densities in most of the small wheel tracks sampled at Willebringen, whereas *Daphnia obtusa* dominated the larger wheel tracks at the Doode Beemde. In most of the small wheel tracks at Willebringen, *Metacyclops gracilis* was the only copepod. In two of these wheel tracks, this species was accompanied by *Paracyclops fimbriatus* (Table 1). In autumn, most ponds sampled only harboured *Metacyclops minutus*. In one of the ponds, this species was accompanied by *Daphnia pulex* and *Cyclops strenuus*.

DISCUSSION

Dumont (1989a) summarized the faunistical knowledge on the non-marine Cladocera of Belgium and listed 74 species. Since then, Beladjal et al. (1992) reported the occurrence of *Alona rustica* Scott, 1895, De Meester & Bosmans (1994) reported on the occurrence of *Eurycercus glacialis* Lilljeborg, 1887 in a pond that had been restored by dredging (thus potentially representing a case of the recovery of a species that occurred in the pristine habitat and hatched from an old resting egg bank), and Ketelaars & Gille (1994) reported on the occurrence of *Bythotrephes longimanus* Leydig, 1860 from Belgium. The present paper adds three new records to this list (*Moina micrura*, *M. weismanni* and *Simocephalus serrulatus*). As a result, the current number of cladocerans recorded from Belgium has increased to 80. Several of the species were anticipated to be discovered based on their known geographic area (Dumont, 1989a). The most surprising and unexpected finding is *M. weismanni*. The previous records from Central and Southern Europe were attributed to an introduction

TABLE 1

List of Cladocera and Copepoda species recorded in 30 temporary pools sampled in Flanders.

	Spring 2000										Summer 2000												Autumn 2000							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Cladocera																														
Daphnia curvirostris Eylman, 1887												+																		
Daphnia obtusa Kurz, 1874	+		+	+	+	+	+	+	+	+			+	+								+	+							
Daphnia pulex Leydig, 1860		+																						+						
Simocephalus vetulus (O.F. Müller, 1776)											+	+																		
Simocephalus exspinosus (Koch, 1841)												+																		
Moina macrocopa (Straus, 1820)														+	+		+	+		+	+									
Chydorus sphaericus (O.F. Müller, 1785)		+					+	+	+				+									+	+							
Copepoda																														
Mixodiaptomus kupelwieseri (Brehm, 1907)	+																													
Macrocyclops albidus (Jurine, 1820)											+	+																		
Eucyclops serrulatus (Fischer, 1851)				+	+		+				+	+																		
Paracyclops fimbriatus (Fischer, 1853)																				+	+									
Cyclops strenuus Fischer, 1851	+	+																						+						
Acanthocyclops robustus (Sars, 1863)											+	+																		
Diacyclops bicuspidatus (Claus, 1857)							+	+	+	+												+	+							
Metacyclops gracilis (Lilljeborg, 1853)													+	+	+	+	+	+	+	+										
Metacyclops minutus (Claus, 1863)																								+	+	+	+	+	+	+

List of pools : Spring samples : 1 : Gent, Bourgoyen Nature Reserve, vernal pool, 25. 03. 2000 ; 2 : Berlare, temporary pond, 25. 03. 2000 ; 3 : Berlare, wheeltrack, 25. 03. 2000 ; 4 : Berlare, forest wheeltrack, 25. 03. 2000 ; 5 : Berlare, forest wheeltrack, 25. 03. 2000 ; 6 : Berlare, forest wheeltrack, 25. 03. 2000 ; 7 : Berlare, forest wheeltrack, 25. 03. 2000 ; 8 : St-Pieters-voeren, forest pool, 07. 04. 2000 ; 9-10 : St-Pieters-voeren, two forest wheel tracks, 07. 04. 2000. Summer samples : 11-12 : Haasrode, two temporary ponds, 04.07.2000 ; 13-21 : Willebringen, nine small wheeltracks, 04.07.2000 ; 22-23 : Sint-Joris-Weert, Doode Bemde Nature Reserve, two large, shaded wheeltracks, 04.07.2000. Autumn samples : 24-26 : Leuven, three temporary pools, 05.11.2000 ; 27 : Erps-Kwerps, temporary pool, 05.11.2000 ; 28 : Nederokkerzele, temporary pool, 05.11.2000 ; 29-30 : Kampenhout, two temporary pools, 05.11.2000.

that was associated with the experimental introduction of rice into Hungary and Slovakia (Hudec, 1990). Brendonck et al. (1989) reported *Leptestheria dahalacensis* from a man-made fish pond near Brussels and presumed the species might have been introduced with fish transport from Hungary in 1984. Thus it is possible that *M. weismanni*, which was first found in Belgium in samples collected in 1988, also arrived to this region by introduction associated with fish transport from Central Europe, but this needs better documentation. Overall, it seems very likely that the frequent translocations of fish in Europe have resulted in numerous artificial introductions of zooplankton species in ponds within as well as outside of the natural geographical range of the species. From a conservation point of view, there is an urgent need to prevent these uncontrolled translocations of organisms. The situation is particularly dramatic with respect to contamination of fish communities themselves (Declerck et al., unpubl. data), but needs also to be documented for the taxa that are by accident translocated together with the fish.

Dumont (1989b) also gave a summary of the free-living fresh- and brackish-water Copepoda of Belgium, and predicted that further species were most likely to be expected from interstitial waters and groundwater. Recently, Fiers & Ghenne (2000) published new records of one cyclopoid and eight harpacticoid species found in moist forest litter, and Alekseev et al. (in press) discovered two new cyclopoid species for the fauna of Belgium. The present paper provides two other unexpected additions to the copepod fauna. Again, it seems that these additions are most likely due to the fact that the habitat studied – temporary waters – has been neglected in earlier work. Altogether, eight calanoid and 46 cyclopoid copepods are known from Belgium.

Species diversity in the temporary pools studied was rather low : none of the pools or wheel tracks sampled harboured more than six crustacean zooplankton species. This may be partly explained by the small size of all of the ponds studied (all less than 0.1 ha, and most less 0.01 ha), but may also reflect the fact that the inhabitants of these short-lived water bodies need special adaptations. The latter is also reflected by the fact that several of the taxa recorded are not often found in other habitats (e.g. *Daphnia obtusa, Moina macrocopa, Mixodiaptomus kupelwieseri, Metacyclops gracilis* and *M minutus*). All five copepod species observed in the temporary pools and wheel tracks studied are known to prefer small waters and are usually observed in spring (e.g. Brtek, 1954 ; Fryer, 1985a). Given this degree of specificity, the temporary pools may be considered valuable from a conservation point of view, even in a region such as Flanders in which many of their more spectacular fauna may have disappeared. *Metacyclops minutus* is listed as an endangered species by Maier et al. (1998).

Our data are suggestive of seasonal changes in the fauna of the pools studied. *Daphnia obtusa* seems to be an important component of temporary waters in spring. This confirms previous literature data (Fryer, 1985a ; Maier 1992b, 1993). In England, *Daphnia obtusa* was commonly found in small ponds (Elbourn, 1966). Fryer (1985b) concluded that it can colonize stressed habitats in Yorkshire, and this observation was confirmed also in Italy by Bachiorri et al. (1991). In summer, the cladoceran fauna of most of the

wheel tracks studied was dominated by *Moina macrocopa, Daphnia obtusa* only being found in relatively large wheel tracks. The copepod fauna in summer was dominated by *Metacyclops gracilis,* whereas the only species occurring in the autumn samples was *M. minutus*, and *Mixodiaptomus kupelwieseri* is a typical spring species. As we sampled different ponds in the different seasons, it is obvious that one should be careful in interpreting these patterns as merely reflecting seasonal changes. Yet, the patterns discussed are in concordance with literature data (Fryer, 1985a ; Maier et al., 1998). Maier (1992b, 1993) describes a replacement of *Daphnia obtusa* by *Moina brachiata* in vernal pools in Germany, whereas Fryer (1985a) reported on the occurrence of both *Daphnia obtusa* and *Moina brachiata* in temporary waters in the U.K. It is striking that we observed only *Moina macrocopa* in our samples, whereas both these earlier reports refer to *M. brachiata.*

Overall, our study underlines the importance of temporary water bodies for the aquatic invertebrate fauna. The fact that an albeit limited sampling effort has resulted in four (two cladocerans and two copepod) taxa new to the Belgian fauna suggests that wheel tracks, temporary waters and wetlands in general deserve more detailed study, and should be incorporated in standard monitoring schemes on nature value.

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Checklist of the Benthic Gammaridea and Caprellidea (Crustacea: Peracarida: Amphipoda) from the Gulf of Mexico Continental Shelf and Slope

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ABSTRACT. The published records of amphipod Crustacea from the Gulf of Mexico continental shelf and slope were reviewed and compiled in a checklist to help the study of this taxonomic group in this large marine ecosystem (LME). Species from both the continental shelf and the upper slope are reported for the diverse habitats that occur in the LME. Unpublished records of amphipod specimens deposited in national collections, validated databases, and reports in the LME have been included. A total of 101 benthic species is recorded in the Gulf of Mexico and is grouped into 55 genera, 26 families and two suborders. The synonymy of the recorded amphipod species of this LME has been included to account for nomenclatural changes in the recent literature. Identifying the components of the amphipod diversity and its occurrence in the different habitats of the Gulf of Mexico provides a useful baseline for both management and conservation.

KEY WORDS : Amphipoda, distribution, diversity, LME, marine habitats.

INTRODUCTION

The order Amphipoda encompasses one of the most diverse peracaridean groups with almost 7000 species (Bellan-Santini, 1999), the gammarid suborder being the most widespread and diverse of the amphipods (Barnard & Karaman, 1991). The other three suborders are highly specialized and restricted in their distribution. The amphipods, together with the polychaetes, constitute 77 % of the total abundance of the macrobenthic community of the coastal ocean (Probert & Grove, 1998). Gammaridean amphipods have been recognized among the six most diverse groups of the macrobenthic assemblages in the northwestern sector of the region (Pequegnat et al., 1990). The amphipods represent a key food resource for cephalopods (Perez & Haimovici, 1995) and for fish and macrocrustacea in the coastal habitats (Bright, 1970; Escobar & Soto, 1997). Amphipods are important components in the regeneration of nitrogen in the sediment-water interface (Gardner et al., 1993) and are sensitive to environmental changes being therefore considered excellent bioindicators (Lear & O'Malley, 1983; Thomas, 1993a; Montagna & Harper, 1996).

The existing documentation on amphipods recorded in the Gulf of Mexico is focussed on the taxonomy of species of the littoral zone (Ortiz, 1979; Ortiz & Lalana1993) and the coastal ocean (Pequegnat et al., 1990; Rabalais et al., 1999). Reviews of the group include Myers (1981) for Aorids, Ortiz (1991) for Bateids, LECROY (1995, 2000) for Colomastigids, and Lowry & Stoddart (1997) for Aristids, Lysianassoids, Scopelocheirids amongst others. The purpose of this work is to compile information on recorded species, genera and families of the suborders Gammaridea and Caprellidea of different marine benthic habitats in the Gulf of Mexico and to provide a baseline of the existing marine amphipod diversity.

METHODS

The records of species herein presented on benthic gammarid and caprellid amphipods were compiled from published references from the Gulf of Mexico continental shelf and slope. New records and some unpublished references, thesis and reports, have been included as well for those specimens available in formal national collections and the validated records of institutional databases.

The classification criteria of Bowman & Abele (1982) was used for the levels of Phylum to superorder, the specific name of the amphipods and their synonymy was validated with the classification of Barnard & Karaman (1991) and in the case of taxa that have not been recorded in the latter reference combined with existing Gulf of Mexico amphipod classification schemes. Every superorder, family, genus and species is presented in an alphabetical rather than phyletic order and follows the taxonomic scheme of Barnard & Karaman (1991), in the case of Lysianassoidea, the classification scheme by Lowry & Stoddard (1997) was followed.

Every recorded species includes the ecological account of the habitat, the association with other species, the depth range, the geographical distribution within the Gulf of Mexico and references to the information sources. An analysis of the occurrence in habitats, depth zones and geographical sectors where the species have been recorded

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was carried out to define the gaps in coverage. A comparison with checklists from other regions in the tropics allowed us to evaluate similarities in representation of families. The rate of discovery of amphipod species for the Gulf of Mexico was described.

SYSTEMATIC ACCOUNT

Crustacea Pennant, 1777
Peracarida Calman, 1904
Amphipoda Latreille, 1816

Caprellidea Leach, 1814

Caprellidae White, 1847

1. *Caprella equilibra* Say, 1818
   Synonyms: *Caprella januari* Kröyer, 1842; *C. esmarkii* Boeck, 1861; *C. laticornis* Boeck, 1861; *C. aequilibra* Bates, 1862; *C. ultima* Bates, 1862; *C. obesa* Haswell, 1880; *C. mendax* Myers, 1903
   Occurrence: in sandy bottoms at 87 m depth on the continental shelf in the western Gulf of Mexico (Borja, 1998), and in seagrass beds, algal mats associated with sponges, hydroids, bryozoan and tunicates in Port Isabel and Port Aransas, Texas and offshore habitats to depths of 300 m (McCain, 1968).

Pariambidae Laubitz, 1993

2. *Deutella californica* Mayer, 1890
   The species inhabits soft bottoms at 17 m depth on the continental shelf in the western Gulf of Mexico (Borja, 1998) and off Port Aransas, Texas (McCain, 1968).
3. *Paracaprella pusilla Mayer*, 1890
   The species inhabits muddy sediments at 21 m depth on the continental shelf in the western Gulf of Mexico (Borja, 1998), and occurs in mangrove roots and seagrass beds associated with hydroids and tunicates in coastal waters of Port Isabel and Port Aransas, Texas (McCain, 1968).

Gammaridea Latreille, 1816

Ampeliscidae Costa, 1857

4. *Ampelisca abdita Mills*, 1964
   The species inhabits soft bottoms of the continental shelf off Terminos lagoon in the southwestern Gulf of Mexico (Molina, 1998) and off Tamiahua lagoon in the western sector at a depth range of 16 to 40 m (Borja, 1998).
5. *Ampelisca agassizi* (Judd, 1896)
   Synonym: *Byblis agassizi* Judd, 1896
   The species inhabits soft sediments on the continental shelf in the western Gulf of Mexico at depths of 16 to175 m (Borja, 1998; Molina, 1998).
6. *Ampelisca bicarinata* Goeke & Heard, 1983
   This species occurs in sandy sediments and has a wide distribution in the Gulf of Mexico with records in the northern Gulf extending from southeastern Florida to Texas in a depth range of 9 to 54 m (Goeke & Heard, 1983), and in the southwestern Gulf of Mexico at a similar depth range (Molina, 1998).
7. *Ampelisca brevisimulata* Barnard, 1954
   The species inhabits soft sediments in the continental shelf of the western Gulf of Mexico at depths of 16 to 36 m (Molina, 1998).
8. *Ampelisca cristata Holmes*, 1908
   Occurrence: in the continental shelf at a depth range of 20 to 40 m in the eastern Gulf of Mexico from Port Charlotte, Florida to the Campeche Bank (Ortiz, 1979).
9. *Ampelisca holmesi* Pearse, 1908
   Occurrence: in sandy sediments and seagrass beds from the northeastern and central Gulf of Mexico from Key West, Florida to the Mississippi Delta at depths of 1 to 54 m (Shoemaker, 1933; Goeke & Gathof, 1983).
10. *Ampelisca lobata Holmes*, 1908
    Occurrence: in the continental shelf on the northeastern Gulf of Mexico off Florida between Sarasota and Naples at a depth range of 20 to 40 m (Ortiz, 1979).
11. *Ampelisca parapacifica* Goeke & Heard, 1984
    Occurrence: in carbonated sandy and silty sediments of the northeast and southeastern Gulf of Mexico at a depth range of 24 to189 m (Goeke & Heard, 1984). Additional records include muddy sediments off Tamaulipas and Veracruz in the western Gulf of Mexico in the inner shelf between 16 and 43 m depth (Molina, 1998).
12. *Ampelisca schellenbergi* Shoemaker, 1933
    Occurrence: from Key Largo and the Dry Tortugas to the Bank of Campeche in shallow waters of 1 to10 m depth (Shoemaker, 1933).
13. *Ampelisca spinipes* Boeck, 1861
    The species is restricted to shallow waters of Key Largo, Florida (Shoemaker, 1933).
14. *Ampelisca vadorum* Mills, 1963
    The species has been recorded in sandy sediments off the Papaloapan river in the western Gulf of Mexico at 27 m depth (Molina, 1998).
15. *Ampelisca venetiensis* Shoemaker, 1916
    Occurrence: in sandy and muddy sediments of the continental shelf in the western Gulf of Mexico at a depth range of 17 to 202 m (Borja, 1998).
16. *Ampelisca verrilli Mills*, 1967
    Occurrence: in muddy sediments, in the western Gulf of Mexico at a depth of 34 m (Borja, 1998).

Ampithoidae Stebbing, 1899

17. *Ampithoe ramondi* Audouin, 1826
    Occurrence: associated with algal mats in reefs of southern Florida in the eastern Gulf of Mexico (Thomas, 1993b) and the Bank of Campeche in the southern Gulf sector (Ortiz, 1979).

Anamixidae Stebbing, 1897

18. *Anamixis covatura* Thomas, 1997
    The species is found in coral rubble and associated with tunicates in the coral reefs of Key West in the eastern Gulf of Mexico and in the Bank of Campeche in the southern Gulf (Thomas, 1997).
19. *Anamixis hanseni* Stebbing, 1897
    Occurrence: in the Florida shelf off Cape Romano in the eastern Gulf of Mexico (Ortiz, 1979).
20. *Anamixis vanga* Thomas, 1997
    The species is found in coral rubble and associated with tunicates in coral reefs of the Florida Keys in the eastern Gulf of Mexico at a depth range of 2 to 20 m (Thomas, 1997).

Aristiidae Lowry & Stoddart, 1997

21. *Aristias captiva* Lowry & Stoddart, 1997
Occurrence: associated with fans, bryozoans and sponges in the northeastern Gulf of Mexico at a depth range of 55 to 73 m (Lowry & Stoddart, 1997).

22. *Boca campi* Lowry & Stoddart, 1997
Occurrence: in sandy and muddy sediments, associated with bryozoan, calcareous algae and shell debris in the northeastern Gulf of Mexico at a depth range of 55 to 73 m (Lowry & Stoddart, 1997).

23. *Boca elvae* Lowry & Stoddart, 1997
Occurrence: in soft sediments in the northeast of Dry Tortugas in the eastern Gulf of Mexico at 180 m depth (Lowry & Stoddart, 1997).

24. *Boca megachela* Lowry & Stoddart, 1997
Occurrence: in coralline algae and seagrass beds, associated with sponges and fans, in the northeastern Gulf of Mexico at a depth range of 18 to 73 m (Lowry & Stoddart, 1997).

Bateidae Stebbing, 1906

25. *Batea bousfieldi* (Ortiz, 1991)
Synonym: *Carinobatea bousfieldi* Ortiz, 1991
This species occurs in the central west shelf of Florida in the northeastern Gulf of Mexico at a depth range of 37 to 73 m (Ortiz, 1991).

26. *Batea campi* (Ortiz, 1991)
Synonym: *Carinobatea campi* Ortiz, 1991
Occurrence: in the central shelf of Florida in the northeastern Gulf of Mexico at a depth range of 1 to 36 m (Ortiz, 1991).

27. *Batea carinata* (Shoemaker, 1926)
Synonym: *Carinobatea carinata* Shoemaker, 1926
Occurrence: in the central west shelf of Florida in the northeastern Gulf of Mexico at a depth range of 55 to 73 m (Ortiz, 1991).

28. *Batea catharinensis* Müller, 1865
Synonym: *Batea secunda* Holmes, 1903
Occurrence: in soft sediments and seagrass beds of the continental shelf of the northern Gulf of Mexico at a depth range of 1 to 45 m (Ortiz, 1991).

29. *Batea cuspidata* (Shoemaker, 1926)
Synonym: *Carinobatea cuspidata* Shoemaker, 1926
Occurrence: off Florida in the northeastern Gulf of Mexico at a depth range of 3 to 49 m (Shoemaker, 1933; Ortiz, 1991).

Colomastigidae Chevreux, 1899

30. *Colomastix bousfieldi* LeCroy, 1995
Occurrence: in coral reefs, algal mats, seagrass beds and mangrove roots, associated with sponges, tunicates in the northern Gulf of Mexico and the eastern Bank of Campeche at a depth range of 1 to172 m (Lecroy, 1995).

31. *Colomastix camura* LeCroy, 1995
Occurrence: in coral reef patches, associated with sponges, in the northern Gulf of Mexico at a depth range of 18 to73 m (Lecroy, 1995).

32. *Colomastix cornuticauda* LeCroy, 1995
Occurrence: associated with the sponges *Agelas dispar* Duchassaing and Michelotti, 1864 and *Aplysina fistularis* (Pallas, 1766) in the Florida Middle Ground in the eastern Gulf of Mexico at a depth range of 24 to 35 m (Lecroy, 1995).

33. *Colomastix denticornis* LeCroy, 1995
Occurrence: associated with the sponges *Agelas dispar* Duchassaing & Michelotti, 1864 and *Aplysina fistularis* (Pallas, 1766) in the Florida Middle Ground in the eastern Gulf of Mexico at a depth range of 55 to 73 m (Lecroy, 1995).

34. *Colomastix falcirama* LeCroy, 1995
Occurrence: in coral reef, associated with sponges and the coral species *Madracis decactis* (Lyman, 1859) in the Florida Middle Ground, Big Pine Key and Dry Tortugas in the eastern Gulf of Mexico at a depth range of 1 to 98 m (Lecroy, 1995).

35. *Colomastix gibbosa* LeCroy, 1995
Occurrence: in coral reef, associated with the sponge *Geodia gibberosa* Lamarck, 1815 and the coral species *Madracis decactis* (Lyman, 1859) in the central West Florida Bank, the Florida Middle Ground and Dry Tortugas in the eastern Gulf of Mexico at 73 m depth (Lecroy, 1995).

36. *Colomastix halichondriae* Bousfield, 1973
Synonym: Colomastix pusilla Pearse, 1932
Occurrence: associated with sponges, corals and bivalves in Texas in the northwestern Gulf of Mexico, the Florida Middle Ground, central West Florida Shelf and Dry Tortugas in the eastern Gulf sector at a depth range of 1 to73 m (Lecroy, 1995).

37. *Colomastix heardi* LeCroy, 1995
Occurrence: associated with sponges and corals in the Florida Middle Ground, the Florida Keys, central West Florida Shelf, in the eastern Gulf of Mexico and south in the eastern Bank of Campeche at a depth range of 10 to 73 m Lecroy, 1995).

38. *Colomastix irciniae* LeCroy, 1995
Occurrence: in coral reefs, associated with sponges of the genus Ircinia and the coral species Madracis decactis (Lyman, 1859) in Florida Middle Ground and Dry Tortugas in the eastern Gulf of Mexico at a depth range of 25 to 36 m (Lecroy, 1995).

39. *Colomastix janiceae* Heard & Perlmutter, 1977
Synonym: *Colomastix pusilla* Pearse, 1912
The species is associated with a large variety of species of sponges and corals in Dry Tortugas, the Florida Keys, the central West Florida shelf and the Florida Middle Ground in the eastern Gulf of Mexico, off Texas in the northwestern Gulf sector and in the Bank of Campeche in the southern sector at a depth range of 1 to 87 m (Lecroy, 1995).

40. *Colomastix tridentata* LeCroy, 1995
Occurrence: associated with sponge and coral species of the Florida Middle Ground, the central West Florida shelf, Dry Tortugas and the Florida Keys in the eastern Gulf of Mexico, off Texas in the northwestern sector and in the Bank of Campeche at a depth range of 1 to 73 m (Lecroy, 1995).

Corophiidae-Ischyroceridae *sensu* Barnard & Karaman, 1991

41. *Acuminodeutopus naglei* (Bousfield, 1973)
Synonym: *Rudilemboides naglei* Bousfield, 1973
Occurrence: in soft sediments with shell debris in the northeastern Gulf of Mexico at a depth range of 1 to 55 m (Myers, 1981).

42. *Bemlos macromanus* Shoemaker, 1925
Occurrence: in the Florida shelf off Cape Romano in the northeastern Gulf of Mexico (Ortiz, 1979).

43. *Bemlos ovalipes* (Myers, 1979)
Synonym: *Lembos ovalipes* Myers, 1979
Occurrence: in the central West Florida shelf in the northeastern Gulf of Mexico at a depth range of 18 to 37 m (Myers, 1981).

44a. *Bemlos spinicarpus inermis* (Myers, 1979)
Synonym: *Lemlos spinicarpus inermis* Myers, 1979
Occurrence: in the central West Florida shelf in the northeastern Gulf of Mexico at a depth range of 6 to 73 m (Myers, 1981).

44b. *Bemlos spinicarpus spinicarpus* (Pearse, 1912)
Synonym: *Lembos spinicarpus spinicarpus* Pearse, 1912
Occurrence: in coral reefs of Key West in the eastern Gulf of Mexico (Myers, 1981; Thomas, 1993b).

45. *Bemlos tempus* (Myers, 1979)
Synonym: *Lembos tempus* Myers, 1979
Occurrence: in sandy bottoms of the central West Florida shelf in the northeastern Gulf of Mexico at a depth range of 37 to 73 m (Myers, 1981).

46. *Bemlos tigrinus* (Myers, 1979)
Synonym: *Lembos tigrinus* Myers, 1979
Occurrence: in sandy sediments of the central West Florida shelf in the northeastern Gulf of Mexico at a depth range of 18 to 73 m (Myers, 1981).

47. *Bemlos unicornis* (Bynum & Fox, 1977)
Synonym: *Lembos uncornis* Bynum & Fox, 1977
Occurrence: throughout the Florida shelf in the northeastern Gulf of Mexico at a depth range of 1 to 20 m (Myers, 1981; Thomas, 1993b).

48a. *Bemlos unifasciatus reductus* (Myers, 1979)
Synonym: *Lembos unifasciatus reductus* Myers, 1979
Occurrence: in soft sediments of the central West Florida shelf in the eastern Gulf of Mexico (Myers, 1981; Thomas, 1993b) and off Veracruz and Terminos Lagoon in the southwestern sector at a depth range of 18 to73 m (Molina, 1998).

48b. *Bemlos unifasciatus unifasciatus* (Myers, 1977)
Synonym: *Lembos unifasciatus unifasciatus* Myers, 1977
Occurrence: in the shallow reefs in southwestern Florida (Myers, 1981; Thomas, 1993b).

49. *Chevalia aviculae* Walker, 1904
Occurrence: in coral reefs, associated with sponges, of Florida (Thomas, 1993b) and in the Bank of Campeche at a depth range of 25 to 40 m (Ortiz, 1979).

50. *Ericthonius brasiliensis* (Dana, 1853)
Synonym: *Pyctilus brasiliensis* Dana, 1853
This tube building species occurs in coral reefs among macroalgae and associated with sponges in the northeastern Gulf of Mexico at a depth range of 10 to 300 m (Barnard & Barnard, 1990; Thomas, 1993b).

51. *Ericthonius rubricornis* (Stimpson, 1853)
Synonym: *Pyctilus rubricornis* Stimpson, 1853
Occurrence: in coral reefs among macroalgae in the northeastern Gulf of Mexico shelf at a depth range of 10 to 235 m (Ortiz, 1979).

52. *Gammaropsis atlantica* Stebbing, 1888
Occurrence: in coral reefs in the northwestern Florida in the eastern Gulf of Mexico (Thomas, 1993b) and in the carbonated sediments from the Bank of Campeche in the southern Gulf sector at a depth range of 20 to 40 m (Ortiz, 1979).

53. *Globosolembos smithi* (Holmes, 1905)
Synonym: *Autonoe smithi* Holmes, 1905
Occurrence: in sandy sediments and shell debris in the Western Florida shelf in the eastern Gulf of Mexico and the Bank of Campeche in the southern sector at 73 m depth (Myers, 1981; Barnard & Karaman, 1991; Ortiz, 1993b).

54. *Lembos websteri* Bate, 1857
Occurrence: between Sarasota and Cape Romano in the Florida shelf in the eastern Gulf of Mexico at a depth range of 20 to 40 m (Ortiz, 1979).

55. *Liocuna caeca* Myers, 1981
Occurrence: in sandy sediments and shell debris of the central West Florida shelf in the northeastern Gulf of Mexico at a depth range of 39 to 73 m (Myers, 1981).

56. *Microdeutopus myersi* Bynum & Fox, 1977
Occurrence: in sandy sediments of the central West Florida shelf in the northeastern Gulf of Mexico and extends its range south to the Florida Keys within a depth range of 1 to 73 m (Myers, 1981).

57. *Photis longicaudata* (Bate & Westwood, 1863)
Synonym: *Eiscladus longicaudata* Bate & Westwood, 1863
Occurrence: in carbonate sediments in the Florida shelf in the eastern Gulf of Mexico (Shoemaker, 1945) and in soft sediments off the Tuxpan river in the western Gulf of Mexico at a depth range of 32 to 40 m (Molina, 1998).

58. *Photis macromanus* McKinney, Kalke & Holland, 1978
Occurrence: in sandy sediments of the western Gulf of Mexico shelf at a depth range of 18 to 24 m (McKinney et al., 1978).

59. *Photis pugnator* Shoemaker, 1945
Occurrence: from the central West Florida shelf in the eastern Gulf of Mexico to the Bank of Campeche in the southern sector at a depth range of 15 to 42 m (Ortiz, 1979).

60. *Rildardanus laminosa* (Pearse, 1912)
Synonym: *Unciola laminosa* Pearse, 1912
Occurrence: in the central West Florida shelf in the eastern Gulf of Mexico at a depth range of 6 to 55 m (Myers, 1981).

61. *Unciola spicata* Shoemaker, 1945
The species has been recorded in the West Florida shelf in the northeastern Gulf of Mexico (Ortiz, 1979).

62. *Unciola serrata* Shoemaker, 1945
Occurrence: in soft bottoms of the continental shelf from Florida to Alabama in the northeastern Gulf of Mexico (Myers, 1981) and in the western and southwestern Gulf of Mexico off Tamiahua and Terminos lagoons at a depth range of 1 to 200 m (Borja, 1998; Molina, 1998).

Cyphocarididae Lowry & Stoddart, 1997

63. *Cyphocaris tunicola* Lowry & Stoddart, 1997
Occurrence: in coral reefs associated with colonies of sea squirts in the northeastern Gulf of Mexico at a depth range of 200 to 500 m (Lowry & Stoddart, 1997).

