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STASE, METAMORPHOSIS AND COMPETITION IN INSECTS AND OTHER ARTHROPODS

par

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SUMMARY

Based on the stase concept and derived from the Gause model, a new and simple model is proposed to describe the competition between species with complex life cycles (CLCs). Two such species are shown to be able to coexist within the same limited space depending on the population parameters and environment capacity. It is suggested that the metamorphosis and other CLCs would have developed as an inevitable effect of minimizing constraints due to competition and would be a key factor in the diversification of insects and other arthropods.

Keywords : Arthopoda, Insecta, model, instar, CLC (Complex Life Cycle), postembryonic development, diversity

Stase, Métamorphose et Compétition

RÉSUMÉ

Stase, métamorphose et compétition ches les insectes et autres arthropodes. A partir du concept de stase et du modèle de Gause, un nouveau modèle simple est construit pour décrire la compétition entre espèces à cycle vital complexe. Le modèle montre que, en fonction des paramètres démographiques et de la capacité de l'environnement, deux espèces de ce type peuvent coexister dans un même espace limité. Dans une telle appproche, l'unité utilisée pour l'analyse des communautés d'arthropodes n'est plus l'espèce, mais la stase. L'hypothèse est avancée, selon laquelle la métamorphose et autres cycles vitaux complexes se seraient développés comme le résultat inévitable de la minimisation de contraintes dues à la compétition et seraient un facteur clé de la diversification des insectes et autres arthropodes.

Where does all this leave the dogma of insect growth by molting ? Obviously, it leaves it in something of a shambles. A much better generalization would be insect development « by molting » for that would bring metamorphosis into the picture.
C. M. WILLIAMS (1980 : 382)
Insect Biology in the Future

During 1980s the challenge is to formulate new models that can serve as useful simplifications for community processes in systems to which current theory is largely irrelevant.
J. ROUGHGARDEN (1983 : 598)
Competition and theory in community ecology

All scientific knowlege can be thought as a model of the reality, a model which is continually updated as new information accumulates.
P. A. KEDDY (1989 : 48)
Competition

INTRODUCTION

The phenomenon of metamorphosis in insects -and also in other animals- has stimulated the thinking of biologists since the time of ARISTOTLE. Even poets (for example, GOETHE who wrote a poem on animal metamorphosis) and philosophers (for example, the chapter « Zur Philosophie und Wissenschaft der Natur » in the book « Vereinzelte, jedoch systematisch geordnete Gedanken über vielerlei Gegenstände » written by SCHOPENHAUER in 1851) have speculated on the role and function of metamorphosis. Paradoxically, even if numerous theories and ideas have been proposed to explain what metamorphosis is (*e.g.* LAMEERE, 1900 ; PÉREZ, 1903 ; BERLESE, 1913 ; POYARKOFF, 1914 ; HENSON, 1946 ; HINTON, 1948 ; SNODGRASS, 1954, etc), it seems that its biological significance is not fully understood. First, metamorphoses must be considered as a special case of a complex life cycle (CLC). As defined by SLADE and WASSERSUG (1975), CLCs occur whenever organisms pass through two or more distinct ecological and morphological phases for each complete generation. Most common hypotheses view metamorphosis and other CLCs either as a mechanism for predator escape (through adult dispersal) or as a mechanism for reducing competition between stages in the life cycle (by evolving distinct ecological stages). SOUTHWOOD (1978) concludes that metamorphosis enables species to avail themselves of a sequence of niches, utilising resources (food, shelter) that may be separated in both time and space and might not, on their own, be adequate for a whole generation or season. More recently, BERNAYS (1986) attributes nutritional advantages to holometabolous development. However, apart from wordy speculations, few models have been proposed to provide alternative hypotheses that can be tested or falsified. ISTOCK (1967) proposed a life table model and concluded that CLCs are inherently unstable over evolutionary time, hence the name of « Istock's dilemma ». Another model was proposed by BRYANT (1969). It is based on habitat selection in a spatially heterogeneous environment and predicts that such a system should favor the evolution of holometabolous development.

Herein a new approach is proposed which relies on two seemingly unrelated observations. First, the number of species in insects and other arthropods seems to be astronomic. As outlined by JANZEN (1977), the number of species of insects is largely a function of how many species can coexist in a habitat. This clearly implies that interspecific competition must be minimized in one way or another if many species are to coexist. Second, insects and other arthropods have a discontinuous development. In primitive forms, the ontogeny is a succession of stages which are more or less similar and their ontogenetic trajectory, *sensu* ANDRÉ (1988), looks like a short, straight line. In most evolved groups, as the ontogeny becomes a complex life cycle, the ontogenetic trajectory is represented by a zig-zag line, more or less lengthened. Therefore, it was tempting to relate the CLC development of insects to their high diversity and the subsequent minimization of interspecific competition.

To set up the new model, two basic approaches have been called forth. The first is the stase concept, which has been used to take account of the discontinuous ontogeny observed in mites and other arthropods. The second will formalize the interspecific competition and is essentially the classical Gause model.

TWO BASIC APPROACHES

The stase concept

As early as 1873, Sir JOHN LUBBOCK drew a fundamental distinction between animals with different terminal or mature forms and animals which pass through a succession of different forms in the course of their development. He proposed to restrict the term polymorphism to the occurrence of different terminal or mature forms and to call polyeidism the succession of different forms in the course of development. As noticed by WIGGLESWORTH (1954), the essential feature of polymorphism is multiple potentiality, but only one form is realized, all the others remaining latent or suppressed. Conversely, multiple potentiality expressed through polyeidism results in the coexistence of different forms in the same individual, these forms succeeding one another during the course of its ontogeny.

These successive morphs succeeding one another during development conform with the concept of stase propounded by the French acarologist, F. GRANDJEAN (1938, 1951, 1957, 1970). A stase is defined as one of the successive forms through which an arthropod passes, these forms being fundamentally different from one another by the criterion of « all or none ». The criterion of « all or none » means that an organ exists in one form but is absent in another. As emphasized in a previous publication (ANDRÉ, 1988, 1989), the stase concept differs in basic ways from those used in the instar-stage-stadium terminology (Table 1).

In actinotrichid mites and in some insect groups (*e.g.* in Thysanoptera), the difference between stase and instar may appear to be subtle since the number of instars is fixed and corresponds to the number of stases. In some other arthropods however, the number of instars is undefined and variable while the number of stases is well-defined and fixed. This is the case in Collembola where the number of stases

is either six or seven depending on the species (ANDRÉ, 1986, 1987) and in Lepidoptera where the number of larval instars may be highly variable although there are only two larval stases (ANDRÉ, 1989).

TABLE 1

Major features discriminating stases from instars
(From ANDRÉ, 1989)

INSTAR	STASE
Defined by reference to molts	No explicit reference to molts (3)
Two successive instars may be quite similar	Any stase is characterized by all or none criteria applied to idionymic characters (1, 2, 3)
Allometric characters suffice to discriminate instars	Only meristic characters may discriminate stases (1, 2)
Often related to growth	Related to development ; a stase is a level of development (2, 3)
Not necessarily homologous from one species to another	Stases of different species may be idionymic (homologous) (2, 4)
Number constant or variable	Number constant (?)
If variable, number environmentally determined	Number genetically fixed

(1) GRANDJEAN (1938) — (2) GRANDJEAN (1957) — (3) GRANDJEAN (1970) — (4) HAMMEN (1966, 1978).

The Gause model

The Gause model (GAUSE, 1935) is based on a simple logistic equation :

$$dN/dt = r \,.\, N \,.\, (K - N)/K \qquad (1)$$

where N represents the number of individuals,
r is the intrinsic rate of increase of the species,
K is the carrying capacity of the environment, i. e. the maximum population size possible.

The equilibrium density, N^*, is observed when the species reaches the carrying capacity, i.e.

$$N^* = K \qquad (2)$$

In the Gause model, also known as the Lotka-Volterra equations, the growth of two species, forced to compete in a limited amount of space and each with a definite K level, is represented by a pair of differential equations similar to (1) :

$$dN_A/dt = r_A \cdot N_A \cdot (K_A - N_A - \beta N_B)/K_A \quad (3)$$

$$dN_B/dt = r_B \cdot N_B \cdot (K_B - N_B - \alpha N_A)/K_B \quad (4)$$

where α and β are the competition coefficients of species *A* and *B*, respectively, and represent the inhibitory effect of one species on the other.

The four possible outcomes of competition for such species that are forced to compete in a restricted space are well-known and can be determined graphically as shown in Fig. 2 . The major conclusion to be drawn is that, except in a special case (case (4) in Fig. 2), only one of the two species will survive.

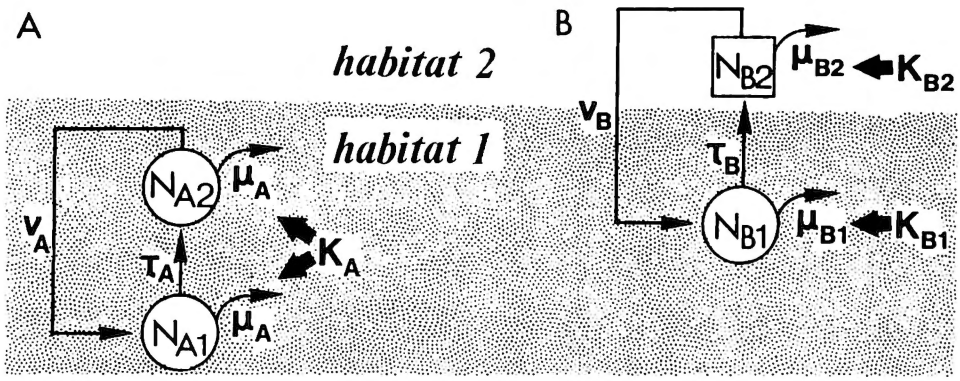

Fig. 1. — Life cycles of one species with two instars (A) and another with two stases (B). Symbols are : *N* : number of individuals, ν : birth rate, τ : transformation rate, μ : mortality rate, *K* : carrying capacity of the environment. The first subscript refers to the species (*A* or *B*), the second designates the level through ontogeny (1 or 2).