Endevouridae Lowry & Stoddart, 1997

64. *Ensayara entrichoma* Gable & Lazo-Wasem, 1990
Occurrence: in the eastern Gulf of Mexico at 73 m depth (Lowry & Stoddart, 1997).

Gammaridae Leach, 1814

65. *Gammarus mucronatus* Say, 1818
This common species of the coastal ocean occurs in sandy sediments of the continental shelf in the western Gulf of Mexico at a depth range of 2 to 40 m (Borja, 1998; Lecroy, 2000).

66. *Gammarus palustris* Bousfield, 1969
Occurrence: in the littoral fringe and in coral reefs, in soft bottoms associated with sponges and shell rubble in the western Gulf of Mexico (Carrera & Vargas, 1997; Lecroy, 2000).

Haustoriidae Stebbing, 1906

67. *Parahaustorius attenuatus* Bousfield, 1965
Occurrence: in coral reefs, associated with sponges, in the western Gulf of Mexico (Carrera & Vargas, 1997).

68. *Pseudohaustorius carolinensis* Bousfield, 1973
The species has been recorded in the Bank of Campeche in the southern Gulf of Mexico at depths of 15 to 40 m (Ortiz, 1979).

Leucothoidae Dana, 1852

69. *Leucothoe spinicarpa* (Abildgaard, 1789)
Synonym: *Gammarus spinicarpa* Abildgaard, 1789
Occurrence: in coral reefs, associated with sponges and tunicates, in the Florida Keys in the eastern Gulf of Mexico (Shoemaker, 1933; Thomas, 1993b; Carrera & Vargas, 1997) and in soft sediments of the western Gulf of Mexico at a depth range of 25 to 30 m (Molina, 1998).

Liljeborgiidae Stebbing, 1899

70. *Liljeborgia dellavallei* Stebbing, 1906
Occurrence: in the central West Florida shelf (Ortiz, 1979).

71. *Listriella carinata* McKinney, 1979
Occurrence: in soft sediments of the Texas shelf in the northwestern Gulf of Mexico (McKinney, 1979) and in the southwestern Gulf of Mexico at depths of 10 to 28 m (Molina, 1998).

Lysianassidae DANA, 1849 s.s. (sensu Lowry & Stoddart, 1997)

72. *Aruga holmesi* Barnard, 1965
Occurrence: in soft sediments of the northeastern Gulf of Mexico at a depth range of 7 to 73 m (Lowry & Stoddart, 1997).

73. *Concarnes concavus* (Shoemaker, 1933)
Synonym: *Socarnes concavus* Shoemaker, 1933
Occurrence: in the eastern Gulf of Mexico at depths from 1 to 80 m (Thomas, 1993b; Lowry & Stoddart, 1997).

74. *Dissiminassa homosassa* Lowry & Stoddart, 1997
The species is found in shell debris, algae, alcyonarians, corals and seagrass beds and sandy sediments of the northeastern Gulf of Mexico at a depth range of 18 to 73 m (Lowry & Stoddart, 1997).

Lysianassidae Dana, 1849 *sensu lato*

75. *Eurythenes gryllus* (Lichtenstein, 1822)
Synonym: *Gammarus gryllus* Lichtenstein, 1822
This scavenger species has been reported from the north central slope of the Gulf of Mexico Lowry & Stoddart, 1997).

76. *Eurythenes obesus* (Chevreux, 1905)
Synonym: *Gammarus obesus* Chevreux, 1905
This scavenger species has been reported from the north central slope of the Gulf of Mexico (Lowry & Stoddart, 1997).

77. *Lysianopsis alba* Holmes, 1903
This species has been reported from Key West and Key Largo in the eastern Gulf of Mexico in a depth range of 1 to 40 m (Shoemaker, 1933; Lowry & Stoddart, 1997).

78. *Lysianopsis ozona* Lowry & Stoddart, 1997
Occurrence: in seagrass beds, algae and bottoms with shell debris, associated with sponges, alcyonarian and corals in the northeastern Gulf of Mexico at a depth range of 18 to 29 m (Lowry & Stoddart, 1997).

79. *Orchomenella perdido* Lowry & Stoddart, 1997
Occurrence: in shell debris, algae and sandy sediments, associated with sponges in the northeastern Gulf of Mexico at a depth range of 1 to 37 m (Lowry & Stoddart, 1997).

80. *Orchomenella thomasi* Lowry & Stoddart, 1997
Occurrence: in sandy bottoms with shell debris, calcareous algae and dead bryozoans in the northeastern Gulf of Mexico at a depth range of 10 to73 m (Lowry & Stoddart, 1997)

81. *Rimakoroga floridiana* Lowry & Stoddart, 1997
Occurrence: in bottoms with shell debris, algae and dead alcyonaria in the eastern Gulf of Mexico at a depth range of 55 to 73 m (Lowry & Stoddart, 1997).

82. *Shoemakerella cubensis* (Stebbing, 1897)
Synonym: *Lysianax cubensis* Stebbing, 1897
Occurrence: in sandy sediments from Dry Tortugas in the eastern Gulf of Mexico to the Mississippi Delta in the central northern Gulf (Thomas, 1993b; Lowry & Stoddart, 1997), and in the western Gulf of Mexico at a depth range of 2 to 69 m (Molina, 1998).

83. *Tryphosella apalachicola* Lowry & Stoddart, 1997
Occurrence: in shell debris and sandy sediments associated with sponges and bryozoans in the northeastern Gulf of Mexico at 55 m depth (Lowry & Stoddart, 1997).

Melitidae *sensu* Jarret & Bousfield, 1996

84. *Ceradocus sheardi* Shoemaker, 1948
Occurrence: in sandy bottoms and hard banks of Dry Tortugas in the eastern Gulf of Mexico and in the Bank of Campeche in the southern Gulf sector at depths of 2 to 80 m (Ortiz, 1979; Thomas, 1993b; Lecroy, 2000).

85. *Elasmopus pocillimanus* (Bates, 1862)
Synonym: *Maera pocillimanus* Bate, 1862
Occurrence: in coral reefs, associated with coral and coral rubble, in seagrass beds and among algae of the southern Florida at a depth of 30 m (Ortiz, 1979; Thomas, 1993b; Lecroy 2000).

86. *Elasmopus rapax* Costa, 1853
Occurrence: in hard bottoms, mangrove roots and associated with sponges, algae and coral in the central West Florida shelf, the eastern Gulf of Mexico and on the Campeche Bank (Ortiz, 1979; Lecroy, 2000).

87. *Eriopisa incisa* McKinney, Kalke & Holland, 1978
Occurrence: in silty sediments of the northwestern Gulf of Mexico at a depth range of 98 to 134 m (McKinney et al., 1978).

88. *Jerbarnia americana* Watling, 1981
Occurrence: throughout the northeastern Gulf of Mexico (Ortiz & Lalana, 1993).

89. *Maera hamigera* Haswell, 1879
Occurrence: from the central West Florida shelf in the eastern Gulf of Mexico to the Bank of Campeche in the southern sector at a depth range of 15 to 40 m (Ortiz, 1979).

90. *Netamelita barnardi* McKinney, Kalke & Holland, 1978
Occurrence: in sandy sediments, coral reefs and algae mats in the northwestern Gulf of Mexico (McKinney et al., 1978), off Florida (Thomas, 1993b), and in the southwestern Gulf of Mexico at a depth range of 15 to 40 m (Molina, 1998).

91. *Netamelita brocha* Thomas & Barnard, 1991
Occurrence: in soft bottoms and associated with coralline algal mats, northeast of the Florida Keys at a depth range of 15 to 76 m (Thomas & Barnard, 1991; Thomas, 1993b; Lecroy, 2000).

Melphidippidae Stebbing, 1899

92. *Hornellia (Metaceradocus) atlanticus* Thomas & Barnard, 1986
Occurrence: in the Florida Keys in the eastern Gulf of Mexico at a depth range of 20 to 40 m (Thomas & Barnard, 1986).

Oedicerotidae Liljeborg, 1865

93. *Monoculodes nyei* Shoemaker, 1933
Occurrence: from Key West in the eastern Gulf of Mexico to southern Texas in the northwestern sector (Shoemaker, 1933; Thomas, 1993b).

Phoxocephalidae Sars, 1895

94. *Eobrolgus spinosus* (Holmes, 1905)
Synonym: *Paraphoxus spinosus* Holmes, 1905
Occurrence: in soft sediments from the continental shelf off Tamiahua lagoon in the western Gulf of Mexico (Borja, 1998).

95. *Metharpinia floridana* (Shoemaker, 1933)
Synonym: *Pontharpinia floridana* Shoemaker, 1933
Occurrence: in sandy sediments and carbonated banks from Florida in the eastern Gulf of Mexico to Texas in the northwestern Gulf sector (Shoemaker, 1933; Barnard & Barnard, 1990; Thomas, 1993b) and the Bank of Campeche (Molina, unpubl.) in the southern Gulf sector at a depth range of 10 to 60 m.

Platyischnopidae Barnard & Drummond, 1979

96. *Eudevenopus honduranus* Thomas & Barnard, 1983
Occurrence: in coral hard bottoms of the Florida shelf and Keys at depths of 1 to 40 m (Barnard & Barnard, 1990; Thomas, 1993b).

Scopelocheiridae Lowry & Stoddart, 1997

97. *Aroui americana* Lowry & Stoddart, 1997
This scavenger species occurs in sandy bottoms of the northeastern Gulf of Mexico at a depth range of 95 to 100 m. It has been often found in stomach contents of fish (Lowry & Stoddart, 1997).

Sebidae Walker, 1908

98. *Seba aloe* Karaman, 1971
This species occurs in the continental shelf off Cape Romano in the eastern Gulf of Mexico (Ortiz, 1979).

Stenothoidae Dana, 1855

99. *Parametopella texensis* McKinney, Kalke & Holland, 1978
Occurrence: in the northwestern Gulf of Mexico at a depth range of 10 to 20 m (McKinney et al., 1978).

Synopiidae Dana, 1855

100. *Synopia ultramarina* Dana, 1853
Occurrence: in soft sediments of the shallow habitats of Key Largo in the eastern Gulf of Mexico (Shoemaker, 1933).

Uristidae Lowry & Stoddart, 1997

101. *Stephonyx biscayensis* (Chevreux, 1908)
Synonym: *Euonyx biscayensis* Chevreux, 1908
The species has been recorded in the northeastern Gulf of Mexico at 494 m depth (Lowry & Stoddart, 1997).

DISCUSSION

This study recognized a total of 101 species of amphipods from the Gulf of Mexico that belong to 55 genera, 26 families and two suborders. The suborder Gammaridea is highly diverse with a total of 97 species that represent 96% of the total species richness recorded in this LME. The most diversified gammarid families in this LME are, in species richness decreasing order, Corophiidae-Ischyroceridae (12 genera, 24 species), Ampeliscidae (1 genus, 13 species), Lysianassidae (9 genera, 12 species), Colomastigidae (1 genus, 11 species) and Melitidae (6 genera, 8 species). In contrast, the suborder Caprellidea is poorly represented and each recorded family in the Gulf of Mexico is represented by one at most two species.

The amphipods have been sampled and recorded in diverse shallow habitats (Table 1), predominantly associated to sessile faunal components in coral reefs (sponges, tunicates, alcyonaria and bryozoa) and to vegetation (calcareous algae, algal mats, seagrass beds, and mangrove roots). The soft sediments are next in species richness (19.13% of records) as are the sandy bottoms (10.43% of records) and bottoms with shell debris (9.57% of records). Only few species occur associated to banks of bivalves (*Colomastix halichondriae*), beds of tunicates (*Leucothoe spinicarpa*), and hydroids (*Caprella equilibra, Paracaprella pusilla*). The large richness of habitats in the Gulf of Mexico promises further species to be recorded in the next years.

TABLE 1

Occurence of Gulf of Mexico amphipods by habitat

Habitat	No. of records	%
Sandy bottoms	12	10.43
Soft sediments	22	19.13
Carbonate bottoms and coral rubble	10	8.70
Shell debris	11	9.57
Associated with vegetation	21	18.26
algae & algal mats	16	
mangrove roots	3	
seagrass beds	9	
Associated with fauna	39	33.91
alcyonarians	5	
bivalves	1	
briozoans	5	
corals	27	
hydroids	2	
sponge	24	
tunicates	7	
Total number of records	214	100

Amphipods have been recorded on the continental shelf and slope (Table 2). The largest number of records has been made on the continental shelf (75% of the records) and the littoral fringe (20% of records, less than 10m depth). Species richness is almost twice as large on the inner shelf (54%, 10 to 50m depth) as records existing for the middle shelf (45, 51 to 100m depth). The outer conti-

nental shelf (101 to 200m depth) and the continental margin and upper slope (201 to 500m depth) have a low number of records (5%). Species from the continental slope and rise and the abyssal plain have not been recorded yet concluding that these depth zones remain gaps in our knowledge of amphipods in the region.

TABLE 2

Occurence of Gulf of Mexico amphipods by depth zone

Depth zone	Depth range (m)	No. of records	%
Littoral		24	20
Continental shelf		90	75
	1 to 50	71	
	51 to 100	45	
	101 to 200	9	
Continental margin & upper slope	201 to 500	6	5
Total number of records		245	100

Amphipods have been recorded throughout the coastal Gulf of Mexico (Table 3). The northern and eastern sectors of the Gulf of Mexico have been studied intensively and accounted for 73% of the total number of amphipod records herein provided, in contrast to 27% of records corresponding to the southern and western sectors. The southeastern and south-western sectors are the least explored with only 11 species.

TABLE 3

Occurence of amphipods by geographic sector in the Gulf of Mexico

Sector	No. of records	%
North	15	11.90
norheastern	28	22.22
northwestern	11	8.73
South	12	9.52
southeastern	3	2.38
southwestern	8	6.35
West	16	12.70
East	33	26.19
Total No of records	126	100

To conclude: the amphipods that inhabit the deep-sea central region remain unknown, and the southern regions have poorly been investigated. A large number species remains to be sampled in a sustained effort to have a better idea of the amphipod diversity in the Gulf of Mexico. The discovery rate in the Gulf of Mexico started in the late 18th century and remained low, with 22 species discovered in the 19th century. The discovery rate increased fivefold in the 20th century. The number of recorded Caprellidea species is low and has remained low since the 19th century with no new records. Future exploratory efforts will lead to more records and discovery of new species in the region as can be derived form Fig. 1. The number of records obtained raised drastically from the 1960's on, when large exploration programs were initiated in the northern Gulf.

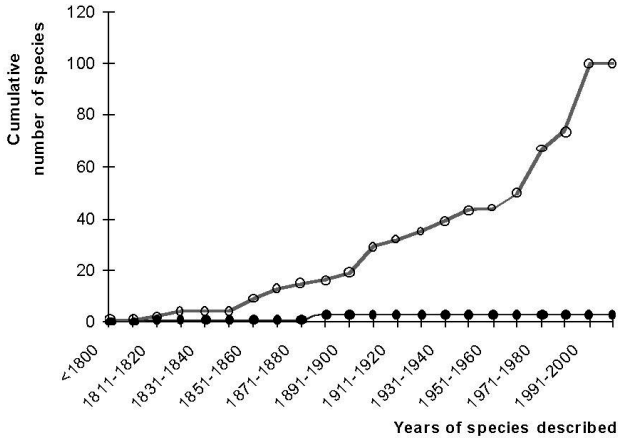

Fig. 1. – Discovery rate of the amphipod species (Caprellidea solid circles, and Gammaridea, open circles) described for the continental shelf and the continental slope of the Gulf of Mexico.

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Rauber's sickle generates only extraembryonic tissues (junctional- and sickle endoblast), and, by positional information, organizes and dominates the whole avian blastoderm (gastrulation, neurulation and blood island formation)

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ABSTRACT. When the Rauber's sickle is (sub)totally mechanically removed from unincubated chicken blastoderms, their further evolution in culture is mostly disturbed. When,after removal of the chicken Rauber's sickle, a quail Rauber's sickle is placed isotopically, the normal development is totally restored. In such quail-chicken chimeras quail cells were found only in extraembryonic tissues, i.e. in junctional- and sickle endoblast and not among cells of the embryo proper (not in upper layer, nor in mesodermal, nor in endodermal cells). Further, we compared the inducing potencies of quail sickle endoblast placed on different regions of unincubated chicken blastoderms, either in the presence or absence of Rauber's sickle material. If a fragment of quail sickle endoblast was placed on the anti-sickle region of an unincubated chicken blastoderm from which the Rauber's sickle was (sub)totally removed, then often starting from this anti-sickle region an embryo presenting gastrulation and/or neurulation phenomena was induced but no blood islands were formed. So our study demonstrates that Rauber's sickle quantitatively and qualitatively dominates or inhibits ectopically-placed sickle endoblast. Earlier studies and the present study indicate the existence of a temporo-spatially bound cascade of gastrulation and neurulation phenomena and blood island formation in the avian blastoderm, starting from Rauber's sickle, the primary major organizer with inducing, inhibiting and dominating potencies.

KEY WORDS : avian blastoderm, Rauber's sickle, junctional endoblast, sickle endoblast, gastrulation, neurulation, blood island formation, positional information.

INTRODUCTION

Our knowledge about the developmental events and the inductory role and function of the different deep layer elements in avian blastoderms has improved only recently (Callebaut 1993a, b, c ; 1994 ; Callebaut & Van Nueten, 1994, 1995, 1996 ; Callebaut et al., 1999a ; Callebaut et al., 2000b). The different components of an unincubated quail blastoderm and surrounding structures are represented in Fig 1. The term Rauber's sickle (Rauber, 1876) is used instead of Koller's sickle (Koller, 1882), since RAUBER was the first to describe it (Callebaut & Van Nueten, 1994). The anti-sickle region was first described by Callebaut (1993a) in gravitationally-oriented quail germs. In this anti-sickle region, an irreversible disruption takes place between the future cranial part of the germ and the underlying subgerminal ooplasm at the moment of bilateral symmetrization (Callebaut, 1993b, 1994). The anti-sickle itself is formed by a sickle-shaped group of loose yolk masses and cells located below the upper layer (UL) in the cranial recessus of the subgerminal space. The upper layer from the anti-sickle region of the unincubated chicken blastoderm is still uncommitted since neither endophyll, nor Rauber's sickle material is present (Callebaut & Van Nueten, 1995 ; Callebaut et al., 1998). Rauber's sickle divides the area pellucida into a peripheral caudal area marginalis and an area centralis. The area centralis contains a subgerminal space filled with liquid. By contrast, Rauber's sickle and the caudal marginal zone are directly in contact with the caudal underlying peripheral subgerminal ooplasm without an underlying cavity. In the caudo-central region of the area centralis, a more or less developed sheet of endophyll can be seen. We use the term endophyll (Celestino Da Costa, 1948) and not endoblast or hypoblast to distinguish it from sickle endoblast (derived later by centripetal outgrowth from Rauber's sickle, Callebaut & Van Nueten, 1994). Endophyll cells and primordial germ cells all contain the same kind of deep central ooplasm (Callebaut & Vakaet : the so-called δ-ooplasm ; Callebaut, 1987) as the nucleus of Pander (Pander, 1817) and are originally localized in the surface of the latter, indicating a common origin (Callebaut, 1984). By contrast, Rauber's sickle cells and sickle endoblast contain more peripherally- and superficially-localized γ- and/or δ- ooplasm (Callebaut, 1987). Thus endophyll and sickle endoblast contain different ooplasms. Previous studies (Callebaut & Van Nueten, 1994 ; Cal-

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LEBAUT et al., 1996a, 1997a) indicated that a function (i.e. definitive endoderm and mesoderm induction in the upper layer) of Rauber's sickle in avian blastoderms is homologous to the function of Nieuwkoop's center (NIEUWKOOP, 1969, 1973) in amphibian blastulas. Recently this has been confirmed by the study of goosecoid genes in avian blastoderms. Indeed LEMAIRE et al. (1997) found strong expression of one of the goosecoid genes (GSX) in the upper layer above Rauber's sickle, suggesting induction by the latter. So, goosecoid expression was not found in Rauber's sickle, but above. Later this upper layer will ingress and form the also-GSX-expressing primitive streak and Hensen's node. The latter are close to the amphibian organizer in SPEMANN & MANGOLD'S definition (1924). The junctional endoblast forms a whitish structure and can easily be seen at the surface of the living blastoderm *in*

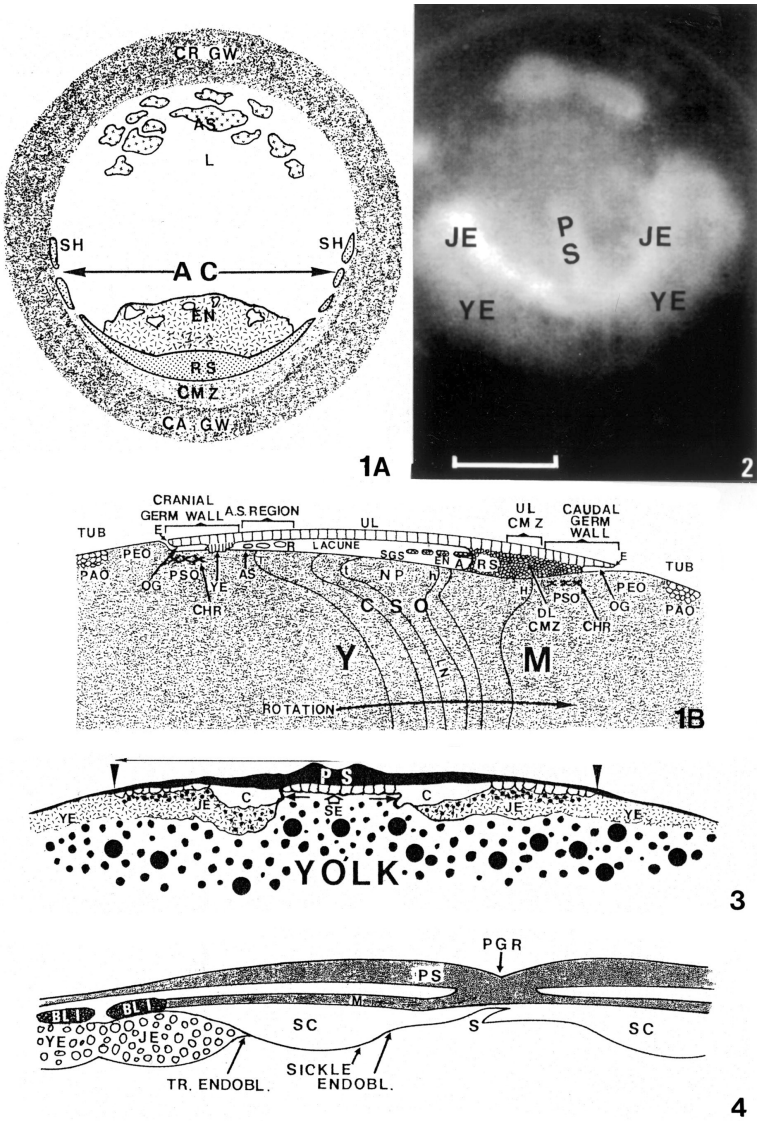


Fig. 1. – A. – Schematic representation of the components of the unincubated chicken blastoderm seen from below after removal of the subgerminal ooplasm, ready for *in vitro* culture. AC, area centralis ; AS, anti-sickle region ; CA GW, caudal germ wall ; CMZ, caudal marginal zone, more or less transparent ; CR GW, cranial germ wall ; EN, incomplete endophyll sheet ; L, lacune in the deep layer ; RS, Rauber's sickle and SH, fragmentary sickle horns enclosing the area centralis. B. – Schematic representation of a mediosagittal section through an unincubated quail blastoderm with surrounding ooplasms after fixation *in situ* on the egg yolk ball ; AS : anti-sickle ; CHR, chromosome clusters (CALLEBAUT, 1994) ; CSO, central subgerminal ooplasm ; DL CMZ, deeper part of the caudal marginal zone ; E, edge of the blastoderm ; EN, incomplete endophyll layer ; h, heel-shaped part of the nucleus of Pander ; H : heel-shaped part of the surrounding yolk layers as result of the rotation *in utero* (the arrow indicates the direction of rotation and compression of the yolk mass under the combined influence of gravity and egg rotation) (CALLEBAUT, 1983, 1993a) ; LN, bended latebra neck. Note that by contrast to the caudal germ wall, the cranial germ wall is disrupted from the underlying peripheral subgerminal ooplasm (CALLEBAUT, 1993 a, b, c) ; NP, nucleus of Pander (1817) ; OG, early overgrowth zone (CALLEBAUT & MEEUSSEN, 1988) ; PAO, paragerminal ooplasm forming a tubulin (TUB) rich ring at distance from the edge of the blastoderm (CALLEBAUT et al., 1996b) ; PEO, perigerminal ooplasm ; PSO, peripheral subgerminal ooplasm ; RS, Rauber's sickle ; SGS, subgerminal space forming a caudal pocket A (axilla-shaped) and a cranial recess (R) in which free yolk masses or sometimes cells are found forming the anti-sickle (AS) ; t, toe-shaped part of the nucleus of Pander ; T : toe-shaped part of the surrounding yolk layers ; UL CMZ, upper layer from the caudal marginal zone ; the caudal marginal zone being a more or less transparent part adherent to the caudal peripheral subgerminal ooplasm ; UL, upper layer ; YE, early development of the yolk endoblast, growing into the peripheral subgerminal ooplasm ; YM, the voluminous yolk mass of the egg yolk ball in which the eccentricity of the successive yolk layers parallel with the eccentricity in the blastoderm is represented.

Fig. 2. – Stereomicroscopic photomicrograph of a living quail embryo (Stage 2-3 of HAMBURGER & HAMILTON, 1951) *in situ* on its egg yolk ball, incubated for approximately 8-10h ; JE, junctional endoblast ; PS, primitive streak ; YE, yolk endoblast. Bar : 1mm.

Fig. 3. – Schematic drawing of a transverse section through the caudal part of the primitive streak region (localized between the two vertical arrowheads as a region where the UL is thicker than laterally) of a stage 3+ quail embryo (HAMBURGER & HAMILTON, 1951). Between the deep side of the primitive streak (PS) and the sickle endoblast (SE) indicated by a vertical hollow arrow, there are cellular extensions passing between small cavities. The horizontal arrows on the left and on the right indicate the transitional endoblast (CALLEBAUT & VAN NUETEN, 1994), which connects the sickle endoblast with the junctional endoblast (JE) ; (C), pararchenteric canals from which the sickle canal will develop. Between the lateral part of the junctional endoblast and the deep side of the primitive streak region, there are numerous extensions and small cavities, while between the more lateral yolk endoblast (YE) and the UL, there are no cavities and no extensions.

Fig. 4. – Schematic representation of the spatial relationship of the first formed blood islands (BL I) with the three germ layers in the caudal part of the chicken blastoderm after approximately one day incubation (stage 7-8 of HAMBURGER & HAMILTON, 1951). M : mesoblast ; PGR : primitive groove ; PS : primitive streak ; S : median septum formed by median sickle endoblast ; SC : sickle canal or sickle cavity localized above the sickle endoblast in the concavity of the sickle-shaped junctional endoblast (JE) ; YE : yolk endoblast.

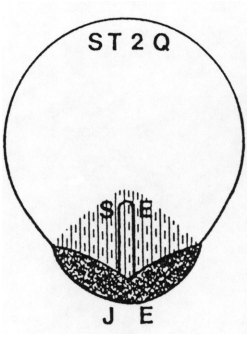


Fig. 5. – Schematic representation of the localization (corresponding to stage 2 of HAMBURGER & HAMILTON, 1951) of the two extraembryonic tissues, derived from Rauber's sickle : SE : quadrant-shaped layer of sickle endoblast extending from Rauber's sickle in a cranial and centripetal direction under the upper layer, below and laterally from the primitive streak (CALLEBAUT et al., 1997b) ; JE : V-shaped junctional endoblast derived from Rauber's sickle by local proliferation into the subgerminal ooplasm (CALLEBAUT & VAN NUETEN, 1994).

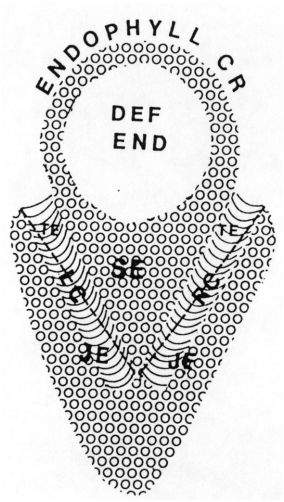


Fig. 6. – Schematic drawing of the anchor-shaped spreading (seen from dorsally after removal of the upper layer and primitive streak region at stage 3-4 of HAMBURGER & HAMILTON in the avian blastoderm) of the two cell lineages (sickle endoblast : SE and junctional endoblast : JE), derived from Rauber's sickle ; the small circles represent cellular connections between the sickle endoblast or junctional endoblast and the removed superficial layer ; LC : left part of the sickle canal ; RC : right part of the sickle canal ; TE : transitional endoblast ; DEF END : place where the definitive endoderm extends radially ; ENDOPHYLL CR : localization of the endophyllic crescent and wall (simplified after CALLEBAUT & VAN NUETEN, 1994).

situ on the egg yolk ball of a quail egg after approximately 9h incubation (Fig. 2). It forms the V- or U-shaped part of the deep layer (visible through the transparent upper layer) in the early avian primitive streak embryo. It is derived from Rauber's sickle cells that migrate into the neighbouring ooplasm during early incubation and has a typical histological aspect (CALLEBAUT & VAN NUETEN, 1994). The angle formed by the junctional endoblast is bisected by the primitive streak formed in the upper layer (Figs 2, 3). Laterally to the convexity of the junctional endoblast, one can distinguish the yolk endoblast (developing in the caudal marginal zone), which has a less dense, white aspect but is more voluminous than the junctional endoblast. The junctional endoblast that forms *in situ* from the Rauber's sickle (CALLEBAUT & VAN NUETEN, 1994) has strong embryo-inducing and -dominating potencies (CALLEBAUT et al., 2000c). In a recent study (CALLEBAUT et al., 2000a), we observed that during avian neurogastrulation, a sickle-shaped canal (called "sickle canal") develops parallel with/and in the concavity of the U-shaped junctional endoblast by caudal fusion of the pararchenteric canals (Figs 3, 4). We observed the existence of a spatial relationship between the sickle canal or sickle cavity (limited below by sickle endoblast with associated junctional endoblast) and the early appearance of blood islands (Fig. 4). We used the term sickle endoblast because we have demonstrated, by using the quail-chick chimaera technique, that this part of the deep layer is directly derived from Rauber's sickle by centripetal and cranial growth and/or migration (CALLEBAUT & VAN NUETEN, 1994 ; CALLEBAUT et al., 1997b) (Figs 5,6). When a Rauber's sickle fragment is placed ectopically on any region of an isolated central part of the area centralis, then a primitive streak, a neural plate (CALLEBAUT et al., 1997a) and blood islands (CALLEBAUT et al., 2000a) are always induced after culture *in vitro*. Recently, we have shown that Rauber's sickle or later junctional endoblast is indispensable for the development of blood islands in the avian blastoderm (CALLEBAUT et al., 2002). A recent experimental *in vitro* study (CALLEBAUT et al., 2003a) demonstrated that a primitive streak (PS) in avian blastoderms is induced by diffusion of signalling molecules emanating from Rauber's sickle according to the basic concept of "positional information" (WOLPERT, 1969, 1981). Indeed, even without direct contact between a quail Rauber's sickle and the adjacent reacting upper layer (by interposition of a vitelline membrane), a primitive streak can be induced in the isolated area centralis or anti-sickle region of unincubated chicken blastoderms. From recent molecular biology studies (KNOETGEN et al., 1999a, b ; FOLEY et al., 2000 ; KNEZEVIC and MACKEM, 2001) it was concluded that the deep layer (containing sickle endoblast) elicits no definitive changes in the adjacent host upper layer after transplantation. However, these authors have not taken into account the fact that Rauber's sickle was always present in their experimental set-up and thus could have an inhibiting effect. Indeed in a recent study (CALLEBAUT et al., 2003b) we observed that if a quail endoblast fragment was placed on the isolated central Rauber's sickle-free part of a chicken area centralis, in culture, then a definitive primitive streak and neural plate were induced. This is confirmed by the present experimental study, since in a whole unincubated chicken blastoderm from which the Rauber's sickle has been (sub)totally removed, a fragment of sickle endoblast can also induce an embryo whilst this

induction is totally suppressed when an intact Rauber's sickle is still present. This indicates that Rauber's sickle dominates and can abolish totally the inducing activity of sickle endoblast. If no Rauber's sickle is present, then sickle endoblast can take over the function of Rauber's sickle, but without blood island formation. Sickle endoblast can thus be considered as a secondary, minor organizer of the avian blastoderm, normally dominated by Rauber's sickle, the primary major organizer.

MATERIAL AND METHODS

We used unincubated chicken (*Gallus domesticus*) blastoderms (or parts of) and sickle endoblast from 7-8h *in ovo* incubated quail (*Coturnix coturnix japonica*) blastoderms. Storage of the unincubated eggs for 1-2 days at room temperature seemed to increase the visibility of the Rauber's sickle. This is probably due to its increase in volume since we observed uptake of voluminous subgerminal "yolk islands" by encircling extensions of Rauber's sickle cells (Callebaut, 1994) and premitotic DNA synthesis in Rauber's sickle cells, even at room temperature (Callebaut, 1989). Stages of embryonic development were indicated according to Hamburger & Hamilton (1951) as (HH) or according to Eyal-Giladi & Kochav (1976) as (EK). After opening of the chicken eggs and removal of the egg white, the egg yolk balls were placed in Ringer's solution. The vitelline membrane was sectioned all around the equator of the yolk ball and removed from the yolk by a slow movement in the direction of/and bending 180˚ over the germ. In this manner, unincubated chicken blastoderms still adhering to the vitelline membrane and underlying subgerminal ooplasm, could usually be separated from the yolk. Normally the required quail sickle endoblast stage, corresponding to Stage 2-3 of Hamburger & Hamilton (1951) in the chicken, is reached in the *in toto* incubated quail egg after approximately 7-8h incubation. This is shorter than for *in toto* incubated chicken eggs, since the latter are approximately six times larger and the time to warm up lasts longer. First, the quail yolk balls are inspected in Ringer solution under a stereomicroscope in order to observe the external morphology of the germ disc. When a short primitive streak and the "legs" of the V-shaped junctional endoblast are visible from the surface, as seen on Figs 2 and 5, the quail germ disc can be used for excision of the sickle endoblast. Each experimental procedure used is described in the legends of the figures or in the text of the results or is represented in a scheme accompanying the microphotographs of the associated blastoderm parts in culture. The blastoderms were cultured according to the technique of Spratt (1947). This semi-solid culture medium allowed microsurgery and further culture on the same substratum. Instead of Petri dishes, the culture vessels described by Gaillard (1949), on which an optical flat glass cover was sealed with hot paraffin, were used. Stereomicroscopic Polaroid photographs were taken in the same direction at the beginning, during and at the end of the culture period (23-32h). Fixation was performed overnight in a modified Heidenhain's fixative (Romeis, 1948) containing 0.5 g sodium chloride, 2 g trichloracetic acid, 4 ml acetic acid, 20 ml formalin and 80 ml water. After rinsing in tap water, the blastoderms were stained *in toto* with Unna to enable their orientation in the paraffin wax to be seen. After rapid dehydration in a graded series of alcohol and embedding in paraffin, the (chimeric) blastoderms were sectioned perpendicularly to the visible or presumed axis. The deparaffinized, 8-µm-thick sections were Feulgen-stained after Demalsy & Callebaut (1967), to enable identification of the origin of the nuclei. This allowed us to observe the typical central or subcentral chromatin granule (most obvious with low power objectives) of the grafted quail cells (Callebaut 1968 ; Koshida & Kosin, 1968 ; Le Douarin & Barq, 1969) as well as to observe their relation with the chicken tissue. In the photographs of the sections, the associated blastoderm parts are represented with their deep side and sickle endoblast material directed upwards, since they were cultured in this orientation.

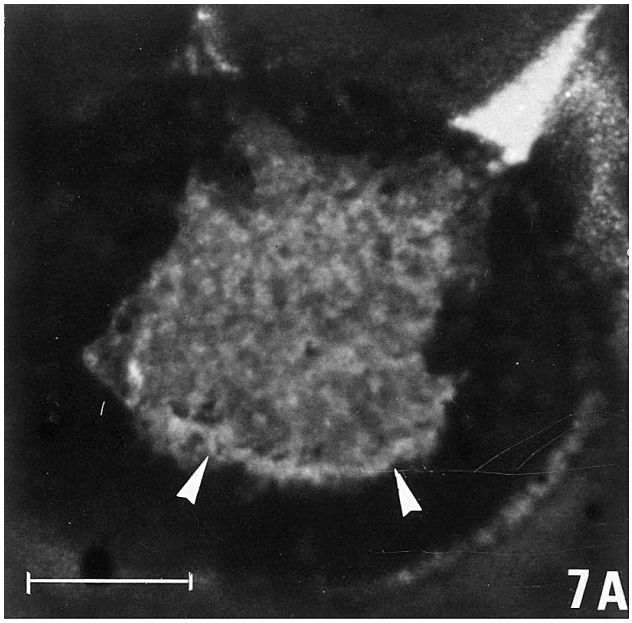
RESULTS

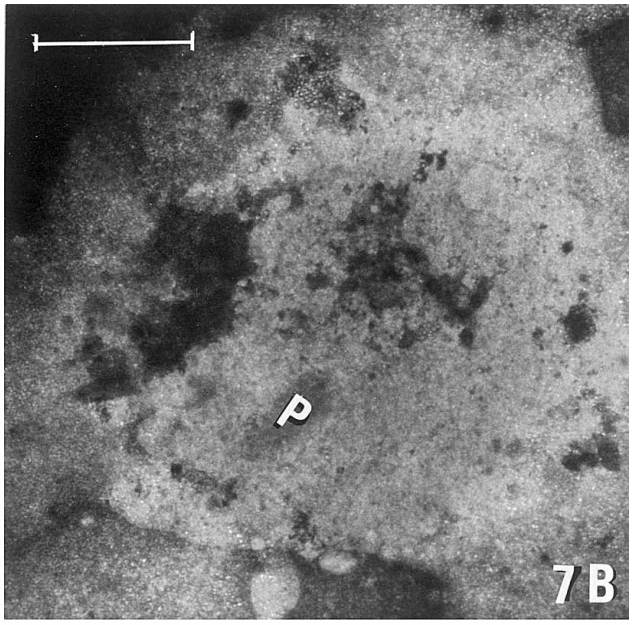
(Sub)total removal of Rauber's sickle (n=15)

After mechanical removal of the Rauber's sickle from an unincubated chicken blastoderm (Fig. 7A) and culture, in about 30% of the cases no complete embryonic development was observed. Sometimes only a preneural plate was formed without primitive streak. The upper layer expanded considerably over the culture medium. In the remainder of cases a small primitive streak with centripetally-directed axis (in the direction of the endophyll), appeared (Fig. 7B). This primitive streak started from a point where formerly the circumference of the autochthonous Rauber's sickle was observed (often from a sickle horn region, since there the Rauber's sickle material is barely visible and difficult to remove). After prolonged culture, a both a primitive streak and a neural plate (confirmed on sections) could be observed (Fig. 7C). Obviously, the development was slower than normal.

Isotopic exchange of a Rauber's sickle from unincubated chicken blastoderms with a quail Rauber's sickle (n=10)

From unincubated chicken blastoderms, the autochthonous Rauber's sickle, was removed. Then a quail Rauber's sickle (also from an unincubated quail blastoderm) was apposed isotopically on this chicken blastoderm (Fig. 8A). Already after 8h of culture a normally-developed primitive streak was observed, starting from the place where the quail Rauber's sickle was placed (Fig. 8B). After approximately 32h of culture, a completely normal embryo corresponding to stage 9 of Hamburger & Hamilton (1951) was seen. On sections through such chimeras quail cells were found only in the form of quail sickle endoblast or junctional endoblast. In the caudal part of the blastoderm, the quail sickle endoblast lines the sickle canal (Fig. 8C). The quail junctional endoblast is found close to the forming blood islands on which it has an inducing effect (Callebaut et al., 2000a, 2002). In the cranial part of the chimeric blastoderm, quail sickle endoblast is found cranially and laterally from the radially expanding definitive chicken endoderm (as was already observed after ectopic placement of a quail Rauber's sickle : Callebaut & Van Nueten, 1994) (Fig. 6). In the remainder of the blastoderm no quail cells were found (not in the superficial layer, nor in the chordomesoblast, nor in the prechordal plate, nor in the definitive gut endoderm) (Fig. 8D).





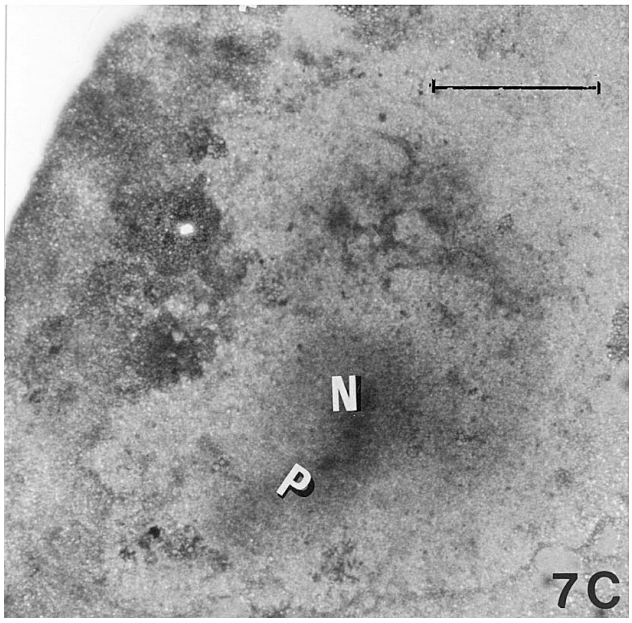


Fig. 7A. – Stereomicroscopic view from the lower side of an unincubated chicken blastoderm from which Rauber's sickle was mechanically removed (indicated by two arrowheads) at the start of the culture period ; bar : 1 mm.

Fig. 7B. – Stereomicroscopic view of the same blastoderm as seen in Fig 7A, after 22h of culture : a small primitive streak (P) has developed from laterally close to the sickle horn region ; bar : 1 mm.

Fig. 7C. – The same blastoderm as in Fig. 7B after 26h of culture before fixation ; besides a primitive streak (P), a neural plate (N) is also visible ; bar : 1 mm.

Placement of a fragment of quail sickle endoblast on the isolated (Rauber's sickle-free) anti-sickle of an unincubated chicken blastoderm (n=12) (Fig. 9A)

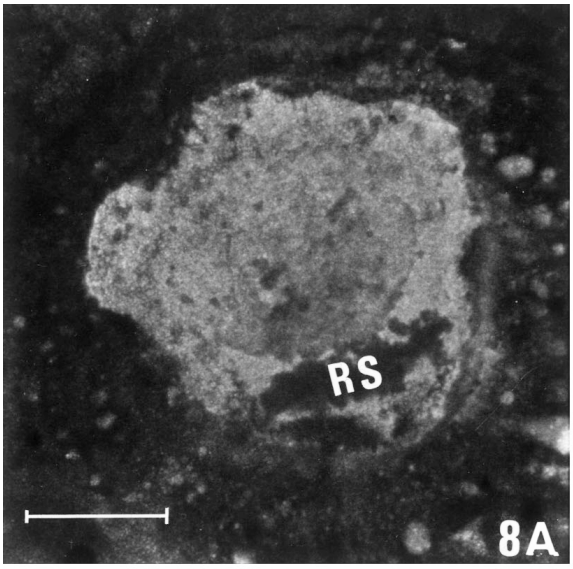
A (pre)neural plate always developed after culture (often with a shallow median neural groove) in the thickened upper layer, adjacent to the quail sickle endoblast (Fig. 9B). No ingression phenomena and no primitive streak were observed.

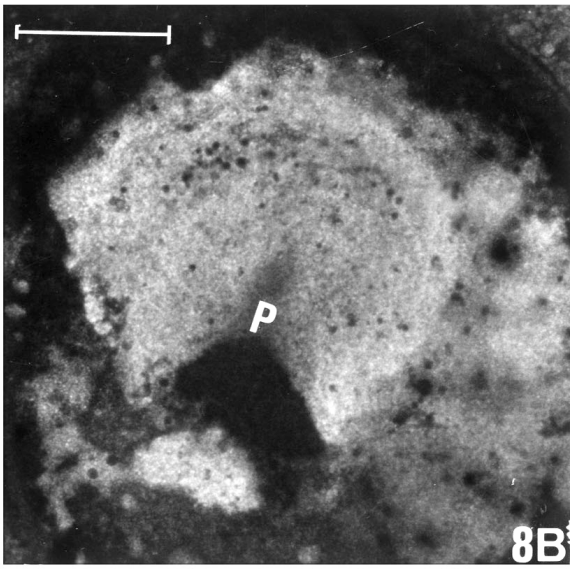
Placement of a fragment of quail sickle endoblast on the anti-sickle region of an intact unincubated chicken blastoderm (n=8)

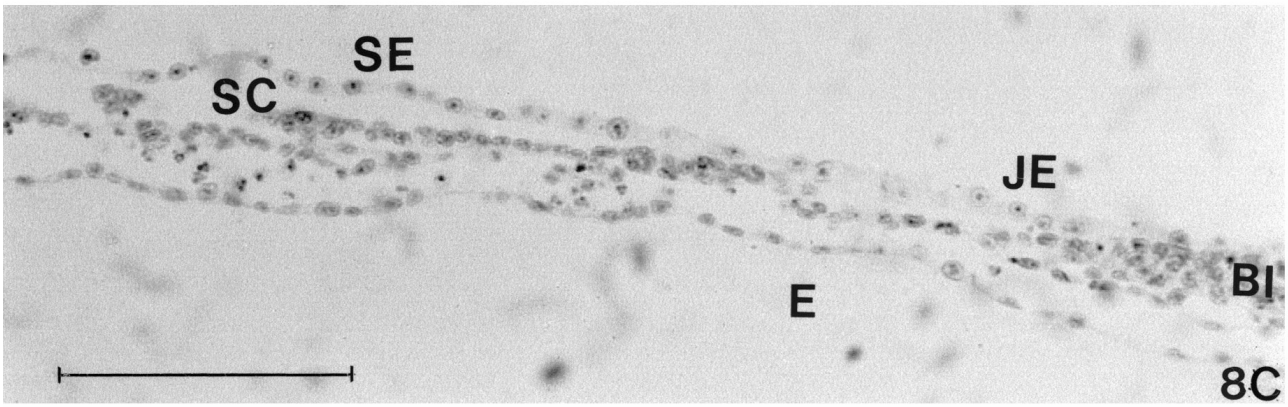
During culture, the quail sickle endoblast rapidly became invisible under the stereomicroscope and no induction phenomena were ever observed. On sections no thickening of the upper layer adjacent to the apposed quail sickle endoblast was seen. Here, clearly neither gastrulation nor neurulation phenomena took place. Also no blood island formation occurred in relation to the apposed sickle endoblast.

Placement of a fragment of quail sickle endoblast on the anti-sickle region of an unincubated chicken blastoderm from which the autochthonous Rauber's sickle was (sub)totally removed (Fig. 10A) (n=20)

After culture, in about half of the cases, a centripetally-oriented embryo was induced in the anti-sickle region by the quail sickle endoblast (independent from its original polarity). When no accessory small embryo developed from the region where the autochthonous Rauber's sickle was removed, then the induced embryo progressively extended over the whole area centralis (Fig. 10B) and, besides a primitive streak, a large neural plate was seen. On sections the quail sickle endoblast was seen medially under the primitive streak region (Figs 10C and C'). Sections







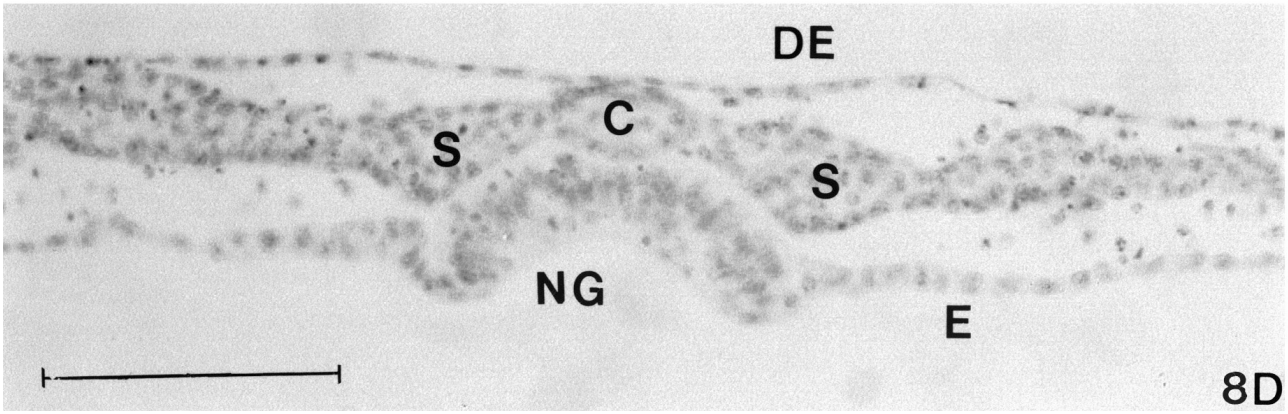


Fig. 8A. - At the start of the culture : a quail Rauber's sickle (RS) was isotopically placed where the autochthonous chicken Rauber's sickle was removed ; bar : 1 mm.

Fig. 8B. – The same chimera as seen in Fig. 8A after 8h of culture : a completely normal blastoderm with primitive streak (P) develops ; bar : 1 mm.

Fig. 8C. – Section through the caudal region of the same embryo after 32h of culture ; SE : quail sickle endoblast ; JE : quail junctional endoblast in the immediate neighborhood of a blood island (BI) ; SC : sickle canal or sickle cavity ; E : epiblast ; Feulgen staining ; bar : 100 μm.

Fig. 8D. – Section through the more cranial part of the same embryo ; DE : definitive endoderm ; C : chorda ; S : somites ; NG : neural groove ; E : epiblast ; no quail cells are visible ; Feulgen staining ; bar : 100 μm.

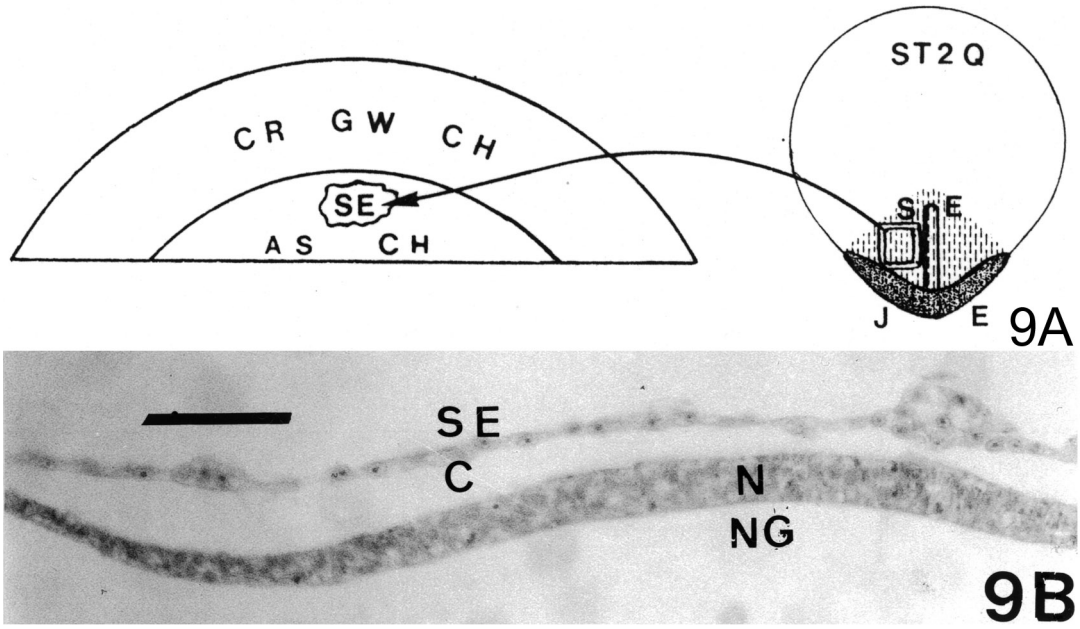
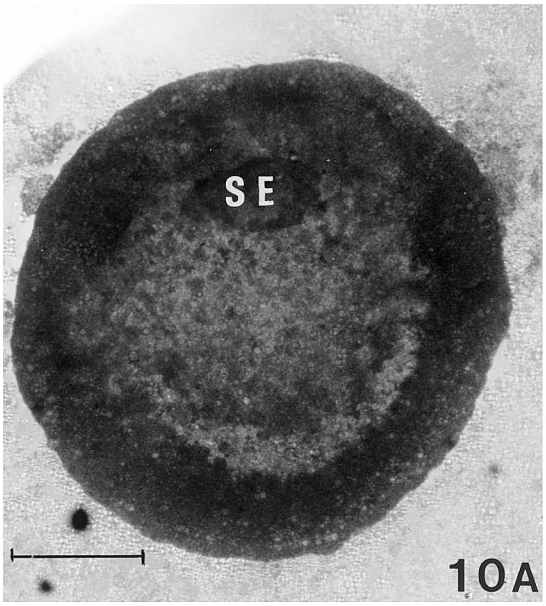


Fig. 9A. – Schematic drawing representing the excision of a fragment of quail sickle endoblast (SE) (at the right) and its transplantation (indicated by a long curved arrow) on the deep side of the anti-sickle region of an unincubated chicken blastoderm (ASCH) ; CR GW CH : cranial germ wall of chicken.

Fig. 9B. – Section through a chimera composed as represented in Fig. 9A, after 24h of culture : the (pre)neural thickening (N) of the upper layer and the parallel inducing quail sickle endoblast layer (SE) have approximately the same extent and are separated by a cavity (C) ; no ingression phenomena are seen ; a broad neural groove (NG) is visible ; Feulgen staining ; bar : 100 µm.



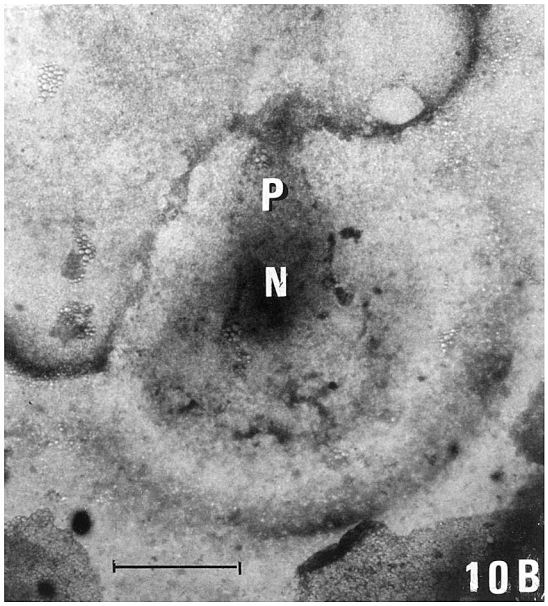


Fig. 10A. – Stereomicroscopic view of the deep side of an unincubated chicken blastoderm from which the Rauber's sickle was removed and on which a fragment of quail sickle endoblast (SE) was placed over the anti-sickle region ; bar : 1 mm.

Fig. 10B. – Stereomicroscopic view of the same chimera as seen in Fig 10A after 26h of culture, before fixation ; an embryo extending from the anti-sickle region over the whole area centralis has been induced by the apposed quail sickle endoblast ; P : primitive streak ; N : neural plate ; no accessory embryo has developed from the region of the removed chicken Rauber's sickle, indicating a successful total removal ; bar : 1 mm.

at the level of HENSEN's node (most cranial part of the primitive streak) revealed the presence of quail sickle endoblast on both sides, separated by the median, radially-expanding definitive endoderm (Figs 10 D and D'). More cranially, the quail sickle endoblast was found medially adjacent to/but separated by a narrow space from the (pre)neural plate (Figs 10 E and E'). No junctional endoblast and no blood islands were observed in the original anti-sickle region. This indicates that sickle endoblast cannot give rise to junctional endoblast even after more than

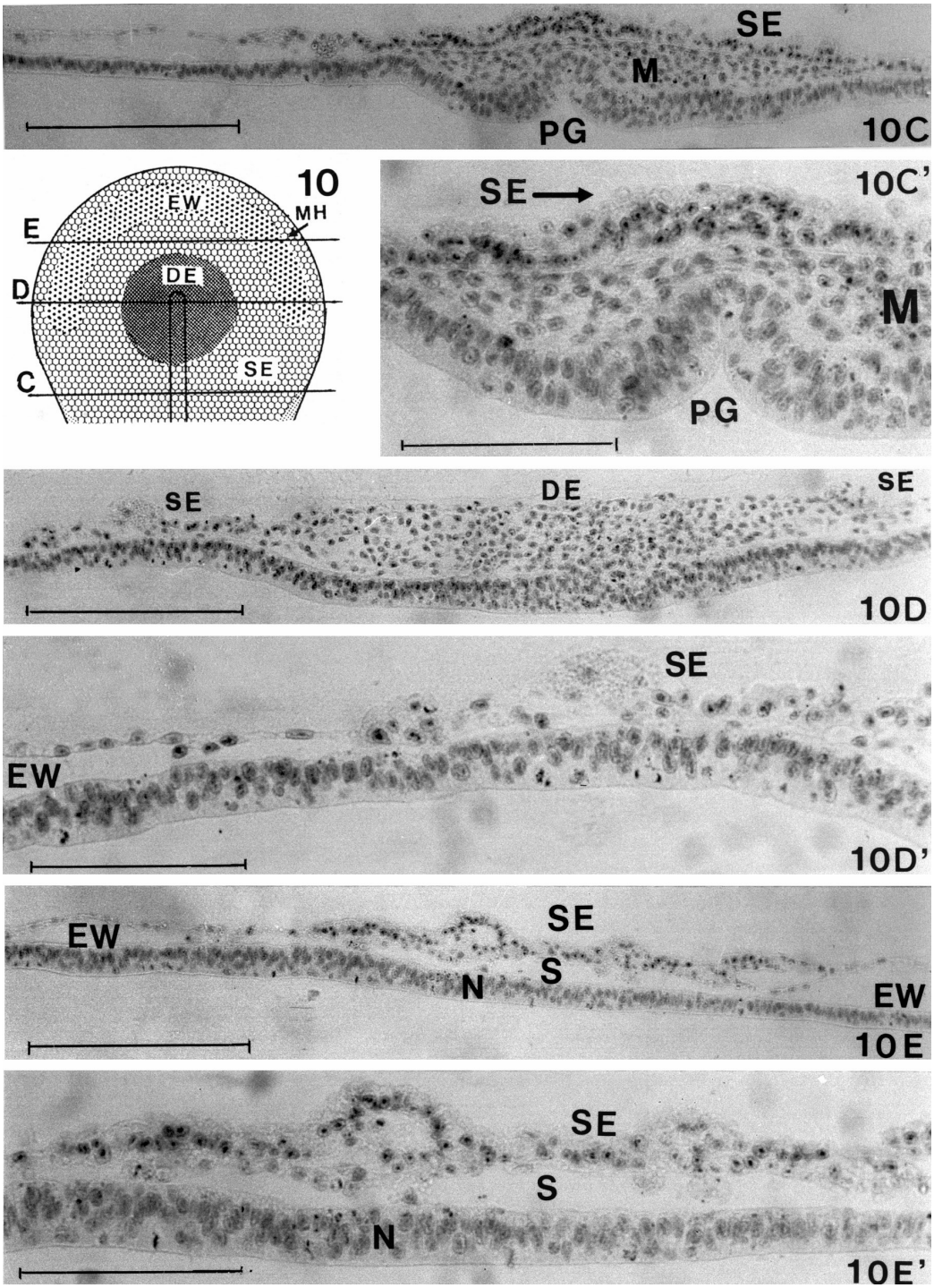


Fig. 10. – Schematic drawing representing the different deep layer components found in the definitive cranial part of the blastoderm of Fig. 10B ; EW : endophyll wall ; MH : marginal hypoblast ; SE : sickle endoblast ; DE : definitive endoderm expanding radially around the top of the primitive streak (Hensen's node) ; the horizontal lines C, D and E indicate the level of the sections seen respectively in Figs 10C and 10C' ; Figs 10D and 10D', Figs 10E and 10E'.