SINGLE SPECIES MODELS

This section is aimed at formalizing the concept of stase in contrast to that of instar and integrating stases into a population dynamics perspective. Practically speaking, the problem consists of determining how these concepts may be conveniently embodied in equations.

Species with instars

Let us suppose a species with merely two instars, or possibly two homeomorphic (similar) stases, as in Fig. 1A . All characteristics of both instars are thus identical except that instar 2 (adult) reproduces while instar 1 does not. Growth equations for a species living alone are then, using the symbols in Fig. 1A,

$$dN_{A1}/dt = (\nu_A N_{A2} - \tau_A N_{A1} - \mu_A N_{A1}) \cdot Z_A \quad (5)$$

$$dN_{A2}/dt = (\tau_A N_{A1} - \mu_A N_{A2}) \cdot Z_A \quad (6)$$

which when added give

$$dN_A/dt = (\nu_A N_{A2} - \mu_A N_A) \cdot Z_A \quad (7)$$

where

$$N_A = N_{A1} + N_{A2} \quad (8)$$

and

$$Z_A = (K_A - N_A) / K_A \quad (9)$$

Z_A thus represents the receptivity of environment to species A. Z_A is maximum in the absence of species A and tends to zero when the density of species A approaches the carrying capacity of the environment.

The equilibrium density, N^*, of such a species is reached at the saturation level of the environment, i.e. when

$$N^*_A = K_A \quad (10)$$

Species with stases

Let us suppose a species having a CLC with heteromorphic (distinct) stases as species B in Fig. 1B. The two stases are supposed to occupy different ecological niches. For instance, larvae may be active wrigglers in the water whereas adults are flying and live far away from water. As stases are distinct, different values are attributed to mortality rates and environmental saturation levels depending on the stases. Growth equations of such a species are (symbols as in Fig. 1B) :

$$dN_{B1}/dt = (\nu_B N_{B2} - \tau_B N_{B1} - \mu_{B1} N_{B1}) \cdot Z_{B1} \quad (11)$$

$$dN_{B2}/dt = (\tau_B N_{B1} - \mu_{B2} N_{B2}) \cdot Z_{B2} \quad (12)$$

where

$$Z_{B1} = (K_{B1} - N_{B1})/K_{B1} \quad (13)$$

$$Z_{B2} = (K_{B2} - N_{B2})/K_{B2} \quad (14)$$

Equations (11), (12) and (13) are isomorphic to equations (5), (6) and (9), respectively.

The maximum density reached by each stase depends on the ratios between the values of carrying capacities compared to the ratio of the birth and transformation rates. If

$$\nu_B/\tau_B > K_{B2}/K_{B1} \quad (15)$$

then, the adult environment is saturated first (see Table 2). At saturation of the adult environment, equations (12) and (11) become, respectively :

$$dN_{B2}/dt = 0 \quad (16)$$

$$dN_{B1}/dt = (\nu_B K_{B2} - \tau_B N_{B1} - \mu_{B1} N_{B1}) \cdot Z_{B1} \quad (17)$$

where the product $(\nu_B K_{B2})$ is constant whereas N_{B1} may still increase. Equation (17) will be equal to zero when

$$N_{B1} = K_{B2} \cdot \nu_B/(\tau_B + \mu_{B1}) = Max_{B1} \quad (18)$$

The value reached by N_{B1} in equation (18) represents the maximum larval density, Max_{B1}, determined from population parameters. If Max_{B1} is smaller than K_{B1}, i.e. if the larval density allowed by population parameters, does not reach the environment saturation level, then the larval habitat will remain unsaturated. Otherwise, the larvae will grow until the habitat is saturated.

The same reasoning applies when

$$\nu_B/\tau_B < K_{B2}/K_{B1} \quad (19)$$

i.e. when the larval environment is saturated first. At saturation of the larval habitat, equations (11) and (12) can be rewritten as :

$$dN_{B1}/dt = 0 \quad (20)$$

$$dN_{B2}/dt = (\tau_B K_{B1} - \mu_{B2} N_{B2}) \cdot Z_{B2} \quad (21)$$

The maximum adult density, Max_{B2}, determined from equations (20) and (21) will be :

$$Max_{B2} = K_{B1} \cdot \tau_B/\mu_{B2} \quad (22)$$

Depending on whether Max_{B2} is greater or smaller than K_{B2}, the adults will or will not reach the saturation threshold of their environment.

TABLE 2

The saturation of environments by a species with two stases

IF		HABITAT OF	
		LARVAE	ADULTS
$\nu_B/\tau_B > K_{B2}/K_{B1}$	$Max_{B1} > K_{B1}$	unsaturated	saturated
	$Max_{B1} < K_{B1}$	saturated	saturated
$\nu_B/\tau_B = K_{B2}/K_{B1}$		saturated	saturated
$\nu_B/\tau_B > K_{B2}/K_{B1}$	$Max_{B2} > K_{B2}$	saturated	saturated
	$Max_{B2} < K_{B2}$	saturated	unsaturated

$Max_{B1} = K_{B2} \cdot \nu_B/(\tau_B + \mu_{B1})$ $\quad$ $Max_{B2} = K_{B1} \cdot \tau_B/\mu_{B2}$

The possible outcomes are summarized in Table 2. It turns out that the outcomes do not depend on the initial densities, but only on the environment capacity and population parameters. For instance, it the larval environment is saturated first, the transformation rate is a key factor regulating the saturation or non-saturation of the adult environment while the birth rate has no effect on the outcome.

Conversely, if the adult habitat is saturated first, the birth rate is an important parameter in determining the saturation or non-saturation of the larval environment.

A major consequence of the occurrence of stases in arthropod ontogeny is that the successive habitats colonized by a species are not necessarily saturated, which means that there is some room available for another species to coexist, as demonstrated in the next section.

TWO SPECIES MODELS

From the basic model described in the previous section are derived models where two species are implicated. Four sets of simulations corresponding to different situations will be analyzed. In the simplest case, two species with mere instars will be forced to compete. In the next set of simulations, one of the species will have a CLC with two stases. The last two sets will treat systems with two species, both of which have two stases.

Two species with instars

In this first set of simulations, the two species have merely two instars as in Fig. 1A. All characteristics of both instars are thus identical except that one reproduces and the other does not (see previous section). If two such species are forced to coexist in a limited space, simultaneous growth equations are :

$$dN_A/dt = (\nu_A N_{A2} - \mu_A N_A) \cdot Z_A \quad (23)$$

$$dN_B/dt = (\nu_B N_{B2} - \mu_B N_B) \cdot Z_B \quad (24)$$

Equations (23) and (24) are similar to equation (7) except that the environmental receptivity, Z, takes into account the interspecific competition :

$$Z_A = (K_A - N_A - \beta N_B) / K_A \quad (25)$$

$$Z_B = (K_B - N_B - \alpha N_A) / K_B \quad (26)$$

where α and β are competition coefficients of species A and B respectively.

The four possible outcomes of the competition between the two species are identical with those predicted by the classical Gause model. The corresponding zero growth isoclines are given in Fig. 2. It must be emphasized that the outcome is again independent of the initial densities, except for case (4).

This first set of simulations is essential. Indeed, it clearly shows that species having mere instars do not differ in their population dynamics from others having a continuous development. The fact of dividing the ontogeny into a succession of instars, i.e. discrete units having similar ecological requirements, does not change anything in the conclusions of the classical Gause model. Provided that the population parameters (natality, mortality rates, etc.) are the same, the behavior of species

(i.e. their variation in density) and the outcome of competition are identical for species with a continuous development or with an ontogeny composed of instars.