Fig. 10C. – Section through the primitive streak region of the chimera of Fig. 10B at the level C as indicated on the schematic drawing ; quail sickle endoblast cells (SE) are seen in the median region above the induced primitive streak and primitive groove (PG) ; M : mesoblast formed by ingression via the primitive streak ; Feulgen staining ; bar : 200 µm.

Fig. 10C' – The same section as in Fig. 10C at a higher magnification with the same indications ; bar : 100 µm.

Fig. 10D. – Section through the nodus region of the chimera of Fig. 10B (at the level D as indicated on the schematic drawing) ; DE : definitive endoderm pushing the quail sickle endoblast (SE) radially to the periphery (visible on both sides) ; Feulgen staining ; bar : 200 µm.

Fig. 10D'. – Enlarged view from part of Fig. 10D ; SE : quail sickle endoblast ; EW : endophyll wall covered by chicken endophyll ; bar : 100 µm.

Fig. 10E. – More cranial section through the region of the neural plate *Anlage* (at the level E, as indicated on the schematic drawing) ; SE : median quail sickle endoblast separated from the neural plate (N) by a space (S) ; EW : endophyll wall on both sides, covered with chicken endophyll ; Feulgen staining ; bar : 200 µm.

Fig. 10E' – Part of section of Fig. 10E at a higher magnification with the same indications ; bar : 100 µm.

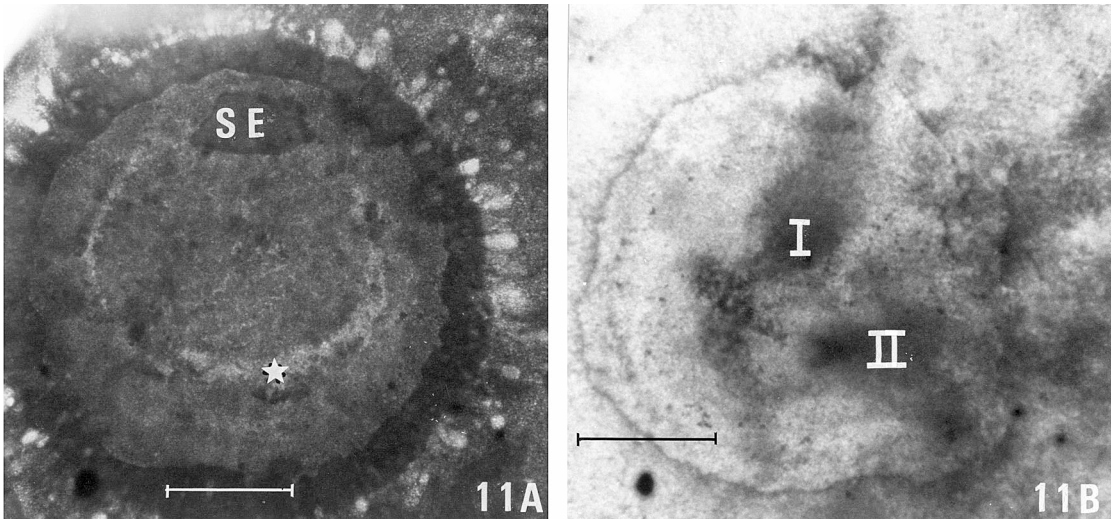


Fig. 11A. – Stereomicroscopic view of a chicken blastoderm from which the Rauber's sickle was mechanically removed (*) and on which a fragment of quail sickle endoblast (SE) was placed on the anti-sickle region ; bar : 1 mm.
Fig. 11B. – The same chimera as seen in Fig 11A, after 25h of culture : two centrally-oriented embryos (with a head against head localization) have formed in the blastoderm ; one (I) is induced by the apposed quail sickle endoblast fragment ; the other (II) is formed in the neighbourhood of the left sickle horn region ; bar : 1 mm.

one day of culture. This experiment also indicates that the caudal marginal zone (which remains present) in the absence of Rauber's sickle material is, on its own, not able to induce a primitive streak nor to inhibit the inducing effect of sickle endoblast apposed on the anti-sickle region. If, after removal of the Rauber's sickle and placement of a fragment of quail sickle endoblast on the anti-sickle region (Fig. 11A), only a small accessory embryo developed from part of the circumference where the autochthonous Rauber's sickle was originally localized before removal, then usually also an embryo appeared under inductory influence of the apposed quail sickle endoblast fragment (Fig. 11B). This embryo also presented a centripetally-directed axis, (independent from the original polarity of the sickle endoblast) with its head region against the head region of the accessory embryo. Both seem to compete for space in the area centralis as is also the case after grafting an ectopic Rauber's sickle on the anti-sickle region (Callebaut & Van Nueten, 1994). However, no quail junctional endoblast and no blood islands were formed in the embryo induced by the sickle endoblast. If a fully developed embryo developed, starting from the sickle-shaped region where Rauber's sickle was (sub)totally removed, then no embryo induction by the apposed quail sickle endoblast was observed. In these cases, on sections, no thickening of the upper layer adjacent to the sickle endoblast was seen, indicating the absence of gastrulation and/or neurulation phenomena. However in some cases only a limited preneurulation with space formation between the thickened upper layer and the quail sickle endoblast was seen. That the chicken Rauber's sickle material was removed incompletely could usually best be seen after culture for 24h or more. Indeed in these cases often a sickle-shaped area vasculosa (visible alive) appeared in the caudal part of the blastoderm behind the chicken embryo proper. On sections, blood islands were then found in the immediate neighborhood of some chicken junctional endoblast, derived from the remnants of the Rauber's sickle. We can conclude that if Rauber's sickle activity is strongly reduced, then sickle endoblast can still induce gastrulation and neurulation phenomena. If Rauber's sickle activity is totally absent, then the inducing power of the sickle endoblast material becomes maximal. If Rauber's sickle is fully present in a blastoderm, then the inducing activity of the sickle endoblast, placed on the anti-sickle region, will always be totally suppressed.

Placement of a fragment of quail sickle endoblast on the area centralis of an intact unincubated chicken blastoderm (Fig. 12A) (n=7)

After culture, the development was always seen to be much slower than normal. Sometimes two embryonic areas were found aligned (Fig. 12B) with a separate primitive streak in each area. In sections, in only one of the areas, the apposed quail sickle endoblast sheet was found.

DISCUSSION

In the past the very existence in unincubated avian blastoderms of a Rauber's sickle (1876) (formely called Koller's sickle : 1882) has been vigourously debated, for decades. Principal opponents to its real existence have been Peter (1938) and Pasteels (1937 ; 1940) who consigned the sickle to oblivion. By their strong scientific impact the sickle has been voluntarily or involuntarily forgotten by most investigators until the 1970s. So the Rauber's (Koller's) sickle was not even mentioned in the detailed description of experiments with avian blastoderms (Bellairs, 1971). The cleavage experiments of avian germ discs *in ovo* (Lutz, 1964) already suggested a functional relationship between the localization of Rauber's sickle fragments and caudocephalic axis formation. Vakaet (1962) concluded that in unincubated chicken blastoderms at least some indication of the existence of a Rauber's sickle was to be found. The reason for the doubt about the real existence of a Rauber's sickle in every unincubated avian blastoderm seems to be that it was only vis-

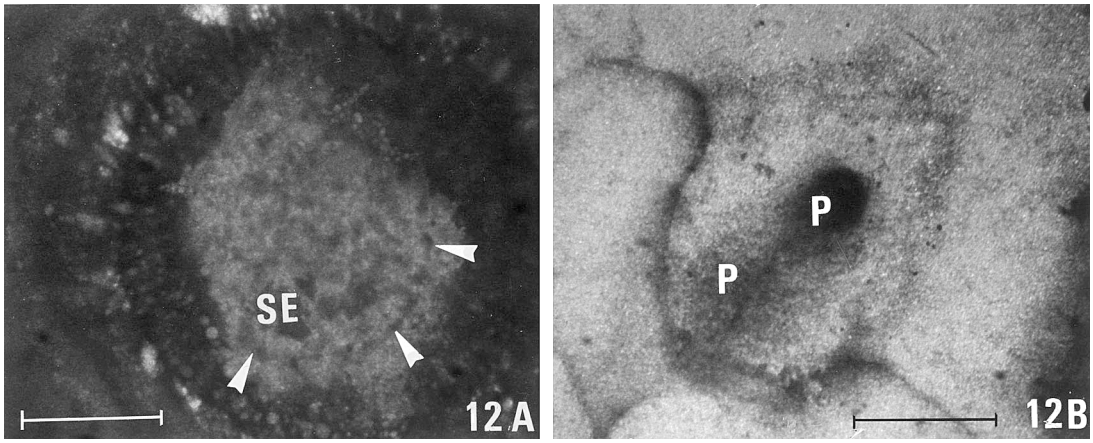


Fig. 12A. – On the deep side of the caudal part of the area centralis of a complete unincubated chicken blastoderm, presenting a narrow fragmentary Rauber's sickle (indicated by arrowheads), a fragment of quail sickle endoblast (SE) was placed at the start of the culture ; bar : 1 mm.
Fig. 12B. – The same chimera as seen in Fig 12A after 27h of culture : the development is much slower than normal ; P and P are two areas with separate aligned primitive streaks (confirmed by sectioning) ; bar : 1 mm.

ible from the surface of the freshly laid quail egg in about 30% of cases (Lutz, 1964 ; Fargeix, 1964). This can be improved with intraoocytal trypan blue labelling to 60% in the quail (Callebaut et al., 1998b). Also the detaching of the unincubated chicken blastoderm together with its covering vitelline membrane from the egg yolk ball can give problems. Indeed it then happened often that Rauber's sickle wholly or partially remained fixed to the subgerminal ooplasm and so was not observed. That a Rauber's sickle is present in nearly every blastoderm of unincubated chicken or quail eggs can be shown in sections (performed perpendicularly to the chalazal rotation axis) through the germ discs (after fixation *in toto* on their egg yolk balls). Indeed in sections after such a fixation, the future caudal part of the germ (with the Rauber's sickle adhering to the subgerminal ooplasm) and the future cranial part of the germ (with the anti-sickle region disrupted from the subgerminal ooplasm) can be easily recognized (Callebaut et al., 1998a). Because during several decades, Rauber's sickle was not considered as a deep layer component distinctly separable from the caudal marginal zone, its function remained unknown, until Callebaut & Van Nueten (1994) first systematically isolated and transplanted (without surrounding tissues) quail Rauber's sickles (which are usually much more voluminous than chicken Rauber's sickles). Thus their powerful inductive activity for the formation of mesoblast and definitive endoderm was shown. The structures and involved early induction phenomena in the avian germ disc present much homology with similar structures and phenomena taking place in the zebrafish egg (*Brachydanio rerio*) (Callebaut et al., 1996b). Recently Koos & Ho (1998) have shown that at the onset of gastrulation nieuwkoid expression becomes localized in a restricted region of the extraembryonic yolk syncytial layer, directly underlying the future dorsal shield. This part of the yolk syncytial layer in teleleosts seems thus to correspond to the Nieuwkoop center in the *Xenopus* embryo and to Rauber's sickle in avian germs. We observed that the tissues in the immediate neighborhood of Rauber's sickle (both the upper and deep part of the caudal marginal zone) have no inductive activity on their own (Callebaut et al., 1998a). In the present study we show once again that it is Rauber's sickle and not the caudal marginal zone that is able to induce a primitive streak, since after mechanical removal of the Rauber's sickle the caudal marginal zone was left intact *in situ*. This seems to be in contradiction with the conclusions of Bachvarova et al. (1998) and Bachvarova (1999). In their study a quail caudal marginal zone was associated *in vitro* culture with a chick cranial half blastoderm. In such associations often a centrally-directed primitive streak was observed starting from the cranial part of this cranial half. However we have shown that this also often occurs spontaneously in the absence of caudal marginal zone material (Callebaut & Van Nueten, 1993) by the presence of far cranially-extending, unrecognized sickle horn material in the cranial blastoderm half. Recently the developmental role of Rauber's sickle became still more fascinating with the discovery that after an initial inducing effect on mesoblast and definitive endoderm formation in the upper layer of the area centralis, it also induces blood island formation in the mesoblast that migrates peripherally over the junctional endoblast, directly below the flat epiblast, in the direction of the area opaca (Callebaut et al., 2000a). Moreover, we found that Rauber's sickle or Rauber's sickle-derived junctional endoblast was indispensable for early formation of blood islands (Callebaut et al., 2002). Although Rauber's sickle and the Rauber's sickle-derived sickle endoblast and junctional endoblast have a very important and indispensable inductive function for the development of the embryonic tissues during gastrulation and neurulation, our study demonstrates that they never give rise to cells of the embryo proper and therefore belong to the so-called extraembryonic part of the blastoderm (as is also the case for the Nieuwkoop center (Guger & Gumbiner, 1995). This is in contrast to the conclusion of the study of Izpisua-Belmonte et al (1993). Indeed, a problem in their experiments was that they could not isolate Rauber's sickle completely neither from the upper layer nor from the deep layer. So, always part of a region in the neighborhood of Rauber's sickle was also taken. The same holds true for their DiI applications to the very narrow and fragile chicken Rauber's sickles after which not only Rauber's sickle cells were labelled, but also upper layer cells, by inevitable leakage of

this stain. In conclusion, the avian Rauber's sickle fulfils the major postulate for homology with a functional Nieuwkoop centre, namely the potential for organizer induction without itself contributing to the new structure. Our present study once again demonstrates the value of the anti-sickle region of unincubated blastoderms as an experimental tool for demonstrating induction phenomena.

In the present study we show that sickle endoblast, if withdrawn from the influence of Rauber's sickle, has gastrulation and/or neurulation inducing potencies on the upper layer of the avian unincubated blastoderm, but it has no influence on blood island formation. Obviously, it also plays a role during normal development in the early organization (primitive streak formation) of the avian blastoderm in combination with its mother tissue, Rauber's sickle. So, the area where a primitive streak-inducing action can take place is considerably extended during early incubation from a sickle shape (Rauber's sickle) to approximately the whole caudal quadrant of the blastoderm (Fig 5), i.e. the area where the sickle endoblast penetrates into the endophyll (Callebaut et al., 1997b). During the first hours of culture, we observed in the latter study that an intimate contact developed (before a real primitive streak with ingressing upper layer cells appears) between the infolded thickened chicken upper layer and the quail sickle endoblast on the midline. This suggested already a primary influence of sickle endoblast on the formation of the primitive groove and primitive streak. These earlier observations can probably been explained as the result of the here described induction phenomena by fragments of quail sickle endoblast. Indeed, our present experiments, where a fragment of sickle endoblast was placed on the area centralis of an intact (Rauber's sickle-containing) unincubated blastoderm, seem to indicate that during early culture a temporary competition takes place between the sickle endoblast and the Rauber's sickle. But there also, Rauber's sickle finally overshadows the early effect of the sickle endoblast. That in the present experiment no junctional endoblast developed from the grafted sickle endoblast seems to indicate that the differentiation of Rauber's sickle material into sickle endoblast is irreversible and that only Rauber's sickle versus junctional endoblast can induce blood islands. The homeobox gene CHex is expressed in Rauber's sickle and sickle endoblast (Yatskievych et al., 1999). CHex transcripts were also detected within blood islands beginning at stage 4 and in extraembryonic and intraembryonic vascular endothelial cells. Since we have shown that Rauber's sickle and junctional endoblast have an inducing effect on blood island formation, we can postulate an unknown relationship with the CHexgene.

The molecular basis of neural induction has been extensively studied in *Xenopus laevis*, and it was found to be tightly coupled to the establishment of the dorso-ventral axis (De Robertis & Sasai, 1996 ; Hemmati-Brivanlou & Melton, 1997). In frogs, the prospective ectoderm is induced by bone morphogenetic proteins (BMPs). In contrast, a neural development requires the inactivation of BMPs and is achieved by direct complex formation between BMPs and neural inducing factors such as chordin, noggin or follistatin (Piccolo et al., 1996 ; Zimmermann et al 1996). In the chick blastoderm at early stages, the prospective epidermis is characterized by the expression of the homeobox gene DLX5, which remains an epidermal marker during gastrulation and neurulation and enables it to be distinguished from the more central neural plate (PERA et al., 1999). That vertical signals from the lower layer are necessary for the establishment of the neural plate has been shown by the latter authors by repeated extirpations of the underlying endoblast. In the absence of the lower germ layers, the epidermis expanded into the region that normally forms the neural plate. Knoetgen et al. (1999a) analysed the GANF (Gallus anterior neural fold) – inducing potential of various tissues at different stages during chick development by transplantation to the outer margin of the area pellucida, where the epiblast cells are fated to become epidermis (Spratt, 1952 ; Rosenquist, 1966 ; Schoenwolf & Sheard, 1990 ; Bortier & Vakaet, 1992 ; Garcia-Martinez et al., 1993). When cranial hypoblast from pre-streak stages (EK XII/XIII) or from mid-streak stages (HH3) or the definitive endoderm from late streak stages (HH4) was grafted on whole blastoderms, neither morphological alteration nor ectopic expression of GANF was elicited. Labelling with DiI demonstrated that the hypoblast cells remained together at the position of grafting during the incubation (which we have also seen in the present study). Transplants of Hensen's node (HH3+/HH4) on whole blastoderms led to the induction of a neuroectodermal structure with a strong expression of GANF in its cranial margin. Grafting of the young head process (HH4+) to the lateral cranial area pellucida caused a thickening of the epiblast and an induction of GANF expression in juxtaposed cells. Formerly, it was generally believed that the neural plate is only formed in the upper layer by an inductive interaction with the underlying chordamesoderm. However, animal caps isolated from amphibian embryos, injected with a dominant negative activin receptor, spontaneously express neural markers such as the neural cell adhesion molecule N-CAM without any signal from the mesoderm (Lemaire, 1992). More recently *in situ* hybridisation studies demonstrated that Otx2 function is required in the murine primitive visceral endoderm for the induction of forebrain and midbrain (Rhinn et al., 1998). A secreted molecule named *Cerberus*, which is expressed in anterior endoderm, has the property to induce ectopic head structures when micro-injected into ventral regions of *Xenopus* embryos (Bouwmeester et al., 1996 ; Bouwmeester, 1997). The patterning of the chick forebrain *Anlage* by the prechordal plate has been described by Pera & Kessel (1997). According to these authors also, the avian neural plate is evident before the first mesendodermal or axial mesodermal cells ingress, excluding the prechordal plate and the notochord as primary sources for neural induction. A previous study indicated that avian endophyll (from unincubated blastoderms) can induce a (pre)neural plate, with or without neural folds in the upper layer of the caudal marginal zone, where normally no endophyll is present (Callebaut et al., 1999a). By interaction with sickle endoblast arising from Rauber's sickle (the early gastrulation organizer : Callebaut & Van Nueten, 1994 ; Callebaut et al., 1997a) or from Hensen's node (a later avian organizer : Waddington, 1932), endophyll orients or re-orients the head region and the caudocranial direction of an induced miniature embryo (Callebaut et al., 1999a). It was proposed by Perea-Gomez et al. (2001) that the anterior visceral endoderm in the mouse embryo protects anterior embryonic regions from signals that promote

posterior development. There seems to be much homology between the visceral endoderm in the mouse and the avian sickle endoblast. Both deep layer structures move actively before and during gastrulation and are progressively replaced by definitive endoderm during gastrulation (in the mouse : Lawson & Pedersen, 1987 ; in the chicken : Callebaut & Van Nueten, 1994 ; Callebaut et al., 1997b). The cranial part of the late avian streak is capable of direct neural induction, and its tip, Hensen's node can induce an anterior neural identity. This latter activity leaves the node together with the cells representing the anterior mesendoderm (Boettger et al., 2001). During early gastrulation, cells invaginate through the tip of the growing streak and spread radially to form the definitive (gut) endoderm (Vakaet, 1970). During this radial expansion, the latter definitive endoderm pushes the sickle endoblast also radially (Callebaut & Van Nueten, 1994) (Figs 6, 10D). The cranial hemi-circular sickle endoblast slides under upper layer cells that will transform into the also hemicircular neural plate *Anlage* (Bortier & Vakaet, 1992). The latter cells are localized close to the former anti-sickle region exactly in the concavity of the cranially-displaced endophyllic crescent (Figs 6, 10E). The remaining more caudal sickle endoblast is localized under the upper layer, which will give rise to the primitive streak-forming area, localized in the area centralis region. This different evolution in the cranial (anti-sickle) region versus the central (area centralis) region can probably been explained by the different reactivity in these two upper layer regions observed in the present study. We cannot exclude the possibility that some endophyll is also included in the transplanted sickle endoblast but surely it contains no definitive endoderm since the sickle endoblast was excised before the definitive endoderm appears. That no inducing effect was observed by apposition of quail sickle endoblast on the anti-sickle region of whole unincubated chicken blastoderms, can probably be explained by the domination and inhibition at long distance (positional information) by the still present autochthonous Rauber's sickle or junctional endoblast (Callebaut et al., 2000c). Indeed in the present study we observed that if a fragment of quail sickle endoblast was placed on the anti-sickle region of an unincubated chicken blastoderm from which the Rauber's sickle was totally or subtotally removed, then often starting from this anti-sickle region an embryo was induced presenting both gastrulation and neurulation phenomena but no blood island formation. Rauber's sickle can thus quantitatively dominate or inhibit the ectopically-placed hierarchically-submitted sickle endoblast (belonging to the same cell lineage). Our study also indicates that via its outgrowth (sickle endoblast), Rauber's sickle also influences at distance the formation of the neural plate. The absence of neural induction after the grafting experiments with hypoblast on whole blastoderms by Galera & Nicolet (1969) and by Knoetgen et al. (1999a, b) can probably also be explained by the full presence of Rauber's sickle material. This indicates also that the earlier conclusions from grafting experiments on whole unincubated blastoderms (containing Rauber's sickle, a primary major organizer) or on primitive streak blastoderms (containing Hensen's node, a secondary major organizer) must be reconsidered. Thus, to study correctly interactions between different combined parts of the avian blastoderm, it is often necessary to culture them in isolation from other parts or cell groups of the blastoderm. Therefore we cannot agree with either of the conclusions of Knoetgen et al (1999b) that the endoblast on its own elicits any detectable change in the adjacent host ectoblast after transplantation, or that the avian organizer is confined to Hensen's node only.

When an anti-sickle region was cultured in isolation no differentiation was seen (Callebaut & Van Nueten, 1995). After placing an endophyll fragment on the naïve upper layer of isolated anti-sickle regions (Callebaut & Van Nueten, 1995), we observed the induction of a (pre)neural plate (often with a median neural groove and lateral neural walls). These experiments incited Foley et al. (2000) to study the eventual role of the early deep layer (endophyll and/or sickle endoblast) on the expression of the molecular markers Sox3 (Uwanogho et al., 1995) and Otx2 (Bally-Cuif et al., 1995) in the upper layer. From stage 6-7 HH on, Sox3 is specifically expressed in the entire chicken neural plate and Otx2 is expressed throughout the forebrain and midbrain. Foley et al. (2000) found that the early deep layer regulates an early transient phase of Otx2 and Sox3 expression in the adjacent upper layer. Therefore they concluded that the early deep layer does not induce neural tissue or forebrain definitively. However, their transplantation experiments were not performed on Rauber's sickle- or junctional endoblast-free blastoderm fragments but on whole blastoderms. As seen in earlier studies and in the present study, both structures have dominating and suppressive potencies (Callebaut et al., 2000c). Recently Knezevic & Mackem (2001) found evidence that two genes, later associated with the gastrula organizer (Gnot-1 and Gnot-2), are induced by the deep layer signals in prestreak embryos. According to the latter authors, these genes could perhaps regulate axis formation in the early embryo, which could also explain the induction of a streak in the isolated central part of the area centralis by sickle endoblast (Callebaut et al., 2003b study). In our fate map of Anlage fields in unincubated chicken blastoderms (Callebaut et al., 1996a), we have shown that the *Anlage* of the future central nervous system extends as a large shield-like surface structure from just caudally from the centre of the area centralis to a short distance from the middle of the concavity of Rauber's sickle (Fig. 13A). This neural plate *Anlage* is thus exactly localized over the endophyll region. During early gastrulation, the cranially-growing sickle endoblast pushes and penetrates the endophyll (Callebaut et al., 1997b). At the same time, the primitive streak-forming upper layer area displaces this predisposed neural plate *Anlage* in a cranial direction (Callebaut & Van Nueten, 1996). So the prediposed neural plate *Anlage* and the associated endophyll (between both exist narrow cellular bridges : Callebaut et al., 1999b) move side by side simultaneously (the so called "mouvements simultanés" reported by Pasteels, 1937). So, both structures remain in prolonged contact during gastrulation. This can probably explain why finally the influenced upper layer, (successively by endophyll and sickle endoblast) during further normal development (after stabilization of its preneural stage by prechordal mesendoderm and cranial head process) will definitively form a central nervous system cranially to the Hensen's node where sickle endoblast is localized in the concavity of the endophyllic crescent (Bortier & Vakaet, 1992) (Fig. 13B) and at distance from the junctional endoblast (Fig. 6). It is indeed remarkable that the shield-like predisposed Anlage field of the whole central

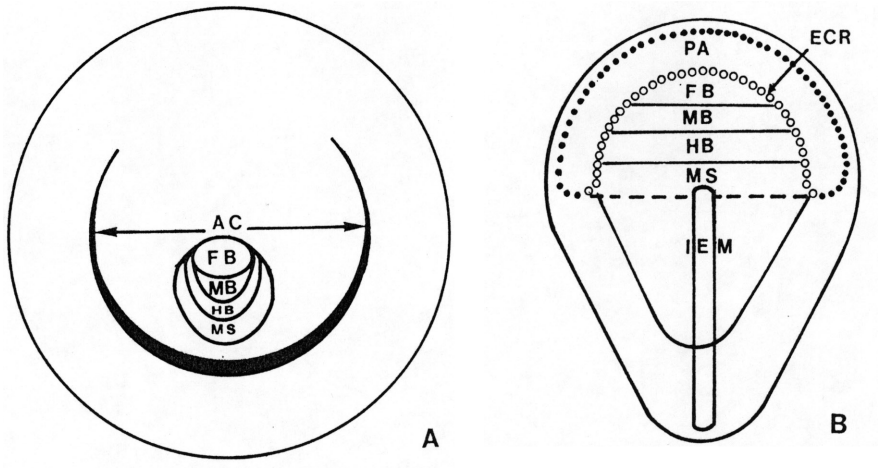


Fig. 13. - Comparison of the disposition of the neural plate Anlage (and its subdivisions), on dorsal views in the upper layer of A. the unincubated chicken blastoderm (according to CALLEBAUT et al., 1996a) and of B. the stage 4-5 (HAMBURGER & HAMILTON) chicken blastoderm (according to BORTIER & VAKAET, 1992) ; FB : forebrain Anlage ; MB : midbrain Anlage ; HB : hindbrain Anlage ; MS : Anlage of the medulla spinalis ; AC : area centralis enclosed by Rauber's sickle ; seen in transparency is the proamnion (PA), a broad sickle-shaped cavity cranially from the endophyllic crescent (ECR) ; IEM : intraembryonic mesoblast Anlage in the upper layer.

nervous system is first localized in the upper layer of the caudal hemicircular half of the blastoderm at the unincubated stage (CALLEBAUT et al., 1996a), whilst after approximately one day of culture (Stage 5-6 of HAMBURGER & HAMILTON) the same upper layer part (but now definitively determined as neural tissue) is localized in the cranial hemicircular half of the blastoderm (cranially from Hensen's node : BORTIER & VAKAET, 1992) (Fig. 13B). A previous study (CALLEBAUT et al., 2003b) and the present one suggest the existence of a temporo-spatially bound cascade of gastrulation and neurulation phenomena and blood island formation in the avian blastoderm, starting from Rauber's sickle, the primary major organizer. Successively, we observed the following steps : 1) induction of a primitive streak in the upper layer by signalling molecules, secreted by Rauber's sickle or by sickle endoblast diffusing over a long distance in the blastoderm eventually through an interposed vitelline membrane (CALLEBAUT et al., 2003a), 2) differentiation of junctional endoblast from Rauber's sickle by interaction with the upper layer and/or endophyll since the interposition of a vitelline membrane inhibits this ; this junctional endoblast is indispensable for blood island formation (CALLEBAUT et al., 2002) ; 3) differentiation of sickle endoblast moving cranially from Rauber's sickle into/and with the endophyll (CALLEBAUT et al., 1997b, 1999a). In the upper layer of the blastoderm, sickle endoblast induces proximally (i.e. close to Rauber's sickle) the formation of a primitive streak, whilst distally (i.e. diametrically opposite to Rauber's sickle) it induces a (pre)neural plate. The ingrowth of sickle endoblast into the endophyll (CALLEBAUT et al., 1999a) seems to be the histological basis by which the caudocephalic orientation of the neural tube and head region is directed towards the endophyll. So, Rauber's sickle material by its cell lineage (sickle endoblast) also influences neurulation at distance (in space and time). Hensen's node must be considered as a secondary major organizer, which becomes functional only after Rauber's sickle.

Already in 1913, SCHOUTTE proposed that the spacing of leaves in plants was the result of mutual inhibition of leaf primordia, such that each new leaf can only appear at a certain distance from the preceding one. Also WIGGLESWORTH (1940) interpreted the spacing of bristles on an insect and the insertion of new bristles in the largest interstices in this way. To account for the long range effect of small specialized regions and for the spatial continuity observed after many experimental interferences in early sea urchin development, hydroid regeneration and development of the chick limb, the "positional information" scheme was proposed by WOLPERT (1969, 1981). As during the spacing of leaves in plants, blastodermal cell groups of the same cell lineage (Rauber's sickle, junctional endoblast and sickle endoblast) have inhibiting effects on their like. So, usually in the limited surface of one and the same blastoderm (3 mm diameter), only one of them may retain its organizing capacities. That after (sub)total removal of the autochthonous Rauber's sickle still an embryo can develop, can be explained by different reasons : 1) the removal of the Rauber's sickle material was incomplete, so from the remnant of a sickle horn often a transversely oriented streak develops ; indeed the extent of the circumference of a complete Rauber's sickle is often considerably underestimated. We have shown that it is not only found in the caudal quadrant of the germ, but also fragmentarily in the whole periphery of the lateral quadrants (CALLEBAUT et al., 2000b) (Fig. 13A). Thus in cases of incomplete removal of Rauber's sickle and after further culture, junctional endoblast accompanied by blood islands will appear in the involved area ; 2) some sickle endoblast derived from the autochthonous Rauber's sickle has already migrated into the caudal part of the area centralis ; 3) signalling molecules secreted by the autochthonous Rauber's sickle before its removal have already diffused sufficiently far enough into the caudal part of the area centralis to start streak formation. Moreover the presence of endophyll in the area centralis can explain why also a neural plate can develop.

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Evolutionary trends in feminization and intersexuality in woodlice (Crustacea, Isopoda) infected with *Wolbachia pipientis* (*α-Proteobacteria*)

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ABSTRACT. Sex ratio distortion (SRD) and intersexuality are common phenomena among Isopoda (Arthropoda, Crustacea), caused by the feminizing action of the α-Proteobacterium *Wolbachia* (F) and/or an F DNA segment (f) integrated into the host genome, probably as a transposon. A dominant autosomal masculinizing allele (M) overrides the primary sex determinants (WZ: females; ZZ: males) and f but not F. The latter can be counterbalanced by the transmission suppressor polygenic system (R). The present study pursued a double object. (i) SRD was found in naturally isolated populations of three out of seven Belgian woodlouse species: *Oniscus asellus*, *Armadillidium vulgare* and *A. pulchellum*. They were (inter)sexed based on external and internal morphology, and specimens of the former two underwent PCR and/or microscopic detection of F. (ii) A deterministic but flexible model was set up to describe SRD changes in an initially F-infested *A. vulgare* population sampled during the last 25 years. Observed and predicted sex ratios fit best if natural transmission rates (i.e., in the absence of M and R) approach 100%, a condition fulfilled if f is carried on a multicopy transposon. Most often, such a population will then gradually lose its invader to the benefit of f. The stable end situation is a host population consisting of ZZ+f individuals of which 44% are neo-females (having mm), and 56% are reversed males (owing to Mm or MM). The model also explains the accelerated SRD evolution under variable degrees of F/f transmission.

KEY WORDS : Isopoda, feminization, intersexuality, sex ratio evolution, *Wolbachia*.

INTRODUCTION

(Abbreviations used: IR, intersex ratio; SR, sex ratio; SRD, sex ratio distortion)

Isopoda (Crustacea, Malacostraca) or woodlice, along with their relatives the Amphipoda, are the only crustaceans of which strictly terrestrial species exist. About 4000 out of a total of 8000 isopod species described are terrestrial; 33 of these belong to the Belgian fauna, which was the starting point of this study.

Sex seems to be determined rather uniformly on a hormonal basis throughout the Crustacea (LEGRAND et al., 1987). However, more than 40% of the terrestrial woodlouse species have been reported to display sex ratio distortion (SRD) towards the female sex (JUCHAULT & LEGRAND, 1989; JUCHAULT et al., 1994; BOUCHON et al., 1998) whereas most aquatic counterparts have a sex ratio (SR) close to 1:1. SRD and intersexuality in isopods have been ascribed to the feminizing action of an obligately-intracellular and maternally-inherited bacterium originally denoted F (MARTIN et al., 1973) but later determined as the α-*Proteobacteria* member *Wolbachia pipientis* (ROUSSET et al., 1992), and also to a wolbachial DNA fragment: f (JUCHAULT et al., 1992). The latter may be unstably integrated into the host genome, maybe as a transposable element; therefore, unlike F it can be partly paternally inherited (JUCHAULT & MOCQUARD, 1993) and so introgress into populations harbouring F (GRANDJEAN et al., 1993).

F can be horizontally transmitted by transplanting infected organs (LEGRAND & JUCHAULT, 1970) or by inoculation (JUCHAULT & MOCQUARD, 1989), whereas f cannot spread horizontally (LEGRAND & JUCHAULT, 1984). Neither can it be eliminated, unlike F, by antibiotics or temporarily elevated temperatures (JUCHAULT et al., 1980b; RIGAUD et al., 1991a, 1997; RIGAUD & JUCHAULT, 1998). A strikingly similar temperature-dependent SRD effect in Amphipoda results from the action of parasitic Microsporidia (GINSBURGER-VOGEL & CARRÉ-LÉCUYER, 1976; BULNHEIM, 1978; GINSBURGER-VOGEL et al., 1980; GINSBURGER-VOGEL, 1991).

The longest and best documented Isopoda case is *Armadillidium vulgare* (LEGRAND & JUCHAULT, 1969; MARTIN et al., 1973; JUCHAULT & LEGRAND, 1976, 1981a,b; JUCHAULT et al., 1980a; RIGAUD et al., 1991b). Here, the female:male ratio in an F-infested population frequently exceeds 10:1. Genetic females are heterogametic WZ, males are homogametic ZZ (JUCHAULT & LEGRAND, 1972). Transformation into so-called neo-females usually takes place in an early embryonic stage: the parasitic sex

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factors inhibit the expression of male genes carried by the Z heterochromosome, thereby preventing the growth of the androgenous gland, which would induce the development of the male gonads (SUZUKI & YAMASAKI, 1991, 1997) through secretion of androgenic hormone (MARTIN et al., 1999).

In some populations SRD is less dramatic. This may result from three possible factors. Firstly, the onset of F expression may occur later in ontogeny, giving rise to incompletely feminized intersexes of one of two categories (MARTIN et al., 1973; LEGRAND et al., 1974): iF having a functional female phenotype with vestigial gonopods, and iM being sterile male-like individuals with reduced gonopods and gonads. There is growing evidence that the dominant allele of an autosomal masculinizing gene (M) slows down *Wolbachia* proliferation (RIGAUD & JUCHAULT, 1993), hence the postponed impact of F. Sometimes this may even happen in the absence of M, albeit in a much less pronounced manner. Secondly, f as well as the primary W female sex determinant, is overruled by M, thus masculinizing even genotypic females provided they are not infected by F (RIGAUD & JUCHAULT, 1993). Moreover, the genetic constitution WZMm may lead to yet another intersex type if the effect of M is delayed: a functional male possessing also two female genital orifices (denoted M_{og} throughout this paper) (LEGRAND et al., 1974; JUCHAULT & LEGRAND, 1976). Furthermore, it is especially M that allows a high paternal transmission rate of f (JUCHAULT et al., 1992). Thirdly, a polygenic "Resistance" (R) system limits the transovarial transmission of *Wolbachia* to offspring. Such infected mothers no longer produce a female-skewed F_1; on the contrary, a few of them are overwhelmingly male-biased, a phenomenon referred to as "ARF (ARrhenogenous Female) trait" (LEGRAND & JUCHAULT, 1972; RIGAUD & JUCHAULT, 1992).

Only in wild infected populations can M, and presumably also R mechanisms, be observed (RIGAUD & JUCHAULT, 1993). Their selection is a naturally genetic response counteracting the selfish wolbachial genes in the struggle for maximal transmission. As a result, males are saved from extinction. An overview of the complex and concurrent sex determination systems in *Wolbachia*-infected woodlice is shown in Fig. 1; the R genes are omitted since these exert only indirect influence on sex development.

The major aim of this work is to examine how far the M and R systems support recovery of the male sex in nature. To achieve this, we resorted to two complementary approaches. (i) We screened populations of seven common Belgian woodlouse species in the suborder Oniscidea for SRD and infections, and compared our results for Belgium with those obtained by BOUCHON et al. (1998) mainly for France. (ii) In the wake of RIGAUD et al. (1992), JUCHAULT et al. (1993), and CAUBET et al. (2000), we present a new mathematical model for the SR changes in *A. vulgare* populations starting from infestation by F. It takes account of

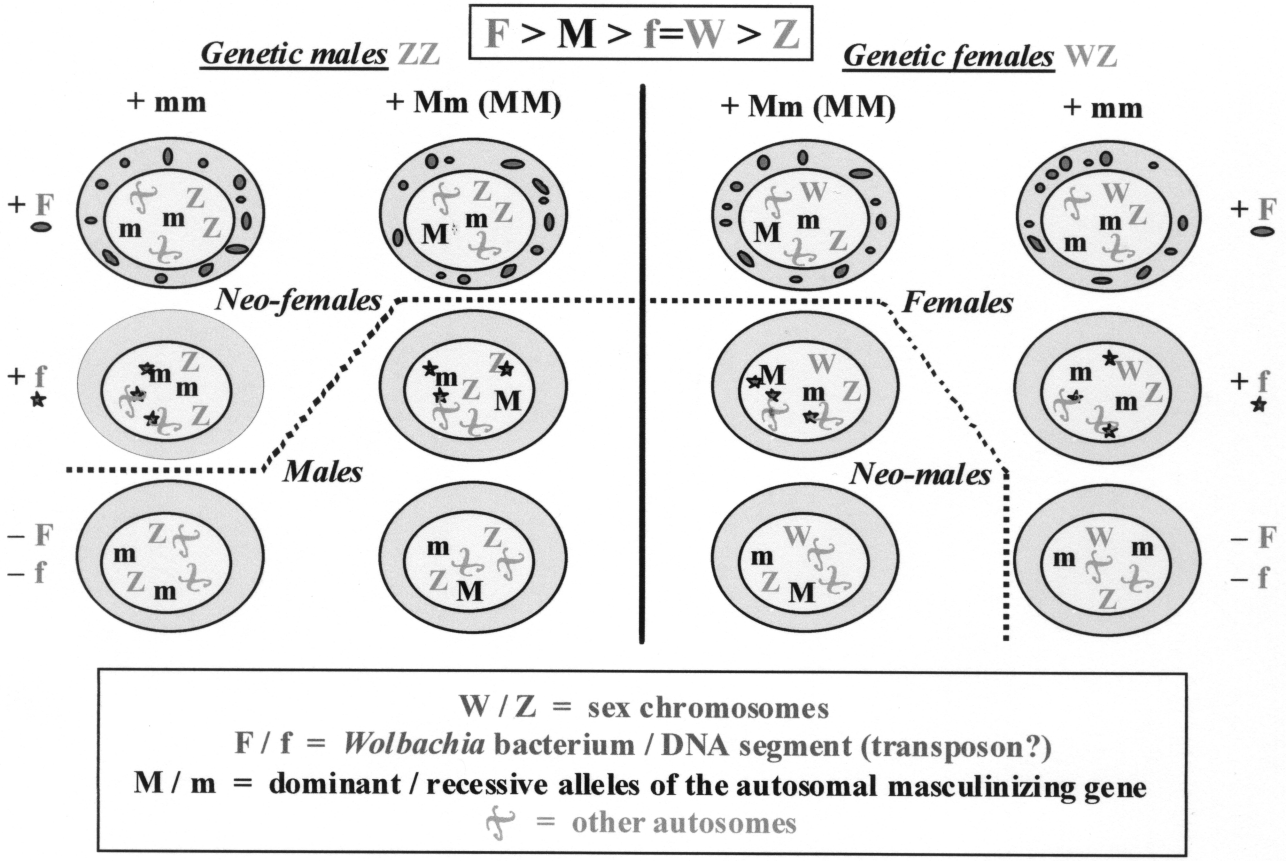

Fig. 1. – Scheme of the concurrent sex determination systems in *A. vulgare*. Note that only three out of six classes of phenotypic males are retained on the whole (but no more than one out of four in the presence of wolbachial factors), whereas only two (one, respectively) classes of genotypic females can be transformed to phenotypic neo-males if M is present. The hierarchical (= epistatic) order of influence of the different factors is also shown.

the M and R genes in addition to f and F, and it is discussed in the context of our observations in nature.

MATERIAL AND METHODS

Isopods

The isopod species were determined using the key of HOPKIN (1991). Populations investigated are displayed in Table 1, along with total numbers of manually-collected individuals, the date of collection (including old samples in addition to recent ones) and their SR. The limit for SRD was arbitrarily drawn at 40% males. The specimens caught before 1995 were fixed and preserved in 70% ethanol with added glycerine, whereas those from 1995 onwards were kept alive upon capture until further specific treatment (see below). They were (inter)sexed by the shape of the endopodites of the first two pleopod pairs, which grow into copulatory organs in males, and/or by internal genital morphology.

Consistently hand-collected samples avoided all noise variation in recorded SR but that originating from the time of the year the isopods were caught. Males are the more active, more exposed sex during the reproductive period (DANGERFIELD & HASSALL, 1994), the start and duration of which depend largely on the latitude with its characteristic photoperiod (for Belgium: May to September) (SOUTY-GROSSET et al., 1994). Thus, especially the observations from May might be an overestimate, and those from autumn or winter a slight underestimate of the real SR. In order to avoid this source of background noise as much as possible, animals were dug out from various depths of sheltered areas and additionally, we carefully rummaged through the soil surface for active individuals.

TABLE1

Isopod species, populations, sampling dates, total numbers of adult individuals and SR (expressed as % males). SRD cases (< 40% males) are typed in boldface.

Species	Populations	Date	#	SR (%)
Armadillidium opacum (Armadillidiidae) (Koch, 1841)	Sint-Genesius-Rode: Zoniënwoud	09/1997	54	51.9 (-)
A. pulchellum (Armadillidiidae) (Zencker, 1798)	Lanaken: Gellikerheide	08/1997	200	**28.5**
		09/1997	73	**28.8**
A. vulgare (Armadillidiidae) (Latreille, 1802)	Nieuwpoort: military domain	05/1977	136	**22.0**
		05/1979	308	**16.9**
		05/1980	132	**25.0**
		10/1980	169	**23.7**
		01/1981	145	**30.3**
		11/1995	100	**34.0** (F)
		11/1997	194	41.8 (F)
	Gent: university, botanical garden	11/1995	196	54.1 (-)
		11/1997	136	48.5 (-)
Ligidium hypnorum (Ligiidae) (Cuvier, 1792)	Waasmunster: spring forest	05/2000	34	41.2 (-)
Oniscus asellus (Oniscidae) Linnaeus, 1758	Waasmunster: spring forest	05/2000	96	**22.9** (F)
	Gent: Bourgoyen nature reserve	05/2000	16	43.8 (-)
Philoscia muscorum (Oniscidae) (Scopoli, 1763)	Nieuwpoort: military domain	03/1980	540	46.9
		10/1980	348	60.9
	Waasmunster: spring forest	05/2000	70	40.0 (-)
Porcellio scaber (Porcellionidae) Latreille, 1804	Nieuwpoort: military domain	03/1980	203	46.3
		10/1980	275	51.6
	Waasmunster: spring forest	05/2000	96	44.8 (-)

(F) Feminizing *Wolbachia* detected by means of DAPI fluorescence microscopy and/or PCR
(-) No F-like bacteria found either by DAPI or PCR; see Table 2 for examples of more detailed (inter)sex analysis

PCR detection of *Wolbachia*

A. vulgare wolbachial DNA was detected as follows. All glassware was sterilized before use. The animals were anaesthetized and surface-sterilized through a three-step 96% ethanol bath (5 min each, followed by rinses in a sterile 0.85% [w/v] NaCl solution), and fixed 30 min in freshly made 50% (v/v) acetic acid. While animals were submerged in the final rinsing solution, the bilateral V-shaped fat bodies and the reproductive organs were dissected out with fine glass needles. Care was taken not to puncture the gut containing a contaminant flora. Whole DNA from the separate organs was prepared and intracellular bacterial 16S rRNA genes PCR-amplified. To determine the presence of *Wolbachia*, partial sequencing of the 16S rDNA was done using two conserved primers: one forward (*Escherichia coli* positions 339-358) and one reverse (*E. coli* positions 536-519). For details on molecular methods, see VANDEKERCKHOVE et al. (2000).

Fluorescence microscopy

The woodlice were anaesthetized, surface-sterilized, fixed, rinsed and dissected as described above. For details on DAPI (4',6-diamidino-2-phenylindole) fluorescence microscopy, see COOMANS et al. (2000) and VANDEKERCKHOVE et al. (2002).

Detection by the DAPI method proved to outperform conventional PCR trials; therefore, most animals were examined using DAPI alone. Although it is not a specific stain, *Wolbachia*-like bacteria could be routinely distinguished by their morphological characteristics (cells are often extremely small, not more than 300 nm, with a marked pleomorphy) once 16S rDNA sequence analysis had unambiguously confirmed the identity of *Wolbachia pipientis* in the organs concerned.

Evolutionary ecological modelling

A simple deterministic model was set up to describe short-term SRD evolution in *A. vulgare*, starting from an F-infested population such as the one from Nieuwpoort in 1977. It must be pointed out that the evolution from the uninfected state (SR 1:1) over F invasion and subsequent sweep to heavy SRD is not applicable here because this part of the micro-evolutionary SR curve obeys quite different dynamics. To test the model on the male recovery side, its mathematical function was cast into a Turbo Pascal program. A whole gamut of parameter values was entered, including figure combinations under several realistic circumstances, and a computer-simulated course of evolution yielded an outcome of the model that could be compared to what was observed in the field.

RESULTS AND DISCUSSION

Prevalence of *Wolbachia* and SRD

From Table 1 it is obvious that SRD exists in *Armadillidium vulgare* (Nieuwpoort), *A. pulchellum* (Lanaken) and *Oniscus asellus* (Waasmunster), as opposed to the remainder of the populations and species included. Over the past 25 years the SRD rate in *A. vulgare* (Nieuwpoort) tended to decrease: the latest survey was even unbiased. Although the 16S rDNA of the *Oniscus asellus* F-like bacteria was not sequenced, it was considered *Wolbachia pipientis* on the grounds that the host behaved exactly like others previously disclosed to harbour *Wolbachia* (BOUCHON et al., 1998), and the bacterial cells had a typically wolbachial size and shape. SRD in *A. pulchellum* was not further studied, but it is most likely that it would be *Wolbachia*-regulated, too.

General observations as to the infection status are shown in Table 1 and Fig. 2. Note that in *A. vulgare*, as opposed to *O. asellus*, the bacteria are better traceable in the fat bodies rather than the adjacent ovaries, a conclusion consistent with that of DOBSON et al. (1999). However, especially in woodlice R gene activity can also underlie the poor, if any, conspicuousness of F within the oocytes.

Detailed external and internal morphological analyses were made of the intersexes (iF, iM and M_{og}) in *A. vulgare* and *O. asellus*. Animals of different categories were tested for cytoplasmic infection of fat bodies and reproductive organs by means of the DAPI fluorescence microscopical method. The results are schematized in Table 2. Interestingly, the fat bodies and the testes of two out of six Waasmunster *O. asellus* males harboured the feminizing endosymbiotic bacteria, albeit at visibly lower densities than females. This is concordant with the results from BOUCHON et al. (1998), where three out of ten males and six out of 13 females were positive for *Wolbachia*. It may seem quite astonishing that functional males be infected with feminizing *Wolbachia*; however, this condition was also observed in two other woodlouse species, *Chaetophiloscia elongata* and *Porcellionides pruinosus* (JUCHAULT et al., 1994). This implies a strong conflict between host and bacterial genomes to restore the male sex.

Fig. 3a shows a.o. the observed course of SRD over the past 25 years in Nieuwpoort *A. vulgare*. This population has a lot in common with the one from Niort in France (JUCHAULT et al., 1980a, 1992). Its 1977 SR of approximately 20% males reflects a sweep by F shortly beforehand. In the following years the SR increased gradually to just exceed 40% males by 1997. As was confirmed by experimental data (Table 2), F infection at that point was not at all that frequent any more. This implies that transmission suppressors (R) and/or male restorers (M) had arisen, whether or not along with the occasional transition of F to f and consequent partial loss of F.

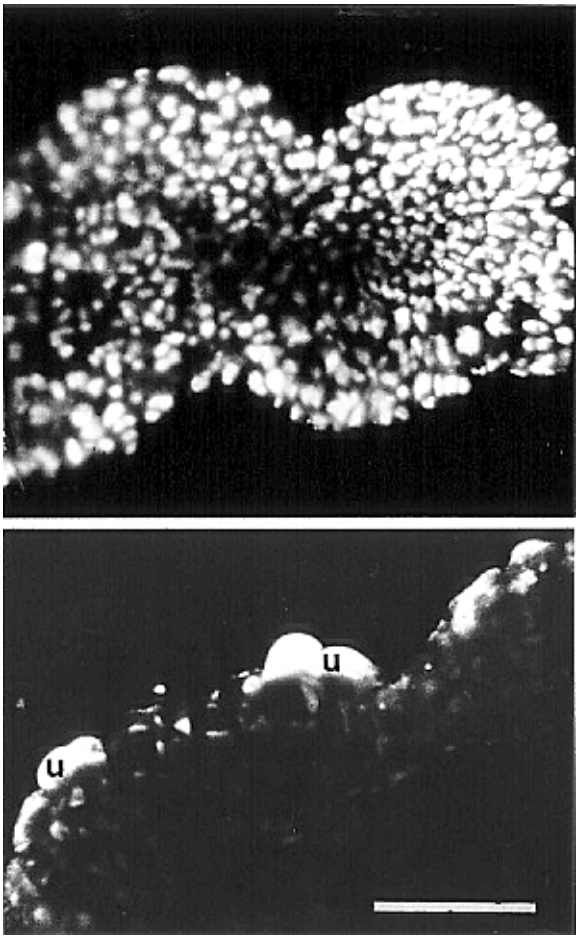

Fig. 2. – DAPI fluorescence micrographs of an uninfected (upper) and an infected (lower) *A. vulgare* fat body. u, ulcer containing numerous intracellular F bacteria appearing as intensely fluorescent clouds at low magnification. The remainder of the objects are host cell nuclei. Bar, 50 μm.

TABLE 2

(Inter)sex compositions and infection levels as determined by the DAPI assay. Fractions of examined animals that were infected by F are given between parentheses. # females excluding iF and iM; # males excluding Mog.

Species	Populations	Date	SR (%)	# females	# iF	# iM	# Mog	# males
A. vulgare	Nieuwpoort	11/1995	34.0	66 (8/18)	2 (1/2)	0	0	34 (0/9)
		11/1997	41.8	100 (3/8)	10 (1/10)	3 (1/3)	0	81 (0/3)
	Gent	11/1995	54.1	90 (0/20)	2 (0/2) †	0	0	106 (0/20)
		11/1997	48.5	67 (0/8)	2 (0/2) †	1 (0/1) †	0	66 (0/2)
O. asellus	Waasmunster	05/2000	22.9	74 (3/6)	1 (1/1)	0	0	22 (2/6)
	Gent	05/2000	43.8	9 (0/6)	0	0	0	7 (0/6)

† All F-uninfected but positive for female intersexes indicates that f is the feminizing factor in Gent; see conclusion section for further explanation on stable equilibrium SR upon recovery from infestation by F

What evidence do the 1997 and other observations provide for these conflicting factors? When carefully contemplating the crossing experiments of RIGAUD & JUCHAULT (1993), one can conclude that the intersex ratio (IR) among female phenotypes (= iF + iM / "normal" females + iF + iM) is <2% in the absence of M, whereas involvement of M in all of the crosses is associated with an IR of 26-46%. In addition, M is more frequent in F/f mixed populations (about 30%) (JUCHAULT et al., 1992) than f-only populations (<10%) (JUCHAULT & LEGRAND, 1981b) since both sexes can transmit M in the former. Extrapolating these percentages to the Nieuwpoort pill bugs in 1997, having an IR of 14% (deduced from Table 2) means that M played a role in roughly 40% of the matings. Thus, the prevalence of M can be estimated to be about 20%; in other words, the study was dealing with a mixed F/f population with preponderating f. The M_{og} phenotype as direct evidence for the M allele was not seen. M_{og} is very rare in nature indeed; even iM usually accounts for <3% of the intersex total (RIGAUD & JUCHAULT, 1993). On the other hand, R gene activity was directly evidenced by the absence of F bacteria in the gonads whereas they did populate the fat bodies.

The summary of the micro-evolutionary SRD traject of Nieuwpoort *A. vulgare* would then look like this. Beginning at a point where F fleeced the population, the R system arose. Soon after the initial transition from F to f, the origin and expansion of M was favoured in the mixed F/f population. The ensuing decrease in SRD brought about an even further reduction of F to the benefit of f, so that by 1995 the SR had gone up to 30-40% males while still growing. In 1997 the population harboured maybe more f and M than F, and the IR was typically over 10%, again in agreement with the Niort population at a corresponding stage (JUCHAULT et al., 1992).

Evolutionary ecological model

The following mathematical model intends to describe the changing SR in an *A. vulgare* population starting at a stage of infestation by F but not f. In such a population, all individuals are genetic males (ZZ) and the female sex is determined by F and f only. It was assumed that (i) neither F nor f have any fecundity effect on the isopods (demonstrated by RIGAUD et al., 1999), (ii) there is no fertility cost in phenotypic females, (iii) the woodlice reproduce once per year with discrete generations, a condition normally fulfilled in Northwestern Europe. Let F_t be the proportion of ZZ+F neo-females, and f_t the proportion of ZZ+f neo-females at time (or generation) t. Then, for the next generation, the following relationships can be expressed:

$$F_{t+1} = T_F F_t.(1 - R_t).(1 - I) \qquad (1)$$

and

$$f_{t+1} = (I.F_t + T_f f_t).(1 - M_t) \qquad (2),$$

R_t representing the instantaneous decrease in F transmission induced by the R polygenic system, I standing for the extent of integration of the wolbachial DNA segment f into the host cell nucleus before loss of the F factor, T_F and T_f being the natural transmission rates (i.e., without any counterweight on the part of the host) of F and f, respectively, and M_t the instantaneous frequency of the M allele causing a decrease in ZZ+f neo-females due to masculinization. Once R and M have originated, their frequencies can be estimated to evolve in concert with F_t and f_t, respectively:

$$R_t \cong I_F^t .(F_0 - F_t) \qquad (3)$$

and

$$M_t \cong T_f^t .(f_t - f_0) \qquad (4).$$

However, substituting (3) and (4) into (1) and (2) yields an unrealistic model in which all situations lead to a heavily male-biased outcome within few generations. Likewise, neo-females are quickly outnumbered if T_F or T_f are not equal to 100%, despite previous estimates for both not exceeding 85% (RIGAUD et al., 1992; but see remarks below). Partial replenishment of the female sex could be accounted for by introgression of (neo-)females from neighbouring populations, but in the case of the fairly isolated Nieuwpoort population this can hardly suffice to warrant persistence of neo-females at the levels observed. Apparently, other conditions are needed to explain the continuity of F/f in Nieuwpoort *A. vulgare*. It is not exceptional to find transovarial transmission rates of 100% among F-like bacteria, e.g. in parasitoid wasps (HUIGENS et al., 2000) and dagger nematodes (COOMANS et al., 2000; VANDEKERCKHOVE et al., 2002). There is reason to believe

that the above T_F of 0.85 is not the natural F transmission rate: on the one hand because it was derived from artificial – not wild – populations stemming from a selection of ten year inbred ZZ+F lines (these were known to lack M, though more importantly, absence of R was not guaranteed), and on the other hand because it is actually termed "primary transmission rate" and represents the aggregate of most mothers transmitting at virtually 100% and a few with very weak transmission (RIGAUD et al., 1992). The latter could be elicited by switching on R expression, and then 0.85 must not be considered the natural F transmission rate indeed. Likewise, the primary T_f would vary a great deal between 0.53 and 0.80 in an artificial ZZ+f population, but a natural T_f close to 1.00 is expected if the transposable element f copies itself without hindrance to several other loci in the host genome. Therefore, we do let T_F and T_f approximate 1.00 in (3) and (4). Moreover, we let R_t and M_t depend differently upon F_t and f_t to obtain the new relationships:

$$R_t \cong F_t - F_{t+1} \qquad (5)$$

and

$$M_t \cong f_{t+1} - f_t \qquad (6).$$

These assumptions can be made on the grounds that R and M are the principal factors responsible for the sink in ZZ+F and ZZ+f neo-females, respectively. The spread of R lags behind as compared to the behaviour of F, whereas M evolves ahead of *f*, thereby determining much of the ZZ+f increase to the detriment of F. Substitution of equation (5) into (1), and (6) into (2) yields, upon rearrangement:

$$F_{t+1} = \frac{1 + (I-1).F_t^2}{1 + (I-1).F_t} - 1 \qquad (7)$$

and

$$f_{t+1} = \frac{T_f.f_t^2 + (T_f + I.F_t).f_t + I.F_t}{1 + I.F_t + T_f.f_t} \qquad (8),$$

so that finally, the SR in generation t+1 can be expressed as the fraction of males by subtracting the fractions of both classes of neo-females from 1:

$$SR_{t+1} = 1 - (F_{t+1} + f_{t+1}) \qquad (9).$$

In 1977 (t = 1) the Nieuwpoort *A. vulgare* SR was calculated to be 22.0% but in reality it was probably lower since two years later it was as low as 16.9%. Therefore, we let e.g. SR_0 = 10%; this means $F_0 + f_0 = 0.90$. In view of the very low SR, most of the females at that time must have been ZZ+F with only a minor fraction being ZZ+f. As such, $F_0 = 0.82$ and $f_0 = 0.08$ would be good starting values to enter, but even if we let F_0 vary between 0.60 and 0.90 (f_0 between 0.00 and 0.30) this has only a minor influence on the issue (Fig. 3a). The same is true for SR_0 (Fig. 3a). Thus, the parameters of importance are *I* and T_f.