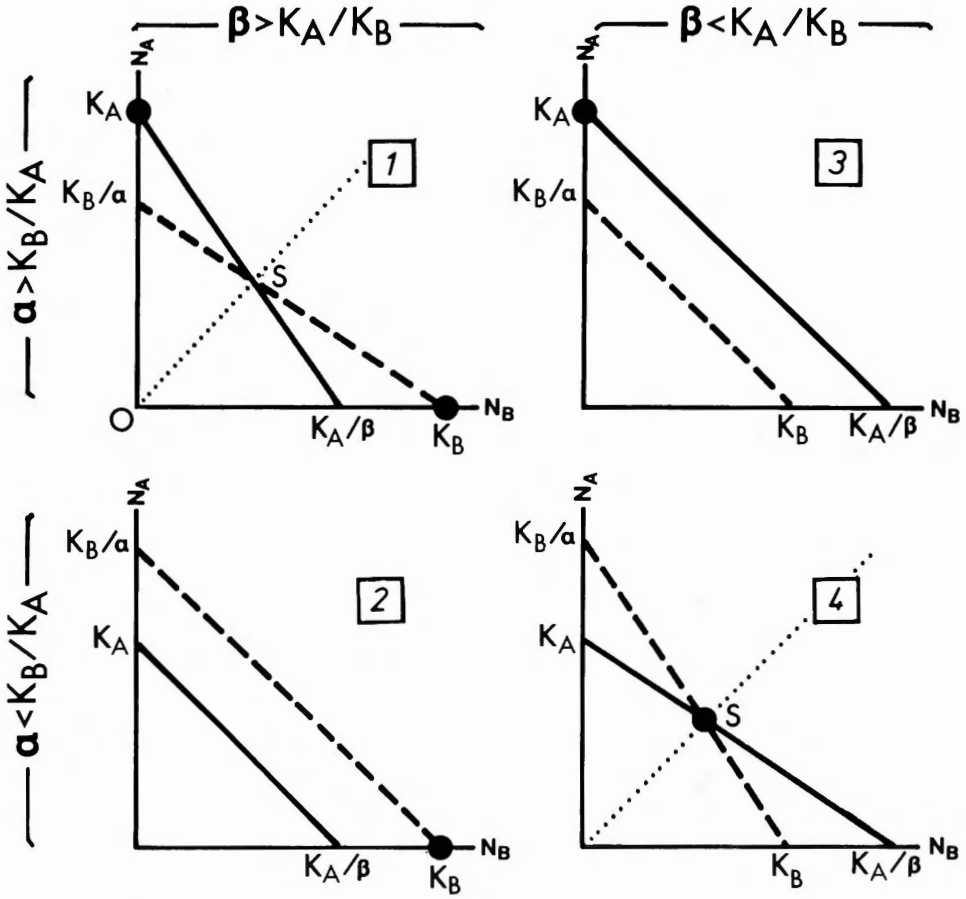

Fig. 2. — Competitive interactions between two species with instars. The four cases and the corresponding conditions are the same as in the Gause model. The solid line represents the zero growth isocline of species *A* while the dashed line refers to species *B*. Black circles represent stable equilibrium points.

One species with instars, another with stases

In this set of simulations, species *A* is supposed to have instars while species *B* develops through two stases. This situation corresponds to Figure 1.

If the maximum density of species *B* larvae, Max_{B1} (see eq. 18), is equal to or greater than the saturation level, K_{B1}, then the outcome is of course similar to that predicted by the classical Gause model. If not, the outcome may be determined by zero growth isoclines as in Fig. 3.

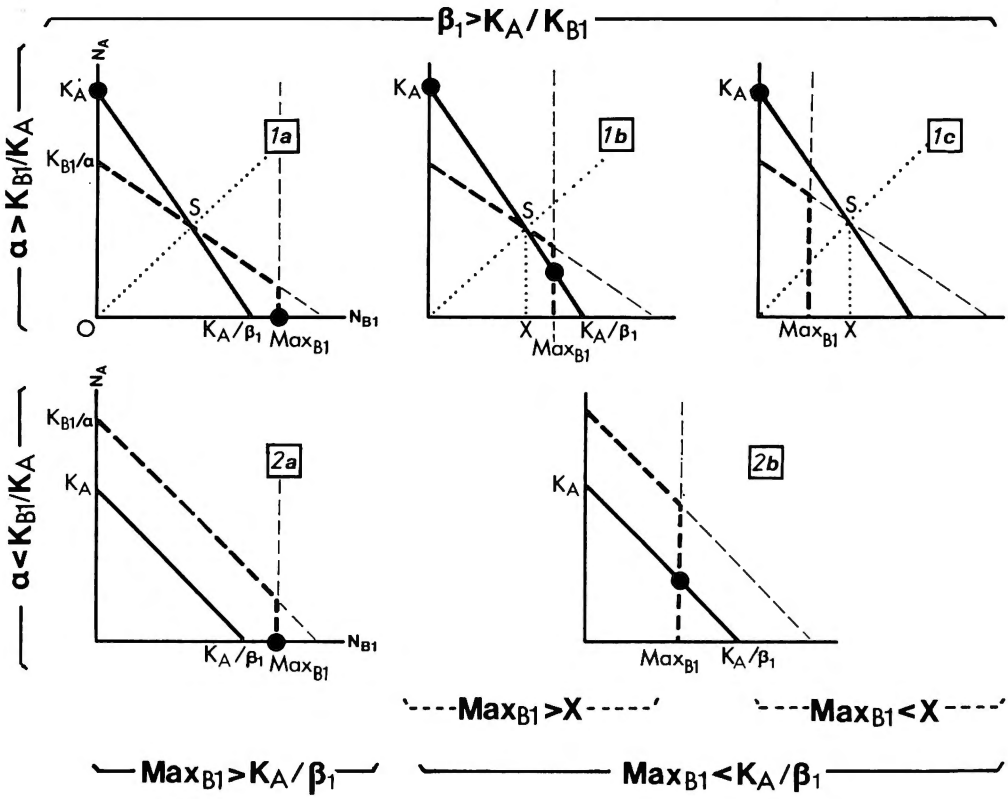

Figure 3 is similar to the zero growth isoclines determined by the Gause model, except that a third isocline, corresponding to Max_{B1} , intercepts the two others. Case (1) of the Gause model (Fig. 2) must be decomposed into three subcases depending on the value of Max_{B1}. If Max_{B1} is greater than K_A/β_1, only one species survives and the outcome depends on the initial densities exactly as in the Gause model. If Max_{B1} is smaller than K_A/β_1 but greater than X (X corresponds to the abscissa of the crossing point S), then an equilibrium is possible depending on the initial densities (case (1b) in Fig. 3). In the triangle S - K_A - K_{B1}/α, species B larvae are above their saturation level while species A is not ; thus if a mixture composed of larvae B and species A is represented by a point located in that triangle, larvae of species B will diminish while species A will continue to increase until species A survives alone. Similarly, if a mixture of larvae B and species A is represented by a point located in the triangle S - K_{B1} - K_A/β, then larvae of species B should continue to increase while species A should vanish. However, the increase of larvae B is limited by the third isocline, the vertical line corresponding to Max_{B1} in Fig. 3. As a result, the growth of larvae B will stop or be brought back at that value and a equilibrium point is determined by the crossing of the vertical isocline and zero growth isocline of species A. The values of that point are :

$$N^*B_1 = Max_{B1} = K_{B2} \cdot \nu_B / (\tau_B + \mu_{B1}) \qquad (27)$$

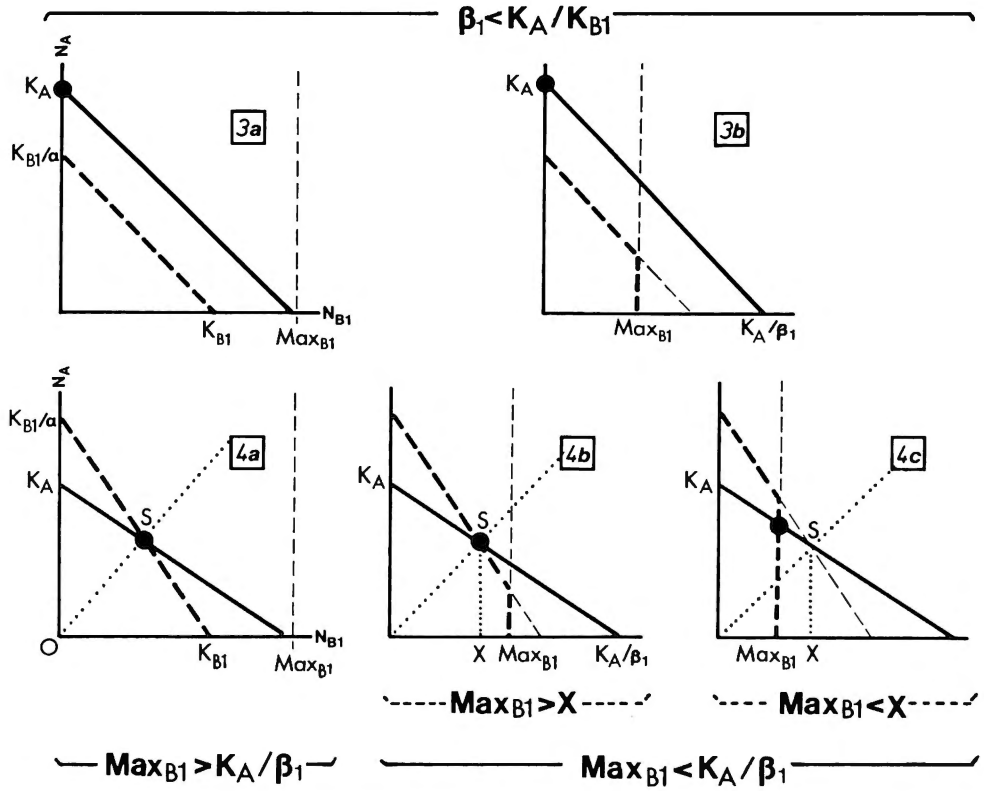

Fig. 3. — Competitive interactions between two species, one with two instars (*A*) and the other with two stases (*B*). Only larvae of species *B* are competing with all individuals of species *A*. The four major cases (1 to 4) answer the same conditions as those defined in Fig. 2. The solid line represents the zero growth isocline of species *A* while dashed line refer to species *B* larvae.

$$N^*_A = K_A - \beta \, . \, Max_{B1} \tag{28}$$

while N^*B_2 will be equal to K_{B2}.

Lastly, if Max_{B1} is smaller than X, then species A will always be the sole survivor (case 1(c) in Fig. 3).

Case (2) of the Gause model must be decomposed into two subcases, again depending on the value of Max_{B1}. If Max_{B1} is greater than or equal to K_A/β_1, then species B will always survive whatever the initial densities (case (2a) in Fig. 3). But if Max_{B1} is smaller than K_A/β_1, then an equilibrium will be reached (case (2b) in Fig. 3).

Similarly to case (2), case (3) of the Gause model may be subdivided into two subcases depending on the value of Max_{B1}. In both subcases (3a and 3b in Fig. 3), only species A will survive.

Lastly similarly to case (1), case (4) of the Gause model may be subdivided into three subcases (cases (4a), (4b) and (4c) in fig. 3). In all subcases, an equilibrium is observed. Note that in case (4c), the equilibrium point does not correspond to the crossing point S contrary to the two other subcases.

In conclusion, there are ten subcases distinguished in Fig. 3. In four subcases, an equilibrium will always be reached while, in one subcase (2b), an equilibrium may be observed depending on the initial densities (Table 3).

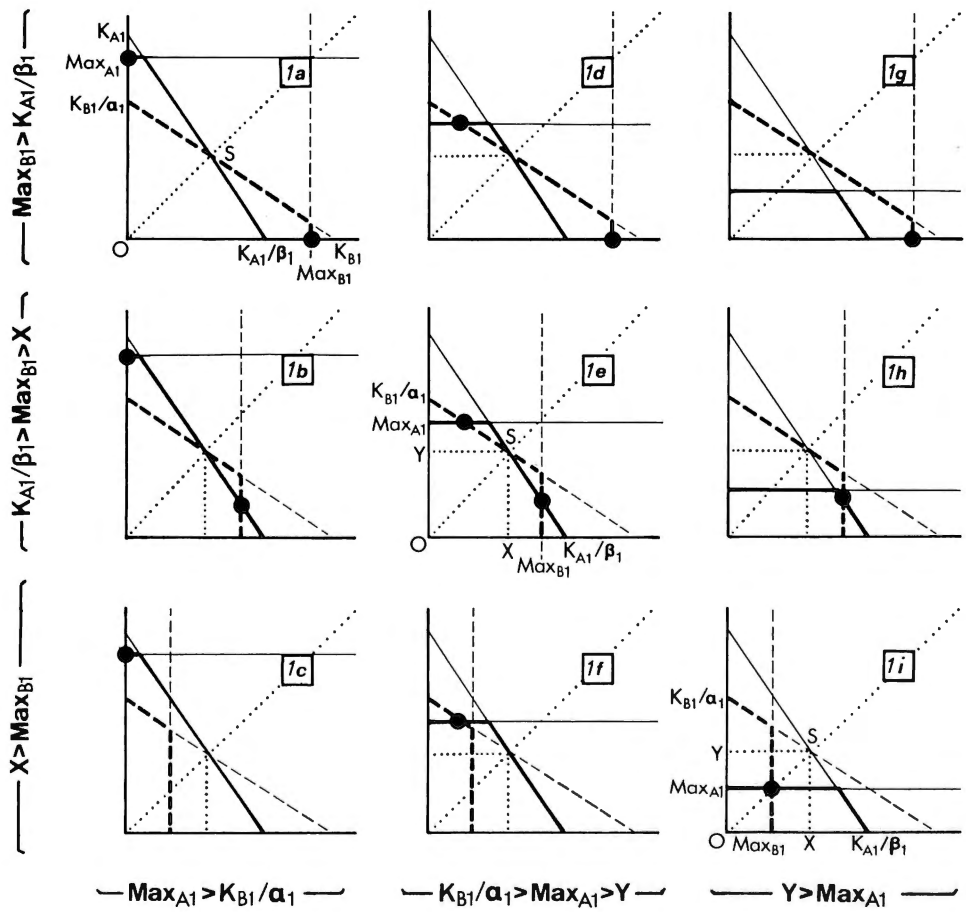

Fig. 4. — Competitive interactions between larvae of two species with stases when $\alpha_1 > K_{B1}/K_{A1}$ and $\beta_1 > K_{A1}/K_{B1}$ (case 1 in Fig. 2). Nine subcases are distinguished depending on the values of Max_{A1} and Max_{B1}. Solid lines represent the zero growth isoclines of species *A* larvae while dashed line refer to species *B* larvae.

Two species with stases (one-level competition)

In this situation, both species are supposed to have stases as in Fig. 1B but the competition is restricted to one stase, for instance to larvae. In parallel to the previous section where case (1) of the Gause model was subdivided into three subcases depending on the value of Max_{B1}, case (1) must be decomposed into nine subcases depending on the values of Max_{B1} and Max_{A1} (Fig. 4). It is easy to see that an equilibrium point is always reached in four subcases (1e, 1f, 1h and 1i), that it may be observed in two subcases (1b and 1d) depending on the initial densities, and that only one species will always survive in the last three subcases (1a, 1c and 1g).

TABLE 3

Number of possible outcomes in the three situations reported in the text. Max_{B1} is supposed to be smaller than K_A/β_1. The same assumption applies to Max_{A1} in the third situation. Unstable equilibriums are not considered.

Situation	Total number of cases	Number of cases where equilibrium is		
		always reached	possible (1)	never observed
Two species with two instars	4	1	0	3
One species with instars, the other with stases	10	4	1	5
Two species with two stases	26	17	2	7

(1) Depending on initial densities.

Similarly, case (2) of the Gause model must be decomposed into four subcases depending on whether Max_{A1} and Max_{B1} are greater or smaller than K_{A1}/β_1 and K_{B1}/α_1, respectively. An equilibrium point will be observed if Max_{B1} is smaller than K_{B1}/α_1 whatever the value of Max_{A1}, otherwise only species B will survive. Case (3) is symmetrical to case (2) and an equilibrium point will be reached in two subcases out of four. Lastly nine subcases may be distinguished in case (4) as in case (1); in all nine subcases, an equilibrium is observed.

It turns out that, among the 26 subcases, an equilibrium is always observed in 17 subcases, i.e. in 65 % of the subcases. Table 3 clearly shows that the more complex are the ontogenies, the more probable will be the equilibrium between the two competing species.

Two species with stases (two-level competition)

In all previous sections, the two species were competing with each other at only one level of their ontogeny, for instance at the larval stase. What happens if both

adults and larvae of the two species are competing ? Due to space limitation, it is not possible to detail all the possible combinations of such a competition. However, it must be stressed that an equilibrium may be reached even if adults and larvae are both competing. An example of simulation is presented in Fig. 5. In this simulation, species *A* is superior in competitive fitness to species *B* at the larval stase but inferior to it at the adult stase. This simulation is important as it corresponds to experimental data presented by AYALA (1969).

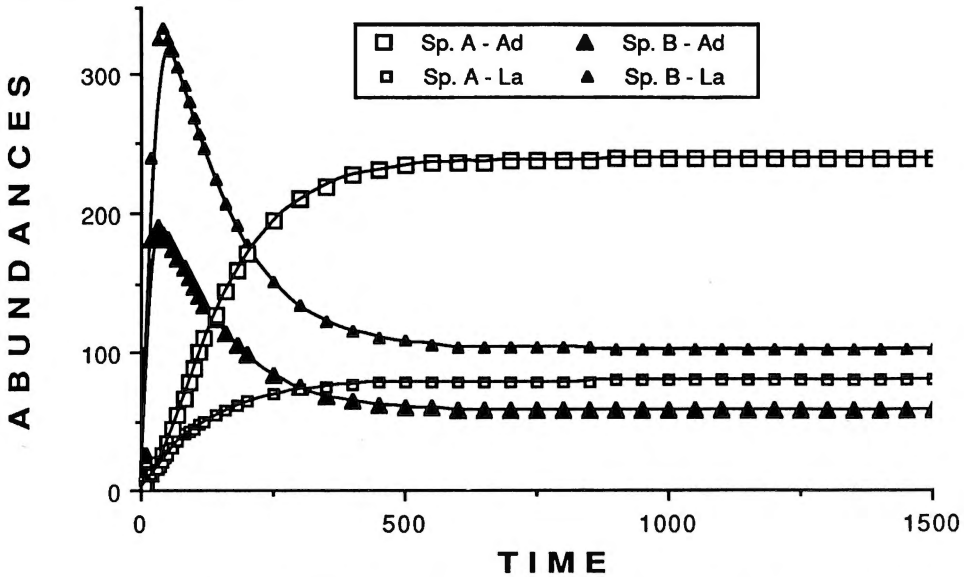

Fig. 5. — Simulation of the growth of two species competing at two levels (larval and adult). Parameters for species *A* are : $\nu = 0.5$; $\tau = 0.3$; $\mu_1 = \mu_2 = 0.10$; $K_1 = 100$; $K_2 = 500$; $\alpha_1 = 2.5$; $\alpha_2 = 0.6$; for species *B* : $\nu = 0.9$; $\tau = 0.45$; $\mu_1 = \mu_2 = 0.05$; $K_1 = 500$; $K_2 = 200$; $\beta_1 = 0.2$; $\beta_2 = 2.0$.