The predictions from the model with a range of values for *I* and T_f are summarized in Figs 3b and 3c. *I* is expected to be very low: when the wolbachial DNA segment f is incorporated into the host nucleus, the bacterium itself usually remains and overrules f as long as R is not involved to reduce the vertical transmission of F. The higher *I*, the sooner the equilibrium SR is attained (Fig. 3b). Further-

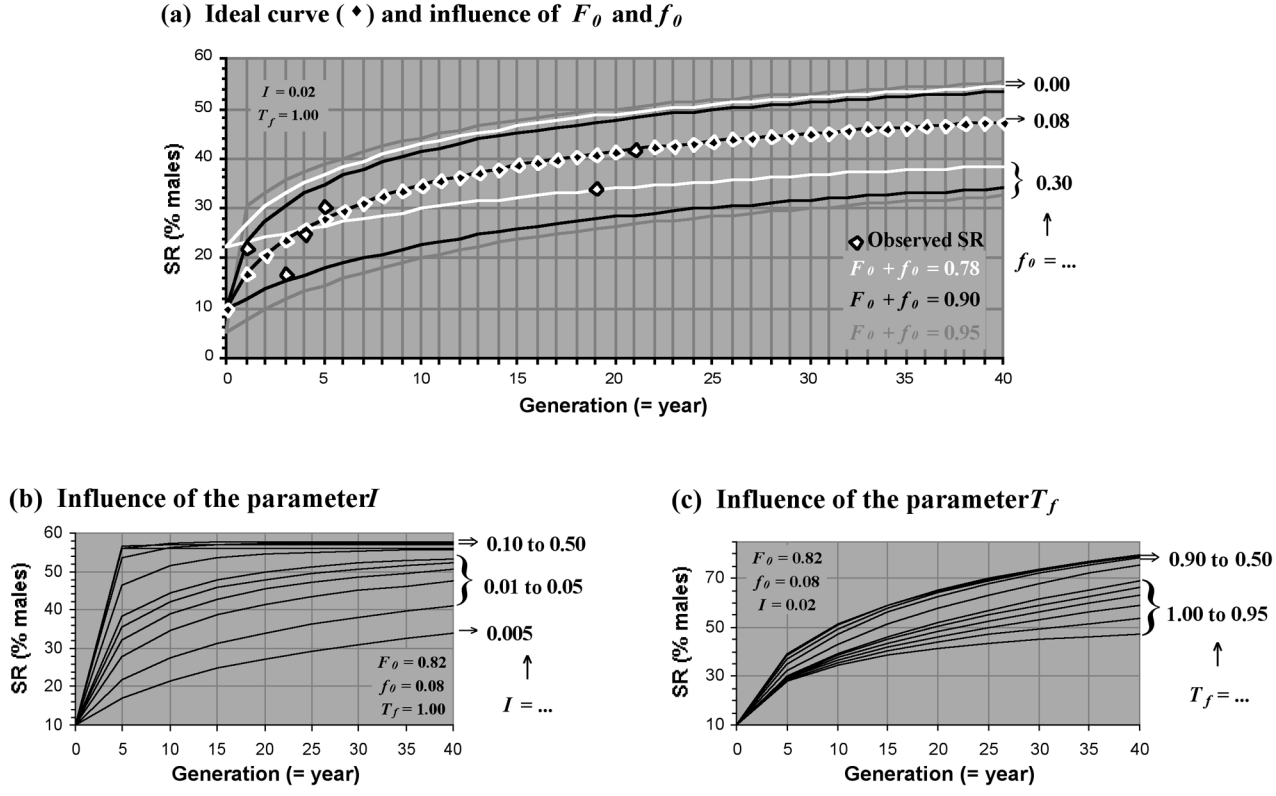

Fig. 3. – Behaviour and predictive power of the deterministic model (described by equations 7-9 in this report) under a range of realistic circumstances.

more, $T_f = 1.00$ yields results that harmonize with the reality of the Nieuwpoort pill bugs. $T_f < 1.00$ (e.g., if f remains a low-copy transposable element) causes an enormous acceleration of the SR evolution with extensive masculinization (Fig. 3c), consistent with the experiments of RIGAUD et al. (1992). It should be pointed out, though, that the present model loses in accuracy under accelerated circumstances. On the whole, $T_f < 0.85$ is attended with relative persistence of ZZ+F instead of ZZ+f neo-females regardless of the presence of R. In the special situation where $0.85 < T_f < 1.00$, ZZ+f neo-females gain in numbers for up to several tens of generations and seem to hold quite comfortably, but this is a pseudo-equilibrium: they fade away eventually after a few hundreds of generations. By that time the whole population would have become male, and have disintegrated consequently.

Altogether, there are several rather well-fitting parameter combinations, the best values being also realistic ones, including the natural transmission rates of F and f that may indeed equal 1.00. This proves the applicability of the deterministic model under the given circumstances in the field. Nevertheless, an inconsistency can be found in that the theoretical proportion of ZZ+F neo-females after 21 generations (using the parameter values from the "ideal curve" in Fig. 3a) equals 33.3%, implying that *Wolbachia* should have been discovered in about 50% of the phenotypic females, whereas the DAPI assay diagnosed only five positive cases out of 21 (Table 2). This is thought to result from overlooking poor infection levels. By contrast, half of the Waasmunster *O. asellus* females were infested, indeed (Table 2).

Outcome of the model – conclusions

What does the model predict for the further evolution of the SR in Nieuwpoort *A. vulgare*? Let us take a look at the following data, computed upon incorporation of the "ideal curve" parameters from Fig. 3a: $SR_{100} = 52.6\%$, $F_{100} = 5.0\%$, $f_{100} = 42.4\%$; and still further: $SR_{200} = 54.0\%$, $F_{200} = 0.6\%$, $f_{200} = 45.4\%$. Clearly, there is a tendency for f_t to increase along with SR_t while F_t decreases continually. It can be proved that the limits for SR_t, F_t and f_t are reached after 326 generations and equal 56.0%, 0.0% and 44.0%, respectively. In such a population, the numbers of both sexes are approximately alike but all females are genetic males carrying the f segment and not M. Most males also carry f, but it is overridden by M which nonetheless enables its transmission to offspring. An *A. vulgare* population where all individuals are genetic males with M and f as principal sex determinants can most probably be found in Ghent: here, the pill bugs still show vestiges of intersexes but not a single specimen was infected by F (Table 2), a status reconcilable only with f's prevalence at a stable equilibrium SR of theoretically 56% (already reached in 1995 – see Table 1). In other words, the chromosomal sex determination system has switched from W/Z to f/M. CAUBET et al. (2000) illustrated nicely how such switches can occur consecutively under external forces by which the sex of organisms is manipulated uninterruptedly. The other F-negative entries in Table 1 were not further investigated, but it is not impossible that some of them share this property with *A. vulgare* from Gent.

The power of this model lies in its simple truth to nature and its flexibility. It describes how *Wolbachia* can either persist for a prolonged period or in many instances be driven out systematically in the long term, often but not always taken over by the wolbachial segment f, the latter being less strongly feminizing in that it is overruled by the masculinizing factor M though taking advantage of the latter's favourable company through the hitchhiking effect (MAYNARD-SMITH & HAIGH, 1974). Hence, it appears that fixation of f is easier than that of F. Similar conclusions were drawn by JUCHAULT et al. (1992) and RIGAUD et al. (1992).

More concretely, this model emphasizes that: (i) SRD evolution is at least in *A. vulgare* reduced to a subtle game between transition (= *I* throughout this report) of *Wolbachia* to a transposable element and the transmissible copy number (= transmission potential, or T_f) of the latter in the host genome; (ii) this f transmission rate is decisive as to whether ZZ+f females will thrive and attain an (either stable or pseudo-) equilibrium, or go extinct quicker than F, with a critical T_f for pseudo-maintenance around 0.85; (iii) the F-to-f transition rate matters mainly to the speed with which the end condition is reached, not to this condition itself; (iv) the initial abundances of both *Wolbachia* (= F_0) and the wolbachial transposon (= f_0) have only a slight influence on the outcome of SR evolution.

The weakness of the model is its inaccurate representativeness in situations where the transmission potential of the wolbachial transposon deviates significantly from 100%, or more generally, under accelerated evolutionary circumstances: the terror of many mathematical models. Besides, the course of an SR curve – however determinative or predictive it may seem – can be disrupted at any point in time by a new invasion, e.g. by a *Wolbachia* strain resistant to the R genes. The ensuing arms race would then comply with entirely different mathematics until the sweep is near completion. By no means do the formulae introduced here advocate a neatly ordered series of events in infected isopods, yet it would be rewarding to see if they can be successfully applied to other populations or further refined from studies of other species (see Table 1 for some good candidates).

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APPENDIX

The Turbo Pascal program implementing the mathematical model in this study. Note that syntax rules do not allow every variable and parameter denomination to be the same as in the discussion text.

```
program WOODLICE (input, output);
uses crt;
var
  Bo, Bt, fo, ft, I, Tf, SEXRAT_M : real;
  Z, ITERATIO : integer;
begin
  clrscr;
  writeln ('  Deterministic model describing sex ratio');
  writeln ('   changes in Wolbachia-infested woodlice');
  writeln;
  writeln ('-----------------------------------------------');
  writeln ('Ft = Fo.(1 - Rt).(1 - I)      [Rt = Fo – Ft]');
  writeln;
  writeln ('ft = (I.Fo + Tf.fo).(1 - Mt)  [Mt = ft – fo]');
  writeln;
  writeln ('SRt = 1 – (Ft + ft)');
  writeln ('-----------------------------------------------');
  writeln;
  writeln ('Enter the values for parameters I and Tf successively: ');
  read (I, Tf);
  writeln ('How many iterations? : ');
  read (ITERATIO);
  writeln ('Start values for Fo and fo? : ');
  read (Bo, fo);
  writeln;
  writeln ('Sex ratio / Ft / ft after iteration');
  for Z := 1 to ITERATIO do
    begin
      ft := (Tf*sqr(fo)+(Tf+Bo*I)*fo+Bo*I)/(1+Bo*I+Tf*fo);
      Bt := ((1+(I-1)*sqr(Bo)/(1+(I-1)*Bo))-1;
      SEXRAT_M := 1-Bt-ft;
      write (Z);
      write (' : ');
      writeln (SEXRAT_M:3:3, '   ', Bt:3:3, '   ', ft:3:3);
      fo := ft;
      Bo := Bt
    end;
  repeat until keypressed
end.
```

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Mono- and oligophagous *Phyllotreta* (Coleoptera: Chrysomelidae) species: the relation between host plant range and genetic diversity

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ABSTRACT. The niche-width variation hypothesis predicts a positive correlation between niche width and genetic variability. Here we evaluated this hypothesis by comparing genetic variability in four monophagous (narrow niche) and six oligophagous (broad niche) species of the beetle genus *Phyllotreta* Chevrolat, 1837 (Coleoptera: Chrysomelidae: Alticinae). The results, obtained over a similar geographic range, demonstrate that for all genetic variability measures used (average number of alleles per locus, percentage of loci polymorphic, observed heterozygosity and expected heterozygosity under Hardy-Weinberg conditions) the monophagous species showed a significantly higher genetic variability compared to the oligophagous species. This result is at variance with the niche-width variation hypothesis.

KEY WORDS : Niche-width variation hypothesis, allozymes, genetic variability, phytophagous insects.

INTRODUCTION

In 1965 VAN VALEN postulated the "niche-width variation hypothesis" stating that "wider niches would permit greater phenotypic variation if this variation is controlled to a significant extent by the adaptive diversity of the niche". Although in his original definition Van Valen does not talk about genetic variation, in later studies the hypothesis was mainly interpreted in a genetic context (SOMERO & SOULÉ, 1974; STEINER, 1977; LACY, 1982; LAVIE & NEVO, 1986; NOY et al., 1987) predicting a positive correlation between niche breadth and genetic variability. Several studies testing the hypothesis have been performed and almost all of them confirm it (e.g. BABBEL & SELANDER, 1974; STEINER, 1977; LAVIE & NEVO, 1981; 1986). The most crucial factor in testing the hypothesis is of course the definition of the niche, and the ability to find a group of closely related organisms in which one subgroup clearly has a broader niche than the other. Although niche components such as geographic (e.g. BABBEL & SELANDER, 1974; LAVIE et al., 1993) or physiological range (NOY et al. 1987) are often used in studies of this kind, it is difficult to really quantify them as niche parameters. In the same way NEVO et al. (1984) compared genetic variation between habitat specialists and generalists over 669 species (literature data) and found more genetic variation in generalists for vertebrates, *Drosophila* and molluscs, and (depending on the combination of categories used to define generalists) also for insects. Several studies testing the hypothesis compared genetic variability between organisms belonging to different genera (e.g. SOMERO & SOULÉ, 1974; MITTER & FUTUYMA, 1979; LACY, 1992; LAVIE et al., 1993). As the amount of genetic variation can differ greatly between animal groups it is preferable only to compare closely related organisms, differing mainly in clearly quantifiable niche components. LAVIE et al. (1993) stated that although hitherto the niche-width variation hypothesis has been supported by many studies, additional critical tests are imperative.

A group of animals that seems to be ideally suited for testing the hypothesis is found in the phytophagous insects. The niche of a phytophagous insect is mainly determined by the number of host plant species it is able to live on. The range of host plants of an insect species will depend on both chemical and morphological characteristics of the plants concerned. Co-evolution, chemical adaptation, fitness, predation and parasitism rates all may have their influence on the effective host plant spectrum (MATSUDA, 1988; SOETENS et al., 1991; JERMY, 1994; METCALF, 1994). Host-associations are the results of complex interactions between the plants' defensive systems and the insects' possibility to survive and reproduce on them (HSIAO, 1969; SIEMENS & JOHNSON, 1990), reaching a climax of adaptation in those insects that are even capable of sequestration of plants' defensive chemicals for use as their own defensive secretions (PASTEELS et al., 1988a, 1988b 1994; ROWELL-RAHIER et al., 1991; PASTEELS, 1993).

Within the phytophagous insects, some genera contain only generalist species, while in others specialisation (on often unrelated plant species) seems to be the rule. However, within certain groups of closely related species, both specialists and generalists are found. These groups provide an excellent opportunity to study the above hypothesis. To our knowledge only two studies testing the hypothesis (and showing contradicting results) on phytophagous insects (MITTER & FUTUYMA, 1979; LACY, 1982) have been performed, unfortunately both using comparisons between species of different genera.

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Within the beetle family Chrysomelidae, the genus *Phyllotreta* (also known as the cruciferous flea beetles) contains both mono- and oligophagous species, all feeding within the plant family of the Brassicaceae or the related families Resedaceae and Caparidaceae (Mohr, 1966; Nielsen, 1978; Doguet, 1995). The only exception in this genus is *Phyllotreta vittula* (Redtenbacher, 1849), which, besides accepting various Brassicaceae, also feeds on grasses and cereals (Kostromitin, 1973; Vig, 1998a, 1998b). Ecology and host plant relationships of several *Phyllotreta* species have been studied extensively (Nielsen, 1978; 1988; Nielsen et al., 1979a 1979b; Lamb & Palaniswamy, 1990; Palaniswamy & Lamb, 1993; Palaniswamy & Bodnaryk, 1994; Milbrath et al., 1995). Both larvae and adults live on the same plants, indicating a close relation between host and parasite. Larvae feed externally on the roots (e.g. *P. cruciferae* (Goeze, 1777), *P. consobrina* (Curtis, 1837)) or mine the leaves (e.g. *P. tetrastigma* (Comolli, 1859), *P. flexuosa* (Illiger, 1794). Cruciferous plants contain several defensive chemicals to protect them from insects, and even inhibit growth of other plants (Bodnaryk, 1991; Dimock, et al. 1991; Ghaout et al., 1991; Bodnaryk, 1992; Yamane et al., 1992). Acceptability of a plant for a *Phyllotreta* species may depend on the presence and/or absence of certain chemicals, which can act as attractants or deterrents (Feeny *et al.,* 1970; Hicks, 1974; Nielsen, 1988; Bodnaryk, 1991,1992), and certain morphological characteristics such as pubescence of the leaves, stems and pods (Lamb, 1980; Palaniswamy & Lamb, 1993; Palaniswamy & Bodnaryk, 1994). This association of closely related insects with closely related host plant species makes *Phyllotreta* a suitable genus for evaluation of the niche-width variation hypothesis.

MATERIAL AND METHODS

We obtained material from 36 *Phyllotreta* populations from six countries (Table 1). All animals were collected from their host plants using an aspirator or a sweepnet. In total about 5000 specimens comprising 10 different species were used for a study of genetic variation using allozyme electrophoresis. Due to the small size of the animals, only three to five loci could be evaluated per specimen. The species were divided into two groups: monophagous (11 populations, 4 species: *P. astrachanica* Lopatin, 1977, *P. dilatata* Thomson, 1866, *P. flexuosa* and *P. tetrastigma*) and oligophagous (25 populations, 6 species: *P. aerea* Allard, 1895, *P. atra* (Fabricius, 1775), *P. consobrina*, *P. cruciferae*, *P. nigripes* (Fabricius, 1775) and *P. ochripes* (Curtis, 1837)). A species was considered monophagous if it only lives on one host plant species or on host plant species belonging to the same genus; it was considered oligophagous if it lives on host plants of several genera (Bernays & Chapman, 1994). Living on a host plant means that in outdoor conditions the species are known to feed and reproduce on the plants concerned. Although for some monophagous species it is known that they survive on other non-host plants in the laboratory (Nielsen, 1978), we do not take this into account. The separation into these two groups does not reflect any phylogenetic relationships (unpublished data). The average distances between two populations of a monophagous species (mean ± st. dev.: 421 ± 241km) and between two populations of an oligophagous species (mean ± st. dev.: 466 ± 264 km) were not significantly different (Mann-Whitney U test, $p > 0.05$).

Vertical polyacrylamide gel electrophoresis (PAGE) was performed for 10 loci: aconitase (ACO, E.C. 4.2.1.3), α-amylase (AMY, E.C. 3.2.1.1), α-glycerophosphate dehydrogenase (GPD, E.C. 1.1.1.8), aspartate aminotransferase (AAT, E.C. 2.6.1.1), isocitric dehydrogenase (ICD, E.C. 1.1.1.42), malate dehydrogenase (MDH, E.C.1.1.1.37), mannose phosphate isomerase (MP1 and MP2, E.C. 5.3.1.8 [2 loci]), peptidase (Leu-Ala) (PEP, E.C. 3.4.-.-) and phosphoglucomutase (PGM, E.C., 5.4.2.2.). Sample preparation, storage, electrophoresis running conditions and buffer systems used are as in Verdyck et al. (1996). As low sample numbers could give rise to aberrations of the variability measures, only populations for which the mean number of specimens per locus was larger than 10 were included in this study.

Genetic variability was studied using four different criteria: the average number of alleles per locus, the percentage of loci polymorphic, the observed heterozygosity (Hobs.) and the expected heterozygosity under Hardy-Weinberg conditions (Hexp.). All calculations were performed using BIOSYS-1 (Swofford & Selander, 1989). For each of the measures a Mann-Whitney U test was used to check for significant differences between monophagous and/or oligophagous species.

Populations were tested for deviation from Hardy-Weinberg equilibrium using exact probabilities, corrected for multiple comparisons using sequential Bonferroni correction. Fixation index (F) and coefficients of heterozygote deviation ($D= H_o-H_e/H_e$) were calculated per locus for each population. To compare between groups, the coefficients were averaged over all polymorphic loci (for which significant deviation from Hardy-Weinberg equilibrium was found) for each of the populations. These averages were compared using a Mann-Whitney U test.

RESULTS

Table 1 gives an overview of all genetic variability measures calculated for all 36 populations. Table 2 gives the minimum, maximum and mean values with standard deviation for each variability measure for the mono- and the oligophagous species. The mean sample size did not differ significantly between the mono- and oligophagous species (Mann-Whitney U test, $p>0.05$). For all the measures, the monophagous species showed a significantly higher genetic variability compared to the oligophagous species (Mann-Whitney U test, $p<0.05$).

The results of the exact probability tests (after sequential Bonferroni correction) showed only significant deviation from Hardy Weinberg equilibrium in the populations *P. aerea* [loc.: Breisach] for MP1, *P. aerea* [loc.: Fulda] for ACO and MP1, *P. aerea* [loc.: Ludwigsberg] for ACO and *P. tetrastigma* [loc.: Zoersel] for MP2. Thirty two out of 36 populations (nine out of ten monophagous and 23 out of 26 oligophagous) showed no deviations from Hardy-Weinberg equilibrium for any of their variable loci. For the mono- and oligophagous species, respectively, one out of 56 and four out of 68 cases of polymorphism were significantly different from Hardy-Weinberg equilibrium (exact probabilities, $p<0.05$).

TABLE 1

Genetic variability (four measures: number of alleles per locus, percentage of polymorphic loci, observed (Hobs) and expected heterozygosity (Hexp)) at 10 loci in all populations for the different *Phyllotreta* species; M= monophagous; O= oligophagous

Species	Population (country)	M/O	Mean sample size/locus	Mean No of alleles/locus	% of loci polym. (no.crit.)	% of loci polym. (95%)	Hobs.	Hexp.
P.aerea	Breisach am Rhein (D)	O	30.5	1.7	40	30	0.058	0.144
P.aerea	Fulda (D)	O	36.9	1.5	30	30	0.066	0.138
P.aerea	Gembloux (B)	O	29.7	1.4	30	30	0.079	0.109
P.aerea	Ludwigsburg (D)	O	29.7	1.6	40	30	0.067	0.139
P.aerea	Wimereux (F)	O	29.5	1.5	30	30	0.083	0.151
P.astrachanica	St.-Aignan-Grandlieu (F)	M	16.4	1.3	30	10	0.059	0.060
P.atra	Celles sur Plaine (F)	O	24.2	1.5	30	20	0.076	0.074
P.atra	Fulda (D)	O	20.6	1.4	40	10	0.065	0.065
P.atra	Stansted (UK)	O	31.3	1.4	30	30	0.050	0.053
P.consobrina	Berchem (B)	O	42.2	1.4	30	20	0.058	0.084
P.consobrina	Gembloux (B)	O	35.4	1.3	20	20	0.066	0.079
P.consobrina	Wimereux (F)	O	28.0	1.3	20	20	0.070	0.083
P.cruciferae	Berchem (B)	O	64.9	1.2	20	10	0.027	0.024
P.cruciferae	Breisach am Rhein (D)	O	37.5	1.4	20	10	0.028	0.028
P.cruciferae	Frederiksberg (DK)	O	43.4	1.3	20	10	0.020	0.019
P.cruciferae	Gembloux (B)	O	83.0	1.3	30	10	0.012	0.014
P.cruciferae	Strasbourg (D)	O	43.8	1.4	40	10	0.027	0.026
P.cruciferae	Wimereux (F)	O	40.8	1.3	20	10	0.023	0.021
P.dilatata	Deurne (B)	M	44.0	1.7	50	30	0.076	0.092
P.dilatata	St.-Aignan-Grandlieu (F)	M	30.0	1.7	60	40	0.062	0.099
P.dilatata	St.-Philbert-de-Grand-Lieu (F)	M	30.1	1.6	50	20	0.059	0.085
P.flexuosa	Udenhout (NL)	M	10.5	1.7	40	20	0.150	0.124
P.nigripes	Berchem (B)	O	35.4	1.2	20	0	0.006	0.012
P.nigripes	Gembloux (B)	O	25.7	1.1	10	10	0.015	0.014
P.nigripes	Fulda (D)	O	39.0	1.5	50	10	0.027	0.029
P.nigripes	Taastrup (DK)	O	16.6	1.1	10	10	0.013	0.012
P.nigripes	Wimereux (F)	O	16.6	1.3	20	10	0.023	0.043
P.ochripes	Deurne (B)	O	35.6	1.1	10	0	0.000	0.008
P.ochripes	Oisterwijk (NL)	O	34.9	1.4	30	10	0.008	0.025
P.ochripes	St.-Philbert-de-Grand-Lieu (F)	O	43.3	1.6	50	0	0.014	0.018
P.tetrastigma	Celles sur Plaine (F)	M	50.8	1.7	40	40	0.119	0.141
P.tetrastigma	Chimay (F)	M	48.7	2.1	80	40	0.114	0.138
P.tetrastigma	Geisenfeld (D)	M	38.2	1.8	50	20	0.103	0.119
P.tetrastigma	Stenholts Vang (DK)	M	27.8	1.5	40	30	0.124	0.127
P.tetrastigma	Udenhout (NL)	M	34.7	1.8	50	30	0.104	0.144
P.tetrastigma	Zoersel (B)	M	73.9	1.9	70	30	0.099	0.130

TABLE 2

Genetic variability in mono- and oligophagous species (four measures: number of alleles per locus, percentage of polymorphic loci, observed (Hobs) and expected heterozygosity (Hexp))

	Mean		St.Dev		Minimum		Maximum	
	M	O	M	O	M	O	M	O
Sample Size	36.827	35.940	17.471	14.086	10.500	16.600	73.900	83.000
No Alleles/locus	1.709	1.368	0.207	0.157	1.300	1.100	2.100	1.700
% loci polym.)	50.909	27.600	14.460	11.284	30.000	10.000	80.000	50.000
Hobs.	0.097	0.039	0.030	0.027	0.059	0.000	0.150	0.083
Hexp.	0.114	0.056	0.027	0.047	0.060	0.008	0.144	0.151

In most populations the expected heterozygosity (Hexp.) was somewhat larger than the observed heterozygosity (Hobs.). The coefficient of heterozygote deviation (D) (Koehn et al. 1976) was negative in all five cases where deviations were found; the fixation index (F) was always positive (Table 3). These results show that, in these few cases, there were fewer heterozygotes than expected under Hardy-Weinberg conditions.

TABLE 3

Values for D (coefficient of heterozygote deviation) and F (fixation index) for loci not in Hardy-Weinberg equilibrium

Population	ACO	MP1	MP2
P. aerea (Breisach)	-	F= 0.755 D= -0.759	-
P. aerea (Fulda)	F= 0.707 D= -0.720	F= 0.908 D= -0.909	
P. aerea (Ludwigsberg)	F= 0.757 D= -0.761	-	-
P. tetrastigma (Zoersel)	-	-	F= 0.733 D= -0.736

DISCUSSION

Hsiao (1989) compared genetic variability studies for 30 Coleopteran species belonging to five families and found a mean heterozygosity (Hexp) of 0.168, amongst the highest recorded in insects. Within the Chrysomelidae he found mean heterozygosities between 0.081 and 0.206. In more recent studies on chrysomelids the following ranges are found: 0.061 to 0.238 in *Diabrotica* species (recalculated from Krysan et al., 1989), 0.003 to 0.195 in *Ophraella* species (recalculated from Futuyma & McCafferty, 1990), 0.077 to 0.624 in *Oreina* species (recalculated from Rowell-Rahier (1992) and Rowell-Rahier & Pasteels (1994)). The heterozygosity values in *Phyllotreta* species seem to be slightly lower than in most other chrysomelid groups (Hexp between 0.060 and 0.144 in monophagous and between 0.008 and 0.151 in oligophagous species). For the monophagous species the mean heterozygosity value (0.114) does not differ very much from the average heterozygosity value in other Coleoptera (0.168) (Hsiao, 1989). For the oligophagous species the mean value (0.056), however, is amongst the lowest found in Coleoptera.

The results clearly indicate that the specialized monophagous *Phyllotreta* species are genetically more variable than generalist oligophagous species. This is at variance with the niche-width variation hypothesis. Until now most of the studies testing this hypothesis confirmed it (e.g. Avise & Selander, 1972; Lacy, 1982; Nevo et al., 1984 (analysing literature data for 669 species); Lavie & Nevo, 1986; Noy et al., 1987; Lavie et al., 1993; Pavliek & Nevo, 1994). However, some studies (Sabath, 1974; Somero & Soulé, 1974; Mitter & Futuyma 1979) did not support the hypothesis.

Although in this study we only tested 10 different loci, representing only a small part of the genome (which makes it impossible to extend our conclusions towards the genetic variability of the entire genome), the finding of strongly significant differences for all genetic variability measures allows us to reject the hypothesis for the *Phyllotreta* species studied.

How can the low genetic variability in the oligophagous species be explained? The monophagous species have always been found in natural habitats, whereas most of the oligophagous populations were found in agricultural or at least cultivated habitats (such as botanical gardens) (except *P. atra* from Celles sur Plaine and the three populations of *P. ochripes*). Populations in agricultural and cultivated habitats may be more subjected to drastic changes in numbers (insecticide use) and more affected by selective pressures (crop rotation), and may be extinguished and recolonized more often than populations in more stable natural habitats. Therefore founder events might be more important in these oligophagous animals, resulting in lower genetic variability. If this were true for many of the populations, we would expect different loci to be variable in different localities. However, our results show that within a species, the variable loci are often the same within different localities (although this may be an indication for selection). Moreover, we did not find higher genetic variability in the four oligophagous populations collected in natural habitats, where founder effects are supposed to be less important.

An alternative explanation for this phenomenon may be found in the host plant use by the different species. The monophagous *Phyllotreta* species studied feed on plants such as *Cardamine* sp. or *Rorippa* sp. These plants are very abundant during a short period of the year, as after flowering they disappear completely. Animals feeding on them have only limited time to complete their cycle. As for the *Phyllotreta* species both larvae and adults live on the same plants, and correct timing is crucial. When the plants will be there is related to weather conditions changing from year to year. This system may cause an evolutionary stress for the monophagous species and, if timing and development are genetically controlled, we can assume an advantage in variable populations, maintaining polymorphism in these species. For the oligophagous species there is always an assortment of cruciferous plants to be found, from early spring to early winter, and timing is supposedly less important, resulting in less polymorphism.

Here we find higher genetic variability for both observed and expected heterozygosity values in the specialist *Phyllotreta* species. Relationships between heterozygosity and several fitness traits have been studied extensively in several organisms, showing that highly heterozygous individuals usually have superior growth rates, more buffered developmental processes and lower morphological variation (Mitton & Grant, 1984; Zouros, 1987; Houle, 1989). In bivalves multiple-locus heterozygosity is positively correlated with growth and viability (Zouros & Foltz, 1984; Holley & Foltz, 1987; Zouros, 1987; Gaffney et al., 1990). Although it has never been studied in chrysomelid beetles, it is possible that also in these animals, under stressful conditions, heterozygous genotypes perform better than more homozygous genotypes.

In conclusion we can state that all alternative explanations for the refutation can be valid. The data are straightforward, and our paper represents a strong case for the rejection of the niche-width variation hypothesis. Although this does not mean that it can not be valid for some organisms, the hypothesis should not be taken for granted in general.

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On the osteology and myology of the cephalic region and pectoral girdle of *Liobagrus reini* Hilgendorf, 1878, with a discussion on the phylogenetic relationships of the Amblycipitidae (Teleostei: Siluriformes)

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ABSTRACT. The cephalic and pectoral girdle structures of *Liobagrus reini* are described in detail and compared with those of *Amblyceps mangois*, as well as of several other catfishes, as the foundation for a discussion on the phylogenetic relationships of the Amblycipitidae. Our observations and comparisons support de Pinna's (1996) phylogenetic hypothesis, according to which the Amblycipitidae, plus the Akysidae, Sisoridae, Erethistidae and the Aspredinidae, form a monophyletic clade, the superfamily Sisoroidea. In addition, our observations and comparisons pointed out a potentially new amblycipitid synapomorphy, namely: the hypobranchial foramen of the parurohyal is markedly enlarged, with the length of the foramen being superior to half of the length of the main body of the parurohyal.

KEY WORDS : Akysidae, Amblycipitidae, catfish, cephalic region, comparative morphology, *Liobagrus*, myology, pectoral girdle, phylogeny, Siluriformes.