DISCUSSION

The stase concept was proposed more than fifty years ago by GRANDJEAN (1938). Although the concept may apply to all arthropods, its use has been confined almost exclusively to mites and its application seems to have been limited to morphology and related evolutionary problems (cf the recent syntheses by ANDRÉ, 1988, 1989). Beyond these limits, the concept might apply equally well to many other domains such as ecology, genetics, and pest control. This paper aims at proposing, for the first time, an hypothesis explaining the existence of stases and the derived phenomena of metamorphosis and CLCs. Specifically, the question of a possible relationship between the discontinuous development of insects and other arthropods and the high species diversity observed in these groups is addressed and explored through a new and simple model derived from the classical Gause model.

The choice of the model

MAY (1973) made a fundamental distinction between « tactical » models which strive for a detailed and pragmatic description of quite specific systems, and « strategic » models which sacrifices precision in an effort to grasp general principles. As emphasized by MAY (1973), strategic models do not correspond in detail to any single real situation but aim to provide a conceptual framework for the discussion of broad classes of phenomena. On the other hand, LEVINS (1968), and later MAYNARD-SMITH (1974), pointed out that we should not look for assertions that are true of all systems. Instead we should look for the causes of differences of behavior between different systems. To answer this type of question, we need models that are as simple as possible (MAYNARD-SMITH, 1974). In other words, the important question is this : does a slightly more complicated model yield significantly more accurate results (GILPIN and AYALA, 1973) ?

Practically speaking, it seems obvious that the new model had to be an extension of the classical Gause model. This model is so popular in ecology that the study of equations themselves is recognized as ecological research (FAGERSTRÖM, 1987). It is the archetype of exploratory models, i.e. models which allow the logical consequences of changes in assumptions or initial conditions to be explored systematically (KEDDY, 1989). In this way, it is possible to meet the first three criteria proposed by GILPIN and AYALA (1973) to arrive at what they call the best model of growth and competition, namely simplicity, reality and generality.

A classical argument advanced against the Gause model is that it is simplistic. Such an argument holds also for the new model. For instance, it assumes that the environment is uniform, which is hardly realistic. It is also simplistic to assume that the environment does not vary in time and there is no delay in the response of each species to the other. It does not account for indirect effects of *n*-species competition, which can make it extremely difficult to extrapolate results from pair-wise experiments to multi-species interactions (MOEN, 1989). Lastly, the new model is simplistic in that it entails only two stases per species while the ontogeny of insects and other arthropods is generally composed of five or more stases. Nevertheless, all of these assumptions have been retained because the more complex a mathematical model becomes, the less generalized is its applicability to ecological situations. Furthermore, in spite of such simplistic assumptions, this study reveals that the coexistence between species forced to live together in a limited space, at least temporarily, is possible provided that their ontogenies comprise distinct stases.

Other weaknesses of the model are that all the immediate and indirect effects of larval competition are not detailed as in studies propounded by NISBET and GURNEY (1984). The adaptive interactions between successive stases of the same species also are disregarded although it is well known that environmental conditions experienced by larvae can affect the adult population (*see e.g.* SIGURJØNSDØTTIR, 1984 ; PROUT and MCCHESSEY, 1985 ; SIMMONS, 1987). However such weaknesses are thought to be inherent to any strategic model; they do not diminish the value of the conclusions drawn from the comparison between the Gause model and the new model derived from it.

Relationships with other models

Comparison with the Gause model

The new differs in basic ways from the Gause model due to the introduction of the stase concept. However, when the differences between stases are reduced to zero, the new model behaves exactly as the Gause model (see the first set of simulation). The latter turns out to be a special case of the former.

There is a second difference. As pointed out by WIENS (1977), in virtually all mathematical treatments of competition, the populations occupying a given environment are supposed to be at their respective carrying capacities, and suitable habitats are thus saturated. WIENS (1977) violently denounces this « assumption which lies at the heart of the classical Lotka-Volterra competition formulation and its extensions ». Obviously, the new model, though derived from the Gause model, escapes this critique.

Age-structure models vs. stase-structure models

Many models of age-structured populations have been proposed (see the synthesis by CHARLESWORTH, 1980). However, it must be emphasized that such models have been designed to apply to both animals and plants with continuous development, as well as to organisms with discrete age classes. It is not by chance that CHARLESWORTH (1980) cited man as one of the two best- known examples of age-structured population.

LEFKOVITCH (1965) was particularly concerned with the population dynamics of insect pests in stored products and noticed that, if it was difficult to estimate the age of these insects, their *stage* could be recognized easily. Accordingly, he proposed, for the first time, a model based on stage groupings rather than age groupings, taking into account the fact that the various stages might have different durations. Despite the numerous models of age-structured populations published subsequent to that of LEFKOVITCH, it was necessary to wait nearly 20 years for the resurrection of the expression « stage structure model » (NISBET and GURNEY, 1984). Nevertheless, the basic assumption underlying most single-species age-structure models still remains that a species has age-specific properties. Even the most recent models applied to insect populations (*e.g.* MACK *et al.*, 1987 ; CROWLEY *et al.*, 1987) are based on arbitrary age classes which have nothing to do with the stase concept, or even with the instar definition. A remarkable exception must, however, be cited : the application and extension of the Leslie matrix model by HADJIBIROS (1975) and CANCELA DA FONSECA and HADJIBIROS (1977) to an oribatid mite population with five active stases.

Age-structure models and competition

PENNYCUICK *et al.* (1968) first designed an age-structure competition model derived from the Leslie matrix model. Their simulations showed that the outcome of competition depended on the values of the competition coefficients, and possibly on the initial density values. In all cases, however, the outcomes were similar to

those predicted by the corresponding Lotka-Volterra equations, even if a time lag was introduced into the system to determine fecundity.

HASSEL and COMINS (1976) explored the general properties of a single age-class model for two-species competition and concluded that it resembled the Lotka-Volterra model in its general properties, i.e. the zero-growth isoclines were linear and the conditions for coexistence of the two competing species were similar. From this basic model, they derived a two age-class competition model. The introduction of such a minimal age-class structure had interesting consequences as it affected both the shape of the zero-growth isoclines, and the number of possible equilibrium points. The Hassel and Comins' model is however fundamentally different from the model presented herein in that it assumes that the generations and stages are discrete and non-overlapping so that adults and larvae are not present at the same time. Another basic difference is the shape of the zero-growth isoclines which are nonlinear in the Hassel and Comins' model and linear in the new model.

More recently, BELLOWS and HASSEL (1984) developed three models for interspecific competition in laboratory populations of two species of bruchid beetles. The first two, the single age-class and the two age-class models, predicted an unstable equilibrium, departures from which could lead to the extinction of either species. The third model, derived from a single-species system model previously described by BELLOWS (1982), predicted the inevitable extinction of the species with the longer generation time. The outcomes of the three models are thus fundamentally different from the predictions obtained with the new model proposed herein.

In conclusion, it seems that the introduction of an age-structure into a model for two-species competition does not fundamentally modify the predictions of the basic model. This is in accordance with the results of my competition model where the two species have instars : the predictions of the Gause model still hold.

Comparison with Istock's model

The new model is fundamentally different from ISTOCK's (1967) model, which was based on a life table approach. If Istock's model predicts that CLCs are inherently unstable over evolutionary time, the new model suggests that species occupying the same environments may coexist when their ontogeny comprises distinct stases. The coexistence is possible even if all distinct stases compete at several levels of ontogeny, provided that the successive stases occupy different ontogenetic niches. This recalls, in some respects, the conclusions of BRYANT (1969) that habitat selection in a spatially heterogeneous environment should favor the evolution of holometabolous development.

Field experiments

Among the 164 field experiments on interspecific competition listed by SCHOENER (1983), only 31 (19 %) bear on arthropods (insects : 21, crustaceans : 5, arachnids : 6). Six additional papers deal with arthropods in competition with other taxa. This survey shows that arthropods are dramatically under-represented ; as

pointed out by KEDDY (1989), there is no correspondence between the abundance of organisms in the biosphere and the effort invested in studying them. Similar conclusions may be drawn from the survey undertaken by CONNELL (1983) : out of 72 studies on interspecific competition, only 13 (18 %) are concerned with arthropods.

All the experiments listed in these surveys offer the same bias. Either the arthropod species are treated as a homogeneous entity (i.e. all stases are confused or supposed to be equally competitive) or the experiments focuse on only one stase without any reference to the others. The same bias is observed in more recent publications.

To explain the species coexistence observed in many cases, authors invoke either the abundance of food resources (TOKESCHI, 1986), or the absence of strong competitive interactions (GIBSON and VISSER, 1982 ; STRONG, 1982 ; TOKESCHI and TOWNSEND, 1987 ; HUNTER and YEARGAN, 1989), or the spatial aggregation (ATKINSON and SHORROCKS, 1984 ; ATKINSON, 1985 ; TOKESCHI and TOWNSEND, 1987). In no case has the existence of successive stases, each with different competitive abilities, been proposed as an explanation to coexistence. However, a recent paper (HOVENKAMP, 1989) is very stimulating, for it shows that the coexistence between *Daphnia* species might be explained in terms of instar-dependent mortality rates.

Other experimental evidences

There is considerable experimental evidence to support the conclusions drawn from the new model. In experiments carried out by PARK (1948) on *Tribolium*, the coexistence of two species was obtained when the system was « open », i.e. when individuals of each species were artificially removed. Similar results were obtained with hydras by SLOBDOKIN (1961). The transformation of individuals from one stase to another (see the transformation rate in Fig. 1B) obviously plays the same role as the artificial removal performed by PARK and by SLOBDOKIN. This transformation through development also recalls the effect of predation which prevent prey species from competing themselves by depressing their populations (cf. WIENS, 1977).

Another famous experiment carried out by AYALA (1969), who succeeded in rearing *Drosophila serrata* and *D. pseudoobscura* together, also supports the new model. AYALA (1969) explained that *D. serrata* was superior in competitive fitness to *D. pseudoobscura* at the larval stage but inferior to it at the adult stage. This explanation fits the case simulated in Fig. 5. After Ayala's demonstration, GAUSE (1970) correctly argued that his experimental environment contained two habitats : a solid phase and an aerial phase. In reply to GAUSE, AYALA (1970) rightly retorted that the *Drosophila* had to compete in both phases. These remarks both apply to the situation simulated in Fig. 5 and emphasize the properties that the new model and AYALA's experiment have in common. As a conclusion, AYALA (1969) stated that the Volterra equations did not give an adequate description of the competition between two drosophilid species. This conclusion may be generalized by stating that, if the Volterra equations describe competition between species having a continuous development, they cannot account for species with CLCs, i.e. for species

having different competition coefficients depending on the stase. However, a slight modification of the original model, i.e. the introduction of a minimal age-class structure based on the stase concept, yields significantly more accurate results and allows the Volterra equations to account for species with CLCs. Therefore, the new model is preferred to other more sophisticated models which have been designed to explain AYALA's data but do not consider the discrete ontogeny observed in insects and other arthropods (GILPIN and JUSTICE, 1972 ; GILPIN and AYALA, 1973 ; AYALA *et al.*, 1973).

CONCLUSIONS

The approach outlined in this paper shows that the development through stases observed in arthropods may, under some conditions, lead to densities which are, for certains stases, well below their respective carrying capacities. As a result, habitats are not necessarily saturated by a given stase.

Due to the non-saturation of habitats by certain stases, the risk of competition between these stases is reduced.

Consequently, the probability of coexistence between species arises with the complexity of development. Two extreme cases are illustrated in Fig. 1 where an ontogeny composed of two similar instars (Fig. 1A) is compared to an ontogeny comprising two quite different stases (Fig. 1B). The outcomes of competition between species with instars (i.e. between age-structured populations) are those predicted by the Gause model. In contrast, the introduction of a minimal stase structure into the Gause model has remarkable consequences since the number of possible equilibrium points increases. As summarized in Table 3, coexistence is all the more probable as the ontogenies become more complex. In this approach, the unit of selective competition is not the species any more, but the stase.

Inasmuch as closely related species are likely to compete, a taxon with complex ontogenies should be more diversified than a taxon with simple development. In primitive insect groups, stases are little differentiated. This is the case in Collembola where stases are very similar except for the first one which may, in some species, occupy an ecological niche quite different from that colonized by older stases. The number of species in Collembola is much lower than that observed in more evolved groups. Most evolved insect orders, such as Coleoptera, in which CLCs and metamorphoses occur, are in fact, extremely speciose. Ontogenies in most evolved groups may become very complex and involve hypermetamorphoses, the presence of several calyptostases (non-feeding and non-walking forms supposed to have low competitive abilities), the heteroxeny observed in many parasites or the alternation of heteromorphic generations as in heterogony.

In conclusion, a new hypothesis is proposed to explain the role and function of CLCs in insects and other arthropods. Metamorphosis and other life cycle complexities observed in arthropods may have developed as an inevitable effect of minimizing constraints due to competition between species living in the same habitat. This minimization of interspecific competition has allowed arthropod species to

diversify to an extreme degree. Although metamorphosis and competition are seemingly unrelated concepts, the former appears to be a way of minimizing the latter and is a prerequisite to the fabulous diversity found in arthropods. This hypothesis supplements SOUTHWOOD's (1978) views mentioned in the introduction but strongly disagrees with MAY's (1978) opinion that the limits to similarity and niche overlap have little to do with the relative diversity of insect faunas.

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STRUCTURE AND FUNCTION OF THE EXOCRINE GLANDS OF THE GENITALIA OF FEMALES OF THE TWO-SPOT LADYBIRD, *ADALIA BIPUNCTATA* (LINNAEUS, 1758) (COLEOPTERA : COCCINELLIDAE)

by

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ABSTRACT

In females of *Adalia bipunctata* (LINNAEUS, 1758), there are exocrine glands associated with the coxites that form part of the genitalia. The coxites are extended anteriorly in the form of a gutter with the concavity orientated towards the vagina. The edges are continuous with a shiny membrane that forms a coxal reservoir. The concave side of this gutter-like extension is lined by a layer of glandular cells. Their ultrastructure is typical of the type 1 insect epidermal cells of NOIROT and QUENNEDEY (1974). The coxal cells undergo a three stage activity cycle that is synchronised with the ovarial activity and correlated with ladybird age. The huge development of the smooth endoplasmic reticulum in the third stage of the activity cycle indicates that terpenoid substances are produced. On the basis of behavioural observations we suggest that these exocrine glands have a role in the sexual behaviour of *A. bipunctata* although we cannot exclude a role in the chemical protection of eggs.

Key-words : Adalia bipunctata, exocrine glands, ultrastructure.

INTRODUCTION

In the course of a histological study of the genital tube in the female *Adalia bipunctata* (LINNAEUS, 1758) to determine the effect of climatic and trophic conditions on reproductive activity, our attention was attracted by a shiny, kidney-shaped organ on either side of the vagina. Preliminary observations indicated that these organs are exocrine glands associated with the genitalia (HEMPTINNE, 1989).

In this paper we describe the morphology of the genitalia, the ultrastructure of the exocrine glands and discuss their role in ladybird reproduction.

This is the first report of exocrine glands in Coccinellidae ; a family for which no pheromone has been reported (BLUM, 1985 ; HABORNE, 1988). A few papers suggest that chemical mediators could play a role in the sexual behaviour of these beetles. RICHARDS (1980) recorded that adult males of *Leptothea galbula* (MULSANT, 1850) guard female pupae and wait for the females to emerge. This guarding behaviour is thought to be linked with the production of a sex pheromone by both the pupal and teneral adult stages of the females. OBATA (1987) suggested that in *Harmonia axyridis* PALLAS, 1773, mating is triggered by chemicals secreted by the females.

MATERIALS AND METHODS

Experimental insects

Observations were made in the laboratory on adults of *A. bipunctata* fed a mixture of instars of the pea aphid (*Acyrthosiphon pisum* HARRIS, 1776). The beetles were housed in an environmental chamber at 21 +/− 1°C and a 16L:8D photoperiod. Fourth instar larvae were isolated in Petri dishes and their sex when teneral adults was determined by checking the shape of their last abdominal segment. Beetles were mated when 24 h old.

HEMPTINNE (1989) has established that there are three main phases of reproductive activity in this ladybird and studied the exocrine cells associated with the genitalia in individuals in each of these phases. In the first phase young adults (age : 1-4 days) did not display any sexual activity ; in the second phase adults (age : 5-10 days) began to copulate and in the third phase adult females (age : 11-21 days) began egg laying.

Light microscopy

General staining method

The abdomens of the female beetles were cut off, fixed in Bouin-Hollande solution and embedded in paraffin wax. After embedding, the specimens were softened in Mollifex® (Gurr) for 24 h and serial sections were cut at a thickness of 7 µm. These sections were successively stained with Heidenhain's iron hematoxylin, phloxine and light green. This technique was used to determine the relationship between the activity of the exocrine cells and the reproductive activity of the females.

Specific staining methods

Lipids : the abdomens of female beetles were cut off, dipped in liquid nitrogen and sectioned in a cold-chamber cryostat. The sections were stained with Sudan

black B. In addition, the genitalia of some females were dissected, pared of fat and directly treated with the same stain.

Mucopolysaccharides : PAS, Alcian blue (pH 1 and pH 2,5) and 7 biotinylated lectins, provided in an ABC kit by Vector Lab. (Burlingame, CA, USA), were applied to serial sections prepared as above. The staining technique based on the glucidic specificities of the lectins involved three steps : first, the sections are incubated with biotinylated lectins that bind to their target glucoside ; secondly, an avidin-biotin-peroxidase complex reacts with the biotin attached to the lectin and thirdly, the peroxydase radicals appear as brown dots after treatment with a mixture of 3-3'-diaminobenzidine (3-3'-DAB) and H_2O_2 (SHARON and LIZ, 1989).

Electron microscopy

The beetles were dissected in Ringer solution and their genitalia prepared for transmission microscopy. The specimens were fixed for 24 h at 4°C in a solution of 2.25 % paraformaldehyde and 2 % glutaraldehyde in a cacodylate buffer (0.1 M ; pH 7.5). They were subsequently rinsed in the buffer before being post-fixed for 1 h at 4°C in a solution of 2 % OsO_4 in the same buffer. Dehydration and embedding in EPON 812 preceded staining with uranyl acetate and lead citrate. The grids were examined with a Philips EM 202 microscope.

RESULTS

General description

The genitalia of female *A. bipunctata* are made of one coxite and one pleurite on either side of the vagina with the 10^{th} arc-shaped tergite positioned between them. The coxites which are roughly discoidal in shape, extend anteriorly in the form of a gutter, which runs in a plane at right angle to the main part of the coxite. The concavity of this gutter-like extension is orientated towards the vagina and its edges continue as a shiny membrane that forms a coxal reservoir, which encloses the convex side of the gutter. This reservoir has an aperture at the point where it meets the main part of the coxite. The concave side of the gutter is lined by a layer of gland cells. Posteriorly each coxite bears a group of chemosensory setae (PL. IA and IB).