INTRODUCTION

The Siluriformes is "one of the economically important groups of fresh and brackish water fishes in the world: in many countries, member species form a significant part of inland fisheries; several species have been introduced into fish culture; numerous species are of interest to the aquarium industry where they represent a substantial portion of the world trade" (Teugels, 1996). The Amblycipitidae, with only three genera (*Liobagrus* Hilgendorf, 1878 *Amblyceps* Blyth, 1858 and *Xiurenbagrus* Chen & Lundberg, 1994) and 15 species is one of the smallest families of catfishes (Chen & Lundberg, 1994). The phylogeny and systematics of the Amblycipitidae were recently revised by Chen & Lundberg (1994). According to these authors, the genus *Xiurenbagrus* is the sister-group of a clade formed by the genus *Liobagrus* and the genus *Amblyceps*, with these three genera being, in turn, the sister-group of a clade containing the two akysid genera, *Akysis* and *Parakysis* (although Chen & Lundberg referred to a monogeneric family Akysidae and to a monogeneric family Parakysidae, the genera *Akysis* and *Parakysis* are nowadays included in a single family, the Akysidae: see Ferraris & de Pinna, 1999). Such a sister-group relationship between the Amblycipitidae and the Akysidae was, however, contradicted in a later study by de Pinna (1996). In fact, according to de Pinna (1996), the Amblycipitidae is not the sister-group of the Akysidae, but instead the sister-group of a clade formed by this latter family, the Sisoridae, the Aspredinidae and the Erethistidae, with all the five families forming the superfamily Sisoroidea, a hypothesis posteriorly reiterated by de Pinna (1998).

It is worthy of note that, despite the large number of studies concerning siluriform morphology (e.g., Regan, 1911; Gauba, 1962, 1966, 1968, 1969; Mahajan, 1963, 1966ab, 1967ab; Alexander, 1965; Chardon, 1968; Gosline, 1975; Ghiot, 1978; Ghiot et al., 1984; Arratia, 1987, 1990, 1992; Arratia & Schultze, 1990; Mo, 1991; He, 1996; Diogo et al., 1999, 2000, 2001ab; Diogo & Chardon, 2000abc; etc.) the only few, somewhat detailed morphological descriptions of the amblycipitid catfishes published so far are those of Tilak (1967), Chardon (1968), Chen & Lundberg (1994) and de Pinna (1996). Moreover, as these descriptions are almost exclusively restricted to the osteology and external anatomy of the amblycipitids, some important aspects of the morphology of these fishes, such as, for example, the configuration of both the muscles and the ligaments of the cephalic region and the pectoral girdle, or the configuration of the structures associated with their mandibular barbels, are practically unknown. This not only complicates the study of the functional morphology of the amblycipitids, but also restricts considerably the data available to infer the synapomorphies and the phylogenetic relationships of these catfishes (see de Pinna, 1996).

The aim of this work is to describe in detail the bones, cartilages, muscles and ligaments of the cephalic region (branchial apparatus excluded) and pectoral girdle of the amblycipitid *Liobagrus reini* Hilgendorf, 1878, and to compare these structures with those of a representative of one of the two other amblycipitid genera, namely *Amblyceps mangois* (Hamilton, 1822), as well as of several other catfishes, as the foundation for a discussion on the phylogenetic relationships of the Amblycipitidae.

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MATERIAL AND METHODS

The fishes studied are from the collection of our laboratory (LFEM), from the Musée Royal de l'Afrique Centrale of Tervuren (MRAC), from the Université Nationale du Bénin (UNB), from the Muséum National D'Histoire Naturelle of Paris (MNHN), from the University of Gent (UG) and from the National Museum of Natural History of Washington (USNM). Anatomical descriptions are made after dissection of alcohol fixed or trypsin-cleared and alizarine-stained (following TAYLOR & VAN DYKE's 1985 method) specimens. Dissections and morphological drawings were made using a Wild M5 dissecting microscope equipped with a camera lucida. The trypsine-cleared and alizarine-stained (c&s) or alcohol fixed (alc) condition of the studied fishes is given in parentheses following the number of specimens dissected. A list of the specimens dissected is given below.

Acanthodoras catophractus (Doradidae): USNM 034433, 1 (alc). *Ageneiosus vittatus* (Auchenipteridae): USNM 257562, 1 (alc). *Ailia colia* (Shilbidae): USNM 165080, 1 (alc). *Akysis leucorhynchus* (Akysidae): USNM 109636, 2 (alc). *Amblyceps mangois* (Amblycipitidae): USNM 109634, 2 (alc). *Amiurus nebolosus* (Ictaluridae): USNM 246143, 1 (alc); USNM 73712, 1 (alc). *Amphilius brevis* (Amphiliidae): MRAC 89-043-P-403, 3 (alc); MRAC 89-043-P-2333, 1 (c&s). *Anadoras weddelii* (Doradidae): USNM 317965, 1 (alc). *Andersonia leptura* (Amphiliidae): MNHN 1961-0600, 2 (alc); *Arius hertzbergii* (Ariidae): LFEM, 1 (alc). *Arius heudelotii* (Ariidae): LFEM, 4 (alc). *Aspredo aspredo* (Aspredinidae): USNM 226072, 1 (alc). *Auchenipterus dentatus* (Auchenipteridae): USNM 339222, 1 (alc). *Auchenoglanis biscutatus* (Claroteidae): MRAC 73-015-P-999, 2 (alc). *Bagarius yarreli* (Sisoridae): USNM 348830, 2 (alc); LFEM, 1 (c&s). *Bagre marinus* (Ariidae): LFEM, 1 (alc); LFEM, 1 (c&s). *Bagrichthys macropterus* (Bagridae): USNM 230275, 1 (alc). *Bagrus bayad* (Bagridae): LFEM, 1 (alc); LFEM, 1 (c&s). *Bagrus docmak* (Bagridae): MRAC 86-07-P-512, 1 (alc); MRAC 86-07-P-516, 1 (c&s). *Belonoglanis tenuis* (Amphiliidae): MRAC P.60494, 2 (alc). *Bunocephalus knerii* (Aspredinidae): USNM 177206, 2 (alc). *Calophysus macropterus* (Pimelodidae): USNM 306962, 1 (alc). *Centromochlus hechelii* (Auchenipteridae): USNM 261397, 1 (alc). *Cetopsis coecutiens* (Cetopsidae): USNM 265628, 2 (alc). *Chrysichthys auratus* (Claroteidae): UNB, 2 (alc); UNB, 2 (c&s). *Chrysichthys nigrodigitatus* (Claroteidae): UNB, 2 (alc); UNB, 2 (c&s). *Clariallabes melas* (Clariidae): LFEM, 2 (alc). *Clarias gariepinus* (Clariidae): MRAC 93-152-P-1356, 1 (alc), LFEM, 2 (alc). *Conta conta* (Erethistidae): LFEM, 2 (alc). *Cranoglanis bouderius* (Cranoglanididae): LFEM, 2 (alc). *Diplomystes chilensis* (Diplomystidae): LFEM, 2 (alc). *Doras punctatus* (Doradidae): USNM 284575, 1 (alc). *Doumea typica* (Amphiliidae): MRAC 93-041-P-1335, 1 (alc). *Erethistes pusillus* (Erethistidae): USNM 044759, 2 (alc). *Franciscodoras marmuratus* (Doradidae): USNM 196712, 2 (alc). *Gagata cenia* (Sisoridae): USNM 109610, 2 (alc). *Genidens genidens* (Ariidae): LFEM, 2 (alc). *Glyptosternon reticulatum* (Sisoridae): USNM 165114, 1 (alc). *Glyptothorax fukiensis* (Sisoridae): USNM 087613, 2 (alc). *Goeldiella eques* (Pimelodidae): USNM 066180, 1 (alc). *Hara filamentosa* (Erethistidae): USNM 288437, 1 (alc). *Helicophagus leptorhynchus* (Pangasiidae): USNM 355238, 1 (alc). *Helogenes marmuratus* (Cetopsidae): USNM 264030, 1 (alc). *Hemibagrus nemurus* (Bagridae): USNM 317590, 1 (alc); *Hemicetopsis candiru* (Cetopsidae): USNM 167854, 1 (alc). *Hepapterus mustelinus* (Pimelodidae): USNM 287058, 2 (alc). *Heterobranchus longifilis* (Clariidae): LFEM, 2 (alc). *Heteropneustes fossilis* (Heteropneustidae): USNM 343564, 2 (alc); USNM 274063, 1 (alc); LFEM, 2 (alc). *Hypophthalmus edentatus* (Pimelodidae): USNM 226140, 1 (alc). *Ictalurus punctatus* (Ictaluridae): USNM 244950, 2 (alc). *Laides hexanema* (Shilbidae): USNM 316734, 1 (alc). *Leptoglanis rotundiceps* (Amphiliidae): MRAC P.186591-93, 3 (alc). *Liobagrus reini* (Amblycipitidae): USNM 089370, 2 (alc). *Loricaria cataphracta* (Loricariidae): LFEM, 1 (alc). *Microglanis cottoides* (Pimelodidae): USNM 285838, 1 (alc). *Mochokus niloticus* (Mochokidae): MRAC P.119413, 1 (alc); MRAC P.119415, 1 (alc). *Mystus gulio* (Bagridae): LFEM, 1 (alc). *Nematogenys inermis* (Nematogenyidae): USNM 084346, 1 (alc). *Nothoglanidium thomasi* (Claroteidae): LFEM, 2 (alc). *Parakysis anomalopteryx* (Akysidae): USNM 230307, 2 (alc). *Paramphilius trichomycteroides* (Amphiliidae): LFEM, 2 (alc). *Pangasius larnaudii* (Pangasiidae): USNM 288673, 1 (alc). *Pangasius sianensis* (Pangasiidae): USNM 316837, 2 (alc). *Paraplotosus albilabris* (Plotosidae): USNM 173554, 2 (alc). *Phractura brevicauda* (Amphiliidae): MRAC 90-057-P-5145, 2 (alc); MRAC 92-125-P-386, 1 (c&s). *Phractura intermedia* (Amphiliidae): MRAC 73-016-P-5888, 1 (alc). *Pimelodus clarias (*Pimelodidae): LFEM, 2 (alc); USNM 076925, 1 (alc). *Plotosus lineatus* (Plotosidae): USNM 200226), 2 (alc). *Pseudeutropius brachypopterus* (Shilbidae): USNM 230301, 1 (alc). *Pseudomystus bicolor* (Bagridae): LFEM, 1 (alc), LFEM, 1 (c&s). *Pseudopimelodus raninus* (Pimelodidae): USNM 226136, 2 (alc). *Pseudoplatystoma fasciatum* (Pimelodidae): USNM 284814, 1 (alc). *Rhamdia guatemalensis* (Pimelodidae): USNM 114494, 1 (alc). *Rita chrysea* (Bagridae): USNM 114948, 1 (alc). *Schilbe intermedius* (Shilbidae): MRAC P.58661, 1 (alc). *Siluranodon auritus* (Shilbidae): USNM 061302, 2 (alc). *Silurus asotus* (Siluridae): USNM 130504, 2 (alc). *Synodontis clarias* (Mochokidae): USNM 229790, 1 (alc). *Tandanus rendahli* (Plotosidae): USNM 173554, 2 (alc). *Trachyglanis ineac* (Amphiliidae): MRAC P.125552-125553, 2 (alc). *Uegitglanis zammaronoi* (Clariidae): MRAC P-15361, 1 (alc). *Wallago attu* (Siluridae): USNM 304884, 1 (alc). *Xyliphius magdalenae* (Aspredinidae): USNM 120224, 1 (alc). *Zaireichthys zonatus* (Amphiliidae): MRAC 89-043-P-2243-2245, 3 (alc).

RESULTS

In the anatomical descriptions, the nomenclature for the osteological structures of the cephalic region follows basically that of ARRATIA (1997). However, for the several reasons explained in detail in our recent papers (DIOGO et al., 2001a; DIOGO & CHARDON, in press), with respect to the skeletal components of the suspensorium we follow DIOGO et al. (2001a). The myological nomenclature is based mainly on WINTERBOTTOM (1974), but for the different adductor mandibulae sections, DIOGO & CHARDON (2000a) is followed. In relation to the muscles associated with the mandibular barbels, which were not studied by WINTERBOTTOM (1974), DIOGO & CHARDON (2000b) is followed. Concerning the nomenclature of the pectoral girdle bones and muscles, DIOGO et al. (2001b) is followed.

Liobagrus reini

Osteology

Os mesethmoideum. Situated on the antero-dorsal surface of the neurocranium (Figs 1,2), with each of its antero-ventro-lateral margins ligamentously connected to the premaxillary.

Os lateroethmoideum. The lateral-ethmoid (Figs 1, 2, 3) presents a well-developed, laterally-directed articulatory facet for the autopalatine.

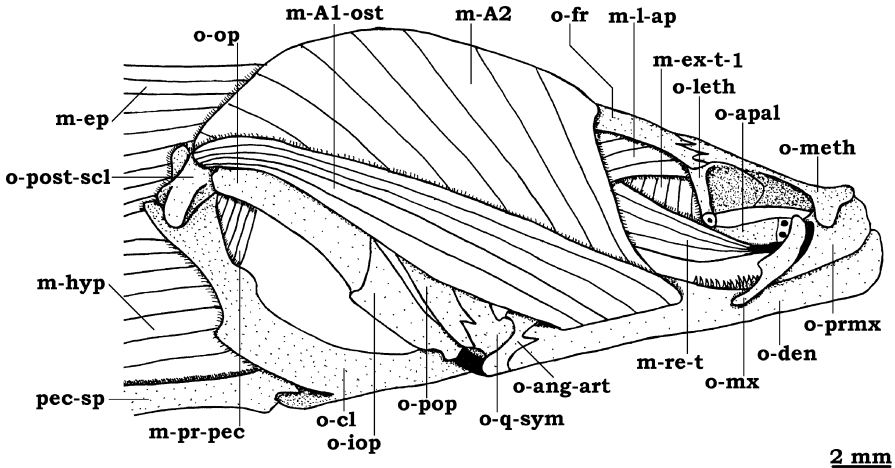

Fig. 1 (fig. above). – Lateral view of the cephalic musculature of *Liobagrus reini*. All the muscles are exposed. *m-A1-ost, m-A2,* sections of musculus adductor mandibulae; *m-ep*, musculus epaxialis; *m-ex-t-1,* section of musculus extensor tentaculi; *m-hyp,* musculus hypoaxialis; *m-l-ap*, musculus levator arcus palatini; *m-pr-pec*, musculus protractor pectoralis; *m-re-t*, musculus retractor tentaculi; *o-ang-art*, os angulo-articulare; *o-apal*, os autopalatinum; *o-cl*, os cleithrum; *o-den*, os dentale; *o-fr*, os frontale; *o-iop*, os interoperculare; *o-leth*, os latero-ethmoideum; *o-meth*, os mesethmoideum; *o-mx*, os maxillare; *o-op*, os operculare; *o-pop*, os praeoperculare; *o-post-scl*, os posttemporo-supracleithrum; *o-prmx*, os praemaxillare; *o-q-sym*, os quadrato-symplecticum; *pec-sp*, pectoral spine.

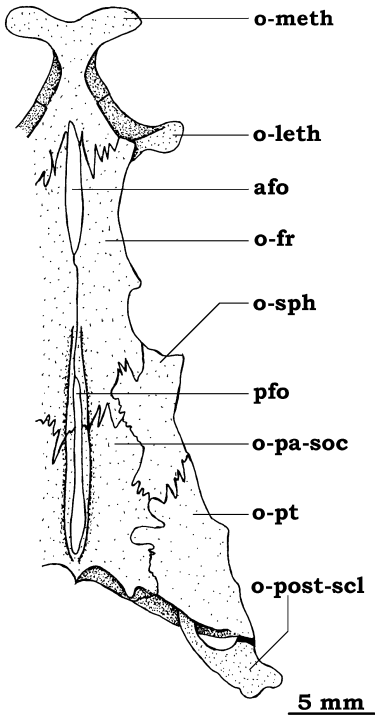

Fig. 2 (fig. to the right). – Dorsal view of the neurocranium of *Liobagrus reini*. *afo*, anterior fontanel, *o-fr*, os frontale; *o-leth*, os latero-ethmoideum; *o-meth*, os mesethmoideum; *o-pa-soc*, os parieto-supraoccipital; *o-post-scl*, os posttemporo-supracleithrum; *o-pt*, os pteroticum; *o-sph*, os sphenoticum; *pfo*, posterior fontanel.

Os praevomerale. Well-developed (Fig. 3), T-shaped bone without a ventral tooth-plate.

Os orbitosphenoideum. Posterior to the lateral ethmoid, with the dorsal edge of its lateral wall being sutured with the ventral surface of the frontal.

Os pterosphenoideum. Posterior to the orbitosphenoid, covering, together with this bone, the gap between the frontals and the parasphenoid.

Os parasphenoideum. The parasphenoid (Fig. 3) is the longest bone of the cranium. It bears a pair of ascending flanges, which suture with the pterosphenoids and prootics.

Os frontale. The frontals (Figs 1, 2) are large bones that constitute a great part of the cranial roof. They are largely separated anteriorly and posteriorly by the anterior and the posterior fontanels, respectively (Fig. 2).

Os sphenoticum. Slightly smaller than the pterotic (Fig. 2), constituting, together with this bone, an articulatory facet for the hyomandibulo-metapterygoid.

Os pteroticum. Well-developed, irregularly-shaped bone situated posteriorly to the sphenotic (Fig. 2).

Os prooticum. Together with the pterosphenoid and the parasphenoid, it borders the well-developed foramen of the trigemino-facial nerve complex.

Os epioccipitale. Situated on the posterior surface of the neurocranium. The extrascapulars are missing.

Os exoccipitale. The well-developed exoccipitals are situated laterally to the basioccipital.

Os *basioccipitale.* Well-developed, unpaired bone, forming the posterior-most part of the floor of the neurocranium. Its ventro-lateral surfaces are ligamentously connected to the ventro-medial limbs of the posttemporo-supracleithra.

Os parieto-supraoccipitale. Large bone constituting the postero-dorso-median surface of the cranial roof, which bears a small, triangular postero-median process (Fig. 2).

Os angulo-articulare. This bone (Fig. 1), plus the dentary, coronomeckelian and Meckel's cartilage, constitute the mandible. Postero-dorsally, the angulo-articular has an articulatory facet for the quadrate-symplectic. Postero-ventrally, it is ligamentously connected to both the interopercular (Figs 1, 4) and the posterior ceratohyal (Fig. 4).

Os dentale. The toothed dentary (Fig. 1) recovers the great majority of the lateral surface of the mandible. The postero-dorsal margin of the dentary forms, together with the antero-dorsal margin of the angulo-articular, a somewhat developed dorsal process (processus coronoideus).

Os coronomeckelium. Small bone lodged in the medial surface of the mandible. Postero-dorsally it bears a crest for attachment of the adductor mandibulae.

Os praemaxillare. The two premaxillaries form a U-shaped structure with its lateral tips curved posteriorly and ligamentously connected, by means of a strong, long ligament, to the antero-lateral tip of the ento-ectopterygoid (Fig. 3). Ventrally, the premaxillaries bear a well-developed tooth-plate with numerous small teeth having their tips slightly turned backward (Fig. 3).

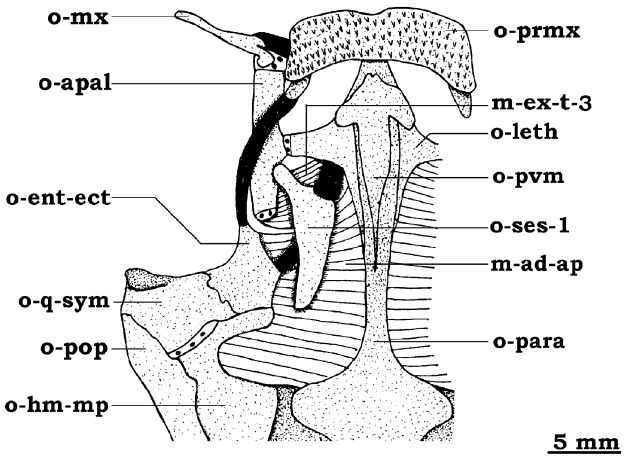

Fig. 3. – Ventral view of the neurocranium of *Liobagrus reini*. The suspensorium, palatine-maxillary system and the muscles and ligaments associated with these structures are also illustrated. *m-ad-ap*, musculus adductor arcus palatini; *m-ex-t-3*, section of musculus extensor tentaculi; *o-apal*, os autopalatinum; *o-ent*, os ento-ectopterygoideum; *o-hm-mp*, os hyomandibulo-metapterygoideum; *o-leth*, os lateroethmoideum; *o-mx*, os maxillare; *o-para*, os parasphenoideum; *o-pop*, os praeoperculare; *o-prmx*, os praeomaxillare; o-pvm, os praevomerale; *o-q-sym*, os quadrato-symplecticum; *o-ses-1*, sesamoidal bone 1 of the suspensorium.

Os maxillare. The well-developed, elongated maxillary is connected to the premaxillary by means of a strong, short ligament (Figs 1, 3). As in most catfishes, the maxillary barbels are supported by the maxillaries.

Os autopalatinum. The autopalatine (Figs 1, 3) is a rod-like, antero-posteriorly elongated bone with its posterior portion somewhat expanded dorso-ventrally. Its posterior end is capped by a small cartilage (Fig. 3). Its anterior end is tipped by a well-developed cartilage with two antero-lateral concavities, which accept the two proximal heads of the maxillary (Fig. 3). Medially, the autopalatine articulates, by means of a small, circular articulatory surface, with the lateral ethmoid (Fig. 32).

Os hyomandibulo-metapterygoideum. The homology, and, thus, the correct denomination, of this bone, as well as of the other suspensorium elements of catfish, has been the subject of endless controversies (McMurrich, 1884; De Beer, 1937; Hoedeman, 1960ab; Gosline, 1975; Arratia et al., 1978; Arratia & Menumarque, 1981; 1984; Howes, 1983ab; 1985; Arratia, 1987; 1990; 1992; Howes & Teugels, 1989; Diogo et al., 2001a; Diogo & Chardon, in press; etc.). As mentioned before, for the several reasons explained in detail in our recent papers (Diogo et al., 2001a; Diogo & Chardon, in press), the nomenclature used here to describe these elements follows strictly that presented by Diogo et al. (2001a). The hyomandibulo-metapterygoid (Fig. 3) is a large bone articulating dorsally with both the pterotic and the sphenotic and posteriorly with the opercular.

Os sesamoideum 1. Well-developed, 7-shaped bone attached, by means of two thick ligaments, to the ento-ectopterygoid and to the prevomer, respectively (Fig. 3). Its antero-dorso-lateral surface is weakly attached, *via* connective tissue, to the postero-ventral surface of the autopalatinum. The sesamoid bones 2 and 3 (see (Diogo et al., 2001a) are absent.

Os entopterygoide-ectopterygoideum. Poorly-developed, with its posterior surface being sutured with both the hyomandibulo-metapterygoid and the quadrate-symplectic (Fig. 3). Antero-laterally, it presents a well-defined thickening for ligamentous connection with the sesamoid bone 1 of the suspensorium.

Os quadrato-symplecticum. The quadrate-symplectic (Figs. 1, 3) presents a well-developed anterior articulatory surface to articulate with the postero-dorsal surface of the angulo-articular.

Os praeoperculare. Long and thin bone (Figs 1, 3) firmly sutured to both the hyomandibulo-metapterygoid and the quadrate-symplectic.

Os operculare. The opercular (Figs 1, 3) is a well-developed, roughly triangular bone attached ventrally, by means of connective tissue, to the interopercular. It presents a well-developed, dorso-ventrally elongated antero-dorsal articulatory surface for the hyomandibulo-metapterygoid.

Os interoperculare. Its anterior surface is ligamentously connected to the postero-ventral margin of the mandible (Figs 1, 4). Medially, the interopercular articulates with the lateral surface of the posterior ceratohyal.

Os ceratohyale posterior. Well-developed, somewhat triangular bone connected, by means of two strong ligaments, to the postero-ventral edge of the mandible (Fig. 4) and to the medial surface of the suspensorium (the interhyal is missing), respectively.

Os ceratohyale anterior. Elongated bone that supports, together with the posterior ceratohyal, the branchiostegal rays.

Os hypohyale ventrale. The ventral hypohyals are ligamentously connected to the antero-lateral edges of the parurohyal (Fig. 4).

Os parurohyale. The parurohyal is an irregular bone presenting two well-developed postero-lateral arms and a poorly-developed postero-median process (Fig. 4). Its hypobranchial foramen (see Arratia & Schultze, 1990) is markedly enlarged, with the length of this foramen being superior to half of the length of the main body of the parurohyal.

Os posttemporo-supracleithrum. This bone (Figs 1, 2), plus the cleithrum and the scapulo-coracoid, constitute the pectoral girdle. Its dorso-medial limb is loosely attached to the neurocranium and its ventro-medial limb is ligamentously connected to the basiocccipital. Its postero-lateral margin is deeply forked, forming an articulating groove for the upper edge of the cleithrum (Fig. 1).

Os cleithrum. The cleithrum (Figs 1, 4) is a large, stout, well-ossified structure forming a great part of the pectoral girdle and the posterior boundary of the branchial chamber. It bears a deep crescentic, medially faced groove that accommodates the dorsal condyle of the well-developed pectoral spine. The two cleithra are attached in the antero-medial line via connective tissue. Antero-ventro-mesially each cleithrum bears a somewhat well-developed, antero-mesially pointed, anterior projection, with the structure formed by the association of the antero-mesial projections of both cleithra having, thus, a triangular shape (see Fig. 4). The poorly-developed humeral process of the cleithrum is surrounded posteriorly by soft tissue, which, in turn, is associated posteriorly with a long, thick ligament attached to the anterior surface of the parapophysis of the sixth vertebra.

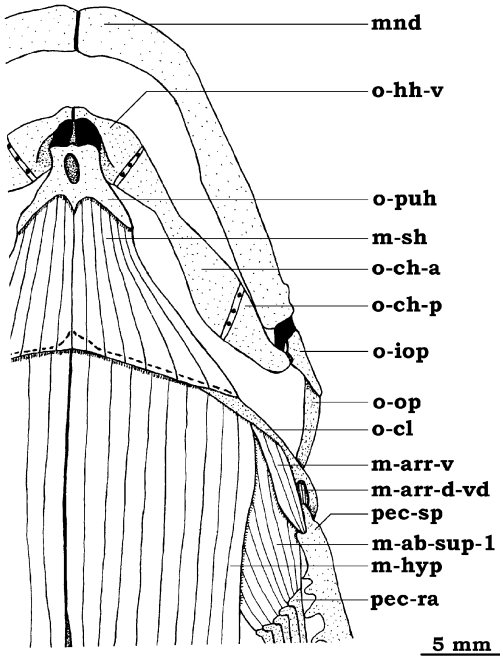

Fig. 4. – Ventral view of the cephalic region and pectoral girdle of *Liobagrus reini*. The muscles associated with the pectoral girdle are also illustrated. *m-ab-sup-1*, section 1 of musculus abductor superficialis; *m-arr-d-vd*, ventral division of musculus arrector dorsalis; *m-arr-v*, musculus arrector ventralis; *m-hyp*, musculus hypoaxialis; *mnd,* mandible; *m-sh*, musculus sternohyoideus; *o-ch-a*, os ceratohyale anterior; *o-ch-p*, os ceratohyale posterior; *o-cl*, os cleithrum; o-hh-v, os hypohyale ventrale; *o-iop*, os interoperculare; *o-op*, os operculare; *o-puh,* os parurohyale*; pec-ra*, pectoral rays; *pec-sp*, pectoral spine.

Os scapulo-coracoideum. Elongated, irregular bony plate suturing with the cleithrum along its antero-lateral edge. Medially it joins its counterpart in an interdigitation of several strong serrations. There is a well-developed mesocoracoid arch.

Myology

Musculus adductor mandibulae. The adductor mandibulae A1-ost (see DIOGO & CHARDON, 2000a) originates on the pterotic, preopercular, hyomandibulo-metapterygoid and quadrate-symplectic, and inserts on the dentary (Fig. 1). The A2 (Fig. 1), which lies dorso-mesially to the A1-ost, attaches postero-dorsally on the parieto-supraoccipital, pterotic, sphenotic and frontal antero-ventrally on the dorso-medial surface of the dentary. The adductor mandibulae A3' originates on the hyomandibulo-metapterygoid and the preoprecular, and inserts tendinously on the coronomeckelian bone. There is no A3'' nor Aω.

Musculus levator arcus palatini. The levator arcus palatini (Fig. 1) is a well-developed muscle situated medially to the adductor mandibulae A3'. It originates on the sphenotic, frontal and lateral-ethmoid and inserts on the lateral face of the hyomandibulo-metapterygoid.

Musculus adductor arcus palatini. This muscle (Fig. 3) runs from the parasphenoid, pterosphenoid, orbitosphenoid and lateral ethmoid to the hyomandibulo-metapterygoid, the ento-ectopterygoid and the sesamoid bone 1 of the suspensorium.

Musculus levator operculi. It originates on the lateral margin of the pterotic and inserts on the dorsal surface of the opercular.

Musculus adductor operculi. Situated medially to the levator operculi, it originates on the ventral surface of the pterotic and inserts on the dorso-medial surface of the opercular.

Musculus adductor hyomandibularis. Small muscle situated mesially to the levator operculi but laterally to the adductor operculi. It originates on the ventral surface of the pterotic and inserts on the postero-dorso-median surface of the hyomandibulo-metapterygoid.

Musculus dilatator operculi. Well-developed, originating on the pterosphenoid, frontal, sphenotic and also on the dorso-lateral surface of the hyomandibula and inserting on the antero-dorsal margin of the opercular.

Musculus extensor tentaculi. This muscle is divided into three bundles. The extensor tentaculi 1 (Fig. 1) runs from both the orbitosphenoid and the lateral ethmoid to the postero-dorsal surface of the autopalatine. The extensor tentaculi 2 originates on the lateral ethmoid and inserts on the postero-medial surface of the autopalatine. The extensor tentaculi 3 (Fig. 3) runs from the lateral ethmoid and the orbitosphenoid to the postero-ventral margin of the autopalatine.

Musculus retractor tentaculi. Well-developed muscle situated medially to the adductor mandibulae (Fig. 1). It originates on the lateral surface of the hyomandibulo-metapterygoid and inserts, by means of a thick tendon, on the maxillary.

Musculus protractor hyoidei. This muscle (Fig. 5) has three parts. The pars ventralis, in which are lodged the cartilages associated with the internal and external mandibular barbels, originates on both the anterior and posterior ceratohyals and inserts on the dentary, meeting its counterpart in a well-developed median aponeurosis (Fig. 5). The pars lateralis runs from the posterior ceratohyal to the ventro-medial face of the dentary (Fig. 5). The pars dorsalis runs from both the anterior and the posterior ceratohyals to the dentary.