Ultrastructure of the gland cells

Whatever the age of the females, the gland cells show the following characters. The apical surface is invaginated to form a prominent cavity lined with microvilli. This cavity is connected to a spherical hollow located inside the 4 µm thick cuticular intima and filled with entangled filaments. These two cavities are joined by a thin neck. The cells have an approximately ellipsoidal nucleus with

euchromatine regularly dispersed in the nucleoplasm. Their lateral membranes are thrown into a series of folds and adjacent cells are attached to each other by junctional complexes (apical septate desmosomes and zona occludens) (Pl. IC). The microvilli, each about 100 nm in diameter, contain microfibrils around a tubular structure (Pl. IIA). The electron dense entangled filaments inside the extracellular reservoir have a diameter of about 16 nm. In the vicinity of the cuticle, they seem

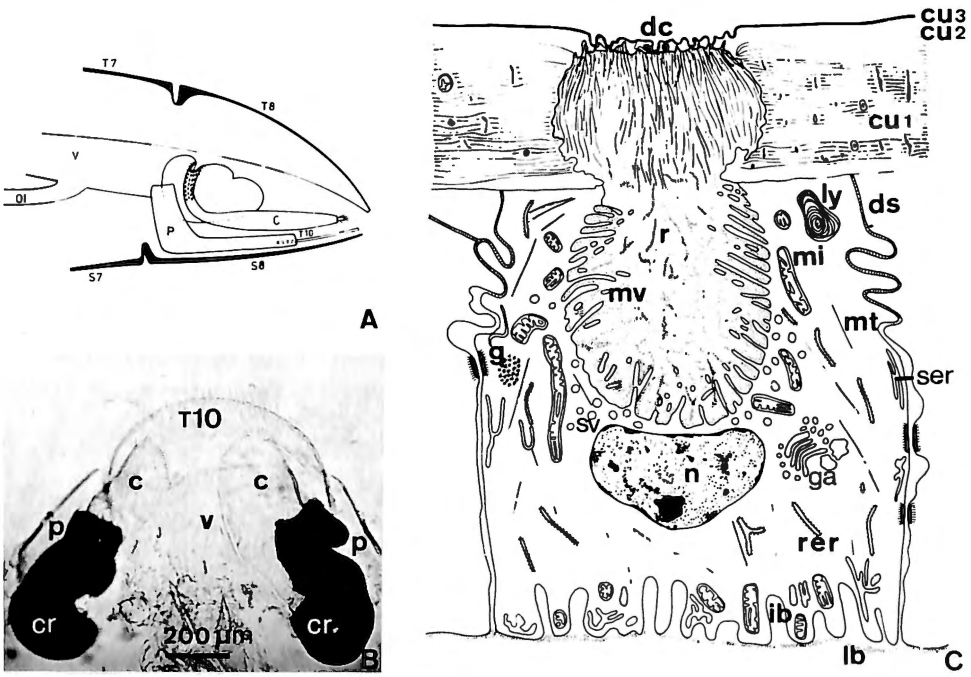


PLATE I

General morphology of the genitalia and associated gland cells in female *Adalia bipunctata*.

A : Diagram of a sagittal section through the abdomen showing the position and the relationship between the different parts of the genitalia.

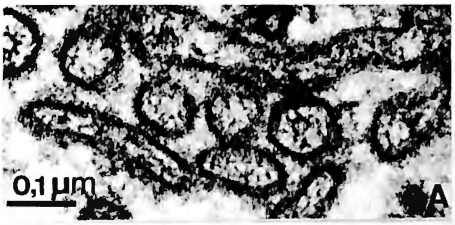
B : In toto preparation of the genitalia with the coxal reservoirs (cr) stained with Sudan black.

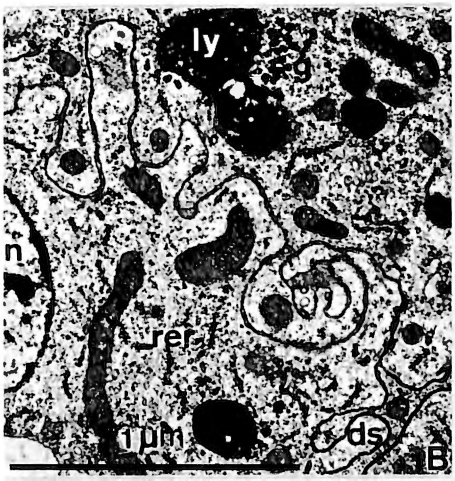
C : Diagrammatic representation of a typical gland cell associated with the genitalia.

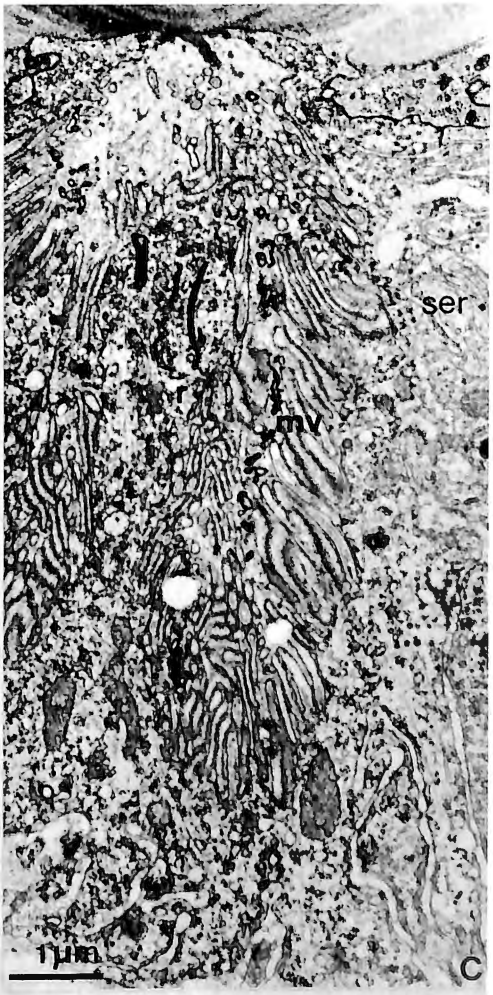
Legend of the symbols

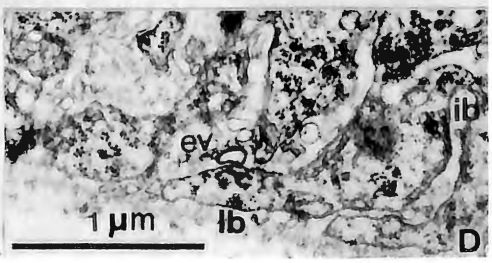
c : coxite ; cr : coxal reservoir ; cu 1, cu 2 and cu 3 : respectively endo-, exo- and epicuticle ; dc : cribellarium ; ds : septate desmosome ; ev : endocytosis vesicle ; g : glycogen ; ga : Golgi apparatus ; ib : infolding of the basal membrane ; lb : basement membrane ; ly : lysosome ; mi : mitochondrion ; mv : microvillus ; mt : microtubule ; n : nucleus ; ol : lateral oviduct ; p : pleurite ; r : extracellular reservoir ; rer : granular endoplasmic reticulum ; ser : smooth endoplasmic reticulum ; sv : secretory vesicle ; s7 and s8 : 7th and 8th sternite ; t7, t8 and t10 : respectively 7th, 8th and 10th tergite ; v : vagina.

PLATE II









more ordered, converging towards the extremities of little tubular infoldings, each about 130 nm in diameter. There are at least 10 of these infoldings forming a permeable area or cribellarium at the outer cuticular surface (Pl. IC and Pl. IIE).

At the base of the epithelium, there are numerous haemocytes, probably spherulocytes in a very active condition with well developed granular endoplasmic reticulum (RER), large Golgi apparatus and many secretory vesicles that are released into the haemolymph inside the coxite gutter.

The general morphology of the secretory cells of the coxites changes with the age of ladybirds.

Stage 1

The cytoplasm has a low electron density and contains the following organelles : elongated mitochondria with a dense matrix, mainly situated close to the extracellular reservoir as well as inside the spaces between the infoldings of the basal membrane. There are many free ribosomes. The RER is wide spread in the cytoplasm whereas the poorly developed smooth endoplasmic reticulum (SER) is located in the vicinity of the basal and lateral membranes. The Golgi apparatus releases some small vesicles containing a flaky electron dense substance. There are also lysosomes and multivesicular bodies, numerous microtubules and granules of stored glycogen (Pl. IIB).

The extracellular reservoir is regularly ovoid. The presence of mitochondria between the infoldings of the basal membrane, evidence of basal endocytosis, and the vesicles from the Golgi apparatus all indicate that the cells are becoming active. In light microscopy, the cells display a basophilic cytoplasm and a small transparent ovoid reservoir.

Stage 2

The cytoplasm has a higher electron density than in the previous stage. The infoldings of the basal membrane are more prominent (Pl. IIC) and the external

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PLATE II

Electron micrographs of gland cells at different stages of maturation.

A : Detail of a cross section of the microvilli lining the extracellular reservoir.
B : Cytoplasm of a gland cell at stage 1.
C : Cytoplasm of a gland cell at stage 2 ; the microvilli (mv) are packed in the extracellular reservoir.
D : Detail of the basal membrane of a gland cell at stage 2. The infoldings of the membrane (ib) are prominent and the external space between them contain vesicles of endocytosis (ev).
E : Cytoplasm of a gland cell at stage 3.