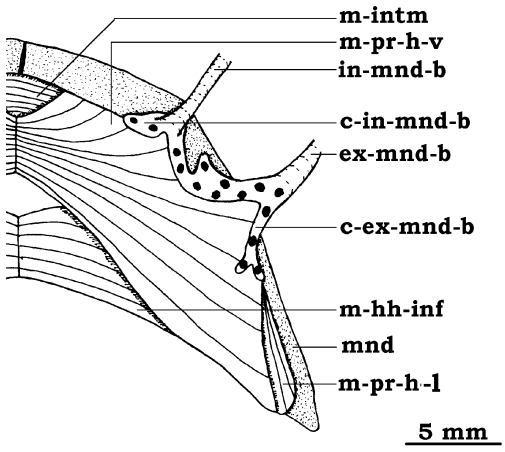

Fig. 5. – Ventral view of the cephalic musculature of *Liobagrus reini*. *c-in-mnd-b*, cartilago internus mandibularis tentaculi; *c-ex-mnd-b*, cartilago externus mandibularis tentaculi; *ex-mnd-b, in-mnd-b*; external and internal mandibular barbels; *m-hh-inf*, musculus hyohyoideus inferior; *m-intm*, musculus intermandibularis; *mnd,* mandible; *m-pr-h-l, m-pr-h-v*, pars lateralis and ventralis of musculus protactor hyoideus.

Musculus retractor externi mandibularis tentaculi. Small muscle situated dorsally to the pars ventralis of the protractor hyodei and running from the dentary to the cartilage associated with the outer mandibular barbel, which is connected with the cartilage associated with the internal mandibular barbel and is markedly bifurcated posteriorly (Fig. 5).

Musculus retractor interni mandibularis tentaculi. Small muscle dorsal to the pars ventralis of the protractor hyodei. It attaches anteriorly to the dentary and posteriorly to the cartilage associated with the internal mandibular barbel.

Musculus intermandibularis. Small muscle joining the two mandibles (Fig. 5).

Musculus hyohyoideus inferior. Thick muscle (Fig. 5) attaching medially on a median aponeurosis and laterally on the ventral surfaces of the ventral hypohyal, the anterior ceratohyal and the posterior ceratohyal.

Musculus hyohyoideus abductor. It runs from the first (medial) branchiostegal ray to a median aponeurosis, which is associated with two long, strong tendons, attached, respectively, to the two ventral hypohyals.

Musculus hyohyoideus adductor. Each hyohyoideus adductor connects the branchiostegal rays of the respective side.

Musculus sternohyoideus. It runs from the posterior portion of the parurohyal to the anterior portion of the cleithrum (Fig. 5).

Musculus arrector ventralis. It runs from the cleithrum to the ventral condyle of the pectoral spine (Fig. 5).

Musculus arrector dorsalis. This muscle, dorsal to the hypoaxialis, the arrector ventralis and the abductor superficialis, is differentiated into two well-developed divisions. The ventral division (Fig. 5), situated on the ventral surface of the pectoral girdle, originates on the ventral margin of both the cleithrum and the scapulo-coracoid and inserts on the antero-lateral edge of the pectoral spine. The dorsal division, situated on the dorsal surface of the pectoral girdle, originates on the dorso-medial edge of the scapulo-coracoid and inserts on the anterior edge of the dorsal condyle of the pectoral spine.

Musculus abductor profundus. It originates on the posterior surface of the scapulo-coracoid and inserts on the medial surface of the dorsal condyle of the pectoral spine.

Musculus abductor superficialis. This muscle is differentiated into two sections. The larger section (Fig. 5: m-ab-sup-1) runs from the ventral margins of both the cleithrum and the scapulo-coracoid to the antero-ventral margin of the ventral part of the pectoral fin rays. The smaller section, situated dorsally to the larger one, runs from the lateral edge of the scapulo-coracoid to the antero-dorsal margin of the ventral part of the pectoral fin rays.

Musculus adductor superficialis. This muscle is situated on the posterior margin of the pectoral girdle and is divided into two sections. The larger section originates on the posterior surfaces of both the cleithrum and the scapulo-coracoid and inserts on the antero-dorsal margin of the dorsal part of the pectoral fin rays. The smaller section runs from both the postero-ventro-lateral edge of the scapulo-coracoid and the dorsal surface of the proximal radials to the antero-ventral margin of the dorsal part of the pectoral fin rays.

Musculus protractor pectoralis. Well-developed muscle (Fig. 1) running from the ventral surfaces of both the pterotic and the posttemporo-supracleithrum to the antero-dorsal surface of the cleithrum.

Amblyceps mangois

The principal differences between the structures of the cephalic region and pectoral girdle of this species and those of *Liobagrus reini* are that in *Amblyceps mangois*: 1) the cartilage associated with the inner mandibular barbel is pierced by a well-developed, triangular foramen; 2) the articulatory surface of the opecular for the hyomandibulo-metapterygoid is not elongated dorso-ventrally; 3) the interhyal is present; 4) the maxillary bone is not elongated proximo-distally; 5) the posterior fontanel of the neurocranium is completely surrounded by the parieto-supraoccipital, and not by both this bone and the frontal; 6) the muscle abductor profundus is significantly more developed than in *Liobagrus reini*, extending mesially to reach the postero-mesial surface of the scapulo-coracoid.

DISCUSSION

Our observations and comparisons support DE PINNA's (1996) phylogenetic hypothesis in that the Amblycipitidae plus the Akysidae, Sisoridae, Erethistidae and Aspredinidae form a monophyletic clade, the superfamily Sisoroidea sensu DE PINNA (1996). In fact, our study not only confirmed the synapomorphies given by DE PINNA to support the monophyly of the Sisoroidea (see DE PINNA, 1996: 59-60), but also pointed out an additional synapomorphy to support this superfamily: *Presence of a well-developed, antero-mesially-pointed projection on the antero-ventro-mesial surface of the cleithrum (Fig. 4)*.

Plesiomorphically in catfishes the cleithrum lacks major projections or processes on its antero-ventro-mesial surface. However, in the catfishes of the superfamily Sisoroidea, with the exception of the Aspredinidae, each cleithrum bears antero-ventro-mesially a well-developed, antero-mesially pointed, anterior projection, with the structure formed by the association of the antero-mesial projections of both cleithra having, thus, a triangular shape (see Fig. 4). The presence of such a projection clearly appears, thus, to represent a Sisoroidea synapomorphy secondarily reversed in aspredinids, since this is by far the most parsimonious hypothesis supporting the phylogenetic position of the Aspredinidae within the Sisoroidea (see DE PINNA, 1996; DIOGO et al., 2001c, 2002).

According to DE PINNA (1996), the Amblycipitidae occupy the most plesiomorphic position within the superfamily Sisoroidea, with the intra-relationships among members of this superfamily being: (Amblycipitidae + (Akysidae + (Sisoridae + (Aspredinidae + Erethistidae)))). However, as noted by DE PINNA (1996: 76) himself, contrarily to the grouping of the Sisoridae, Aspredinidae and Erethistidae, which is very well supported (and was subsequently also confirmed by DIOGO et al., 2001c, 2002), the proposal of a sister-group relationship between the Akysidae and these three families is based on scarce evidence.

In fact, this proposal relied "on three synapomorphies, one of these (supratemporal fossae present) shows reversals, and the other two (supracleithrum strongly attached to skull; posterior nuchal plate with anterior process facet for articulation with anterior nuchal plate) have a number of putatively homoplastic occurrences elsewhere in siluriforms" (De Pinna, 1996: 76). The evidence presented by de Pinna (1996) to support a sister-group relationship between the Akysidae and the clade (Sisoridae + (Aspredinidae + Erethistidae)) was not significantly stronger than the evidence supporting the alternative, most traditional (see, e.g., Reagan, 1911; Chen & Lundberg, 1994) hypothesis of a sister-group relationship between the Akysidae and the Amblycipitidae (with the clade formed by these two families being, in turn, the sister-group of the clade formed by the Sisoridae, Erethistidae and Aspredinidae).

Our observations and comparisons confirmed the three synapomorphies provided by de Pinna (1996) to support the grouping of the Akysidae, Sisoridae, Aspredinidae and Erethistidae, and, in addition, pointed out an additional synapomorphy to support the grouping of these four families. This additional synapomorphy is described below: *Coronoid process of the mandible exclusively formed by the dorsal margin of the dentary.*

Plesiomorphically in catfishes the coronoid process of the mandible is constituted by the dorsal surfaces of both the dentary and the angulo-articular bones. However, in the Akysidae, Erethistidae and Sisoridae the coronoid process is exclusively formed by the dorsal margin of the dentary (see, e.g., Diogo et al., 2002: Fig. 3A). Therefore, this character appears to represent a synapomorphy of the clade constituted by the Akysidae, Sisoridae, Aspredinidae and Erethistidae, which was secondarily reversed in the aspredinids (see Diogo et al., 2001c).

Altough the present study supports, thus, De Pinna's (1996) grouping of the Akysidae, Sisoridae, Erethistidae and Aspredinidae, we consider, as did De Pinna, that it is important to point out that the evidence to support this grouping is scarce and that there are some conflicting characters with this hypothesis. One of these characters is the peculiar 7-shape of the sesamoid bone 1 of the suspensorium (Fig. 3). De Pinna (1996: 70) mentioned this peculiar feature, mentioning that it was present in the amblycipitids and in *Parakysis*. However, this peculiar feature is also present in the other akysid genus, *Akysis*, as it was clearly noted by Chen & Lundberg (1994: 795) and confirmed in the present study. The presence of this peculiar, derived feature in the Amblycipitidae and Akysidae genera thus conflicts with the grouping of the Akysidae with the families Sisoridae, Aspredinidae and Erethistidae. The other conflicting character concerns the bifurcation of the basal cartilages of the external mandibular barbels. Contrary to the situation in most catfishes, in both the akysids and the amblycipitids (see Fig. 5), as well as in the glyptosternin Sisoridae (see Diogo et al., 2002: Fig. 5), the basal cartilages of the outer mandibular barbels are bifurcated posteriorly. Attending to the well-supported grouping of the Sisoridae, Aspredinidae and Erethistidae in a monophyletic clade (see De Pinna, 1996; Diogo et al., 2001c, 2002), the taxonomic distribution of this character would support a sister-group relationship between the Akysidae and the Amblycipitidae, with an independent, homoplastic acquisition in the glyptosternin Sisoridae. It is also important to refer to three other characters discussed by de Pinna (1996: 69), namely the "morphology of the first proximal pectoral radial", the "presence of a spur-like process on the quadrate" and the "state of the humerovertebral ligament". These refer to derived features present in the amblycipitids and in *Parakysis*, but not in *Akysis*, which, thus, could either be interpreted as independently acquired in the amblycipitids and in *Parakysis*, or as acquired in the amblycipitids plus akysids and subsequently reversed in *Akysis*. In this latter case, they would support a sister-group relationship between the families Amblycipitidae and Akysidae.

In summary, it can be said that although this study supports, with an additional synapomorphy, De Pinna's (1996) grouping of the Akysidae, Sisoridae, Erethistidae and Aspredinidae, the phylogenetic position of the Amblycipitidae remains a problematic topic within the interrelationships of the superfamily Sisoroidea.

With respect to the synapomorphies of the Amblycipitidae, six characters were presented by de Pinna (see De Pinna, 1996: 60). Our observations and comparisons pointed out a quite peculiar, derived character that is found in both *Liobagrus* and *Amblyceps*, and in no other catfish examined, which, thus, could eventually constitute an additional amblycipitid synapomorphy: the hypobranchial foramen of the parurohyal is markedly enlarged, with the length of the foramen being superior to half of the length of the main body of the parurohyal (Fig. 4). However, since it was not possible to dissect an exemplar of the other amblycipitid genera, *Xiurenbagrus* (see Introduction), and since the parurohyal of *Xiurenbagrus xiurensis*, the only species of this genus, is not described in Chen & Lundberg (1994), it was not possible to confirm if such a feature is, or not, present in this species. Therefore, only when a more detailed osteological description of this species becomes available will it be possible to confirm if this feature represents, or not, an unambiguous synapomorphy of the family Amblycipitidae.

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SHORT NOTES

Mutualism between the sea anemone *Antholoba achates* (Drayton, 1846) (Cnidaria: Actiniaria: Actinostolidae) and the spider crab *Libinia spinosa* Milne-Edwards, 1834 (Crustacea: Decapoda, Majidae)

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Associations between sea anemones and other invertebrates have been widely studied (1, 2, 3, 4, 5, 6). The relationships between actiniarians and spider crabs (family Majidae) are of two sorts: in some cases crabs are found on the column or among the tentacles of the sea anemones (7); in the second case the sea anemone, as well as other invertebrates and algae, are located on the spider crab carapace. Most of those studies were based on species from the northern hemisphere, and the knowledge about actiniarian relationships from the austral hemisphere is scarce. Bürger (8) described the association between the crab *Hepatus chilensis* Milne-Edwards, 1837 and *Antholoba achates* (= *A. reticulata* Couthouy in Dana, 1846) in Chile. Boschi (9) and Olivier (10) observed the presence of sea anemones on the spider crab *Libinia spinosa* Milne-Edwards, 1834 from the southwestern Atlantic Ocean, but the actiniarians were not identified, while Zamponi (11) mentioned the relationship between *Phlyctenanthus australis* (Carlgren, 1950) and *L. spinosa.* This note reports the association between the sea anemone *A. achates* and *L. spinosa* in the Argentinian sea with an analysis of the sea anemone´s position on the spider crab, food items and the size relationships of the two species.

Material was collected from Mar del Plata (38°05'S, 57°32'W), Buenos Aires Province, Argentina, during shrimp captures by means of a beam-trawl at a 5-10 m depth on soft muddy-sand during fall 1999 and 2001, and preserved in 5% saline formaldehyde solution. In *L. spinosa,* carapace maximum width was measured and the blotted individuals weighed (without considering appendages since most of them were incomplete or lost). Each individual was sexed and maturity condition determined from abdominal morphology. The position of each anemone on the carapace of the spider crab was recorded on three zones: anterior third, middle third and posterior third of the carapace. The diameter of the basal disk of each anemone was measured in situ using a calliper, and it was then detached, blotted and weighed. Reproductive condition was checked by examining the mesenteries for gonadal tissue. The gut contents of 167 individuals of the spider crab and 102 individuals of the sea anemone were analyzed and each piece of each food item was considered as one score.

Ninety three individuals of *A. achates* were found on 35 individuals of *L. spinosa* (Fig. 1). The anemones were mainly found on the posterior third of the carapace (55 %), 29 % in the middle and 16 % on the anterior part of the carapace. The *L. spinosa* individuals had a carapace width between 29.3 and 58.5 mm (mean of 42.56 ± 6.1 mm) and a weight between 9.57 and 67.89 g (mean of 35.42 ± 14.58 g); both variables correlated (R^2= 0.87, F= 304.29, $p<0.05$ after log10 transformation). Eighty percent of them were males; only one immature female and six immature males were found. Individuals of *L. spinosa* carried one to seven specimens of *A. achates* on the carapace with an average of three. The mean basal disc diameter of the actiniarians, varied from 4 to 37 mm (mean of 16.03 ± 7.86 mm), and weight varied between 0.02 and 12.74 g (mean of 1.69 ± 2.12 g). The regression analysis between basal diameter and wet weight of sea anemones, after log10 transformation, showed a good linear fit (R^2= 0.947 F= 1568.15, $p<0.05$). All individuals of *A. achates* were immature.

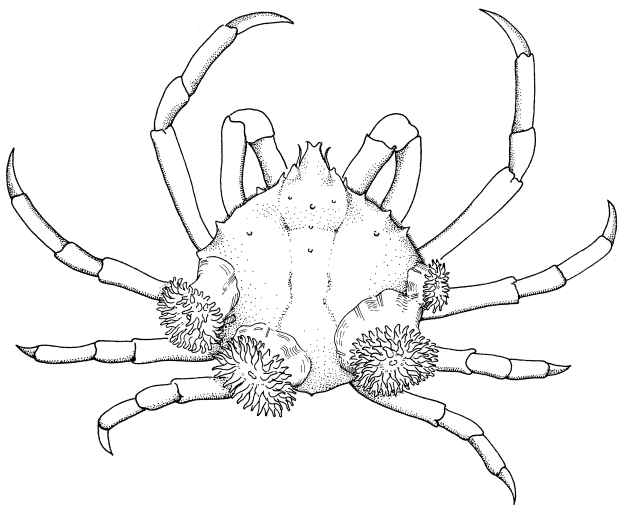

Fig. 1. – Individuals of *A. achates* on the carapace of *L. spinosa*.

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There was no relation between the number of anemones and the size of the crabs, but there was a moderate correlation between the size (weight) of the anemones and the size (weight) of the crabs (R^2= 0.449; F= 26.12, p<0.05), indicating that the larger crabs carried the larger anemones. This can be expected, since the larger the crab, the longer the intermoult, and the longer the period during which the anemone can grow on the crab.

The contents of the stomach of *L. spinosa* and of the gastro-vascular cavity of *A. achates* were similar in the kind of food items, but the proportions differed. In the spider crab stomachs (Fig. 2) we mainly found unidentifiable detritus, pieces of crustaceans such as the shrimp *Artemesia longinaris* Bate, 1888; the prawn *Pleoticus muelleri* (Bate, 1888); hermit crabs *Pagurus exilis* Benedict, 1892, *P. criniticornis* Dana, 1852 and *Loxopagurus loxochelis* (Moreira, 1901); harpacticoid copepods; juvenile gastropod molluscs such as *Buccinanops monilifer* (Kiener, 1834) and *Adelomelon brasiliana* (Lamarck, 1811); valve pieces of bivalve molluscs; scales and vertebrae of bony fish, hydropolyps and pieces of macroalgae. The main content of the sea anemone gastral cavities was composed of unidentified flesh material (probably molluscs) and also pieces of crustaceans, mainly penaeid shrimps and scales of bony fish. Some nematodes were also present.

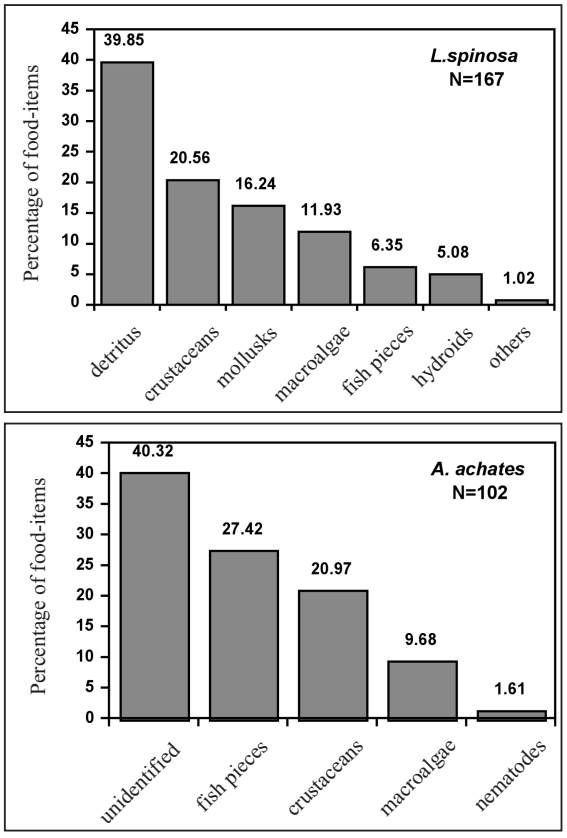

Fig. 2. – Percentage of food items in the spider crab *L. spinosa*, and the sea anemone *A. achates*.

Ectosymbioses between sea anemones and decapod crustaceans may be commensal or mutualistic in nature. In some cases, host and guest demonstrably protect each other from predators, and some anemones are fed by their mutualistic partners (12). In our study, specimens of *A. achates* were located mainly on the posterior part of carapace, so it is improbable that they have any benefit from the crab feeding, and the food items we found in the guts were quite different. According to the analysis of food items, the spider crab *L. spinosa* may be classified as detritivorous-omnivorous and the sea anemone *A. achates* as a polyphagous opportunist (13).

The relationship between *A. achates* and *L. spinosa* is probably mutualistic. The crab is camouflaged by the anemones and protected by the anemone's cnidocysts. The carapace of the spider crab forms a hard substrate where the sea anemone can attach, important for the anemone, since the specimens were captured in a sandy-muddy, soft substrate. The anemone also acquires a greater mobility with additional benefits such as food availability and also gains protection against predators (such as aeolidians).

However, this association is not specific nor obligatory and may be temporary. In captivity, the sea anemone moved from the carapace of the spider crab to the glass wall of the aquarium, but no direct action of the spider crab was observed involving direct transference as does happen in other majid species during the decorating activities (14). *A. achates* occurs frequently on gastropods and other substrates (15). Many other actiniarian species are known to change attachment site, and e.g. the site of larval settlement usually differs from the habitats eventually settled by adults (16). In the studied association, anemones were generally small and all immature, so the relationship clearly starts at the earliest stages of *A. achates*. Chintiroglou et al. (17) observed that the sea anemone *Sagartiogeton undatus* (Mueller, 1778) establishes symbiotic relationships with pagurids at an early age, while Christidis et al. (18) found a similar behaviour in *Calliactis parasitica* (Couch 1838) living on anomuran crabs.

As far as we know, *L. spinosa* is the only crab living in the Argentine Sea that carries sea anemones on its carapace. Its association with *A. achates* is mutualistic, probably temporary and restricted to the young stages of the anemone. Future studies with experimental designs will allow us to establish the mechanism by which the mutualistic relationships begin, and the role of both species.

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Spatial memory in a solitary subterranean rodent *Ctenomys talarum (Rodentia : Ctenomyidae)*

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Almost all research on spatial orientation in animals has been conducted on surface-dwelling species (9), however, more recently studies have focused on spatial orientation in subterranean mammals (10,11). Spatial orientation in underground tunnels must be based on restricted sensory input, because of the limited use of auditory and olfactory cues, and the impossibility of using visual cues. This restriction of sensory stimuli necessitates the development of a mechanism for orienting efficiently within the burrows to reduce the energetic costs of digging (12).

Ctenomys talarum (Thomas, 1898) is a small subterranean rodent found in southern parts of South America (13), that inhabits a system of closed galleries parallel to the soil surface. Animals of both sexes and all ages maintain exclusive territories (5). The complex burrow system of *C. talarum* has a branching structure, consisting of a main axial tunnel and a variable number of lateral branches and feeding tunnels, all of them plugged with soil (1). Although individuals of *C. talarum* forage within their tunnels, the majority of foraging bouts occur above ground, and animals must venture away from their tunnels for brief periods to gather vegetation growing on the soil (5). A highly developed spatial orientation capacity and the ability to learn and memorize a tunnel system is crucial for *C. talarum* to achieve successful foraging, reproduction and to defend a home range within their complex subterranean burrows. The objective of this study was to investigate the ability of *C. talarum* to learn and memorize an artificial maze.

Adult tuco-tuco (*Ctenomys talarum*) of both sexes were trapped in Mar de Cobo (Buenos Aires Province, Argentina ; 37˚45´S, 57˚56´W) using plastic live traps set at fresh surface mounds, carried to the laboratory and housed in individual plastic cages (25 cm x 32 cm x 42 cm) and maintained in an animal room with photoperiod and temperature automatically controlled (12 :12 L : D ; 25 ± 1˚ C). Relative ambient humidity ranged from 50 to 70 %. Animals were fed a vegetarian diet. Since *C. talarum* do not drink free water, this source was not provided to the animals. The ability of a total of nine individuals (three males and six females) to learn and memorize a pathway through a burrow system was studied. To investigate the phenomenon of spatial orientation, and following KIMCHI & TERKEL (11) methodology, we built a labyrinth with white PVC tubes (10 cm in diameter) that comprised a series of dead-end paths with one correct path to the goal (Fig. 1).

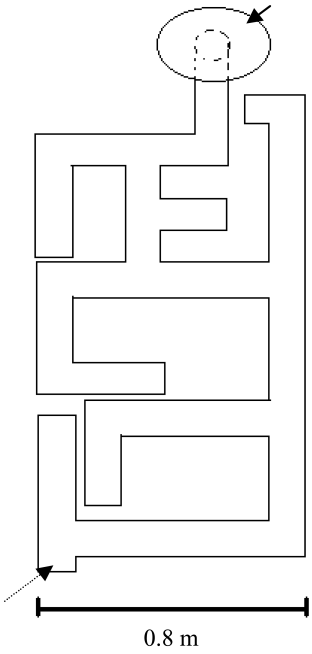

Fig. 1. – Complex labyrinth used for testing spatial memory in *Ctenomys talarum*. The dotted arrow indicates the entrance and the solid arrow the resource goal.

Animals were maintained at 75 and 80 percent of initial body weight for three days prior to the trials to increase their motivation to explore and learn the complex maze. Individuals were introduced into the labyrinth at the entrance, which was subsequently closed. Sweet potato and lettuce were put into the goal cage, the entrance to which was closed with a removable paper lid.

The time spent and the numbers of errors made by individuals to reach the goal cage were recorded for each individual during learning trials (days 1 to 6) and memory trials (15, 30, and 60 days after the learning experiment). In the memory trials each animal was tested only once at only one of the three time intervals. At the end of each trial, the labyrinth was thoroughly washed to ensure that no odors from the previous trial remained. At the end of the experiments animals were fed *ad libitum*, recovered initial body weight after a few days, and remained in good physical condition. They were then returned to their site of capture.

Since both time and error values did not fit a normal distribution, we applied logarithmic transformation to the data. Since error values were zero in some trials we decided to add a constant to this variable before transformation. A repeated measures ANOVA design was used to test the null hypothesis of no differences in time spent to reach the food resource cage and number of errors made before reaching the resource cages during the first to sixth day of

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the learning experiments. A paired t-test was used to test the null hypothesis of no difference in time spent to reach the food resource cage and number of errors made before reaching the resource cages on day 6 of the learning experiment and on days 15, 30 and 60 of the memory experiment.

Concerning spatial learning, individuals of *C. talarum* markedly improved their performance (measured as the number of errors and time spent to reach the resource cage) within the complex maze after the first trial and maintained a similar performance during consecutive trials (Fig. 2). Similar results have been reported for the solitary subterranean rodent, *Spalax ehrenberghi*, which exhibited a clear improvement in performance after the initial trial, and learned a complex maze significantly faster than did surface dwelling rats and voles (11).

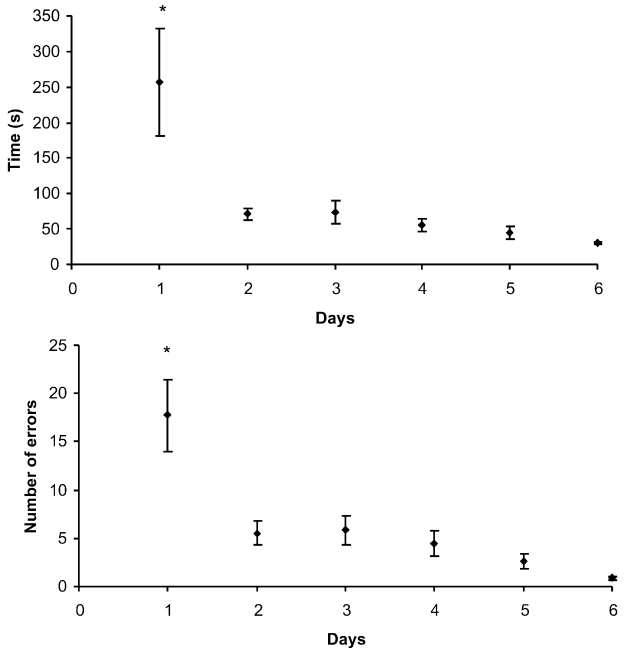

Fig. 2. – Time ± SEM (standard error of the median) spent (A), nd number of errors ± SEM made (B) by *Ctenomys talarum* to reach the resource cage in the complex labyrinth during learning experiment (n = 9). * indicates statistical differences between the first and the other trials ($p < 0.05$).

Sex differences in spatial learning abilities have been documented for several species of rodents (2, 7, 14), while no sex differences were found in the learning performance in other species (8, 11). *C. talarum* exhibited no sex difference in learning ability ($F_{time} = 0.12$, $F_{errors} = 0.15$, d.f. = 7, n = 9, P_{time} and $P_{errors} > 0.5$). This lack of difference in learning performance between males and females of *C. talarum* may be due to the similar size of their burrows (1), since sex differences in learning abilities between male and female rodents have been associated with differences in their home range sizes (8).

The orientation abilities exhibited by many species that are able to avoid or return to particular places in their home ranges involve some kind of spatial memorization (3). *Ctenomys talarum* was able to memorize the complex maze for a period of between 30 and 60 days after the last trial of the learning process, showing a highly developed spatial memory Comparable results were recorded for *S. ehrenbergi* (11) (Table I).

TABLE 1

Time that animals spent traveling trough the labyrinth to reach the resource cage (Time) and number of errors made to reach the resource cage (errors) for the sixth day of the learning trial and for all the days of the memory trial. Values are showed as mean ± SD. (RM ANOVA $t_{time\ 6\text{-}15} = -0.36$, $t_{time\ 6\text{-}30} = -2.92$, $t_{time\ 6\text{-}60} = -6.58$, $t_{error\ 6\text{-}15} = -0.5$, $t_{error\ 6\text{-}30} = -2.52$, $t_{error\ 6\text{-}60} = -4.45$; d.f = 2, n = 3 ; $P_{time\text{-}error\ 6\text{-}15}$, $P_{time\text{-}error\ 6\text{-}30} > 0.05$ and $P_{time\text{-}error\ 6\text{-}60} < 0.05$).

day	Time (seconds)	errors
6	30.78 ± 6.8	0.89 ± 0.6
15	28.67 ± 3.05	1.33 ± 0.58
30	91.33 ± 40.45	4.67 ± 2.31
60	195.67 ± 39.55	13.67 ± 4.16

The restricted sensory input existent in the subterranean ecotope (4) together with the inability of *C. talarum* to use the earth's magnetic field to obtain spatial information (unpubl. data), suggest that *C. talarum* may also rely on internal cues for accurate spatial orientation in their subterranean environment. Internal cues or path integration is a navigational process by which signals generated during locomotion allow the individual to establish its location in relation to its point of departure. Path integration may occur without the help of external references (6). Future studies should focus on determining if *C. talarum* is really able to measure self-generated information without external references to orientate in their subterranean environment.

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