Legends of the symbols as in Plate I.

spaces between them contain small vesicles (Pl. IID) that apparently resulted from the fragmentation of bigger ones produced by the spherulocytes. The intensity of the basal endocytosis is greater and the SER is more extensive. The small vesicles released by the Golgi apparatus (see stage 1) are more abundant and aggregate at the base of the microvilli. These are longer than in the first stage and are tightly packed in the extracellular reservoir into which they extend (Pl. IIC). Under a light microscope, the cells have a basophilic cytoplasm but the extracellular reservoirs are no longer visible. The cellular secretion begins to reach the coxal reservoir through the cribellarium.

Stage 3

During this stage, the cells reach their maximum electron density and the extracellular reservoirs again become visible because they are dilated by the secretion (Pl. IIE). As a consequence of the increase in volume, the microvilli become separated. The basal endocytosis remains intense but more striking is the extensive SER and large lysosomes filled with lamellar structures. Under a light microscope, the extracellular reservoirs are visible and full of secretion but the cytoplasm is no longer basophilic.

The extensive development of both the SER and RER suggests that the coxal cells produce lipid-like substances, proteins or glycoconjugates. The specific dyes used strengthened this hypothesis. The cytoplasm reacted positively to the PAS test and to tests with Alcian blue at pH 1 and 2.5 indicating the presence of neutral and acid mucopolysaccharides. Among the lectins, only Con-A and SBA reacted with the cells. The former, which reveals the presence of α-D-mannose or α-D-glucose, was strictly located near the basal membrane infoldings while the latter which reveals α-D-galactose or N-acetylgalactosamine groups, was associated with the filaments inside the extracellular reservoir. The coxal cells of complete genitalia stained strongly with Sudan black (Pl. IB). Frozen sections always shrank badly probably because we did not find the equivalent of Mollifex® for cuticle softening for cryostat. These sections were unsuitable for pinpointing cells with an affinity for the dye.

Correlation between the morphology of the glands and the reproductive activity of the females

Young females (1 to 4 days old) do not show any sexual activity and their coxal cells are mainly at stage 1. Females from 5 tot 10 days old mainly have stage 2 cells while older ones (11 to 21 days old) are at stage 3 (Tab. 1 ; contingency coefficient $c = 0.63$, $P < 0.001$). HEMPTINNE (1989) also found that ovarial activity also showed three stages. In the first the gonads had differentiated or growing oocytes, the second, oocytes engaged in vitellogenesis and in the third chorionated oocytes in the oviducts. As for the coxal glands, ovary maturation is positively correlated with female age ; the third stage is significantly more frequent in 12-21 days old beetles (Tab. 1 ; contingency coefficient $c = 0.56$, $P < 0.001$). In addition, the fre-

quencies of beetles with immature ovaries and stage 1 coxal cells in the three age groups of ladybirds are identical as are those with mature ovaries and stage 3 coxal cells (Tab. 1, $P > 0.05$). This highlights synchronisation between ovarial and coxal gland activity.

TABLE 1

Frequencies (in %) of the maturation stages (1, 2 and 3) of the ovaries and the coxal glands in females of A. bipunctata 1-4, 5-10 and 11-21 days old

Age group (in days)	Ovaries (%)				Coxal glands (%)			
	n	1	2	3	*n*	1	2	3
1-4	15	66.7	26.6	6.7	15	80.0	13.3	6.7
5-10	43	16.3	32.5	51.2	37	13.6	48.6	37.8
11-21	50	2.0	10.0	88.0	43	4.7	9.3	86.0

Legend : n = number of coccinellid beetles observed ;
maturation stage : see text for description ;
frequencies of ovary and coxal gland stage 1, 2 and 3 do not differ significantly in each age group (two by two proportion comparisons, $P > 0.05$).

DISCUSSION

The morphology of the gland cells associated with the coxites in female *A. bipunctata* is characteristic of the type 1 insect epidermal cell of NOIROT and QUENNEDEY (1974). They release their secretion into an extracellular reservoir which does not communicate directly with the outside. The integument above this reservoir is, however, modified into a thin permeable area (the cribellarium), which facilitates the export of the cells' products into the coxal reservoir. This latter encloses the convex side of the coxal gutter.

Three stages of cell activity were recognised. In the first stage, the cells displayed a low level of activity. During the second stage, the microvilli became so prominent that they completely invaded the extracellular reservoir. This resulted in a further increase in the surface area of the reservoir in order to accommodate the secretion released during the third stage. A similar concomitant development of microvilli and reservoir has also been described in *Periplaneta americana* (LINNAEUS, 1758) (GUPTA and SMITH, 1969) and *Schistocerca paranensis* (BURMEISTER, 1861) (HAWKES *et al.*, 1987). The most important organelle in the gland cells in the third stage is the SER. This feature generally indicates the production of terpenic or steroidic substance as has been observed in the *corpora allata* (SMITH, 1968), the Leydig cells, the hepatocytes and the yellow bodies (ALBERTS *et al.*, 1983 ; PORTER and BONNEVILLE, 1969 ; FAWCETT, 1966 ; SCHOENMAKERS *et al.*, 1977 ; THREADGOLD, 1976) as well as oenocytes that secrete cuticular lipids (LOCKE, 1969). Moreover, the SER is also well developed in the defensive cells of *Ocypus olens* MÜLLER, 1764 and

Drusilla canaliculata (FABRICIUS, 1787), where it is involved in the synthesis of hydrocarbons and aldehydes (ARAUJO, 1978). The activity of the coxal cells and of the ovaries is synchronized and correlated with ladybird age.

The morphology of the genitalia is used as a taxonomic character in coccinellid beetles (SASAJI, 1968) but there is no report of exocrine glands associated with the genitalia in any other species than *A. bipunctata.* However we have observed similar structures in *Coccinella septempunctata* LINNAEUS, 1758 and *Propylea quatuordecimpunctata* (LINNAEUS, 1758).

Our observations combined with information on ladybird behaviour suggest two possible roles for the coxal glands : they could be involved, firstly in egg laying, and secondly in mating behaviour.

Coxal glands and egg laying

The eggs of *A. bipunctata* are bright yellow and laid in batches on plants where aphids are thriving (HODEK, 1973). Thus, the eggs are exposed to predators that forage on the leaves as do the ladybirds (PRICE *et al.,* 1980 ; CARTER *et al.,* 1984). In such circumstances the evolution of a chemical protection for the eggs would be favoured. This kind of defence has been widely recorded in the chrysomelid beetles (PASTEELS *et al.,* 1986, 1988) and the seven-spot ladybird (TURSCH *et al.,* 1971 ; PASTEELS *et al.,* 1973) where the eggs are defended by a maternal allomone produced and incorporated into the yolk during oogenesis (PASTEELS *et al.,* 1986). This however does not rule out the possibility of there being a protective coating in some species (HINTON, 1981). Thus the coxal gland secretion in *A. bipunctata* may serve as glue to attach the eggs to plants and/or as a defensive coating. Of these two hypotheses, the second is more likely because terpenoid substances are better suited to a protective function than to an adhesive one (HARBORNE, 1988 ; PASTEELS *et al.,* 1986, 1988). Moreover, the bursa copulatrix epithelium secretes huge quantities of mucopolysaccharides (HEMPTINNE, 1989) which could act as glue.

Coxal glands and mating behaviour

The description of the mating behaviour of *H. axyridis* by OBATA (1987) suggests that males recognize females by mean of a chemical cue. Similarly during the courtship of *A. bipunctata,* the male generally palpates the apex of the abdomen of its mate before attempting copulation. Palpation occurs less frequently when males are offered freshly killed males (3 h in a freezer) or freshly killed females washed in ethanol for 24 h than with freshly killed females. The application of an ethanol extract of the coxal glands to the females previously washed in ethanol restored their attractiveness to males (HEMPTINNE and MABILDE, own unpublished observations). Although this needs to be confirmed, it is put forward as evidence that coxal cells may synthesize a sexual pheromone. In *A. bipunctata,* these cells commence their secretory activity when the females are approximately 5 to 10 days

old (Table 1), at the time when the first copulations are recorded (HEMPTINNE, 1989).

Further research is needed to discriminate between these two hypotheses, i.e. whether the coxal glands are involved in egg protection or in the production of a sex pheromone. Nevertheless, our observations indicate that various chemicals are important in ladybird ecology and behaviour. An accurate knowledge of the nature of these signals and of their role will improve our understanding of ladybird population ecology and, hopefully, have a positive effect on the design of aphid biological control programs.

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ULTRASTRUCTURAL SURVEY OF TUNIC MORPHOGENESIS IN THE LARVAL AND YOUNG ADULT ASCIDIAN *ASCIDIELLA ASPERSA* (TUNICATA, ASCIDIACEA)

by

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SUMMARY

In *Ascidiella aspersa,* tunic development begins during the tail-bud stage. In the trunk, the larval cuticle and three tunic layers arise consecutively. In the tail, just the larval cuticle and one tunic layer are formed. Shortly before metamorphosis, a new very thin layer arises limiting the tail tunic interiorly. The tunic is composed of granular and fibrillar material embedded in an electron-transparent ground substance. In the course of metamorphosis, the tail is retracted. The larval cuticle and the first tunic layer are discharged. The young ascidian is just surrounded by a cuticle and one tunic layer.

Key words : Ascidian, *Ascidiella,* tunic.

INTRODUCTION

For a long while the ascidian tunic, that covers the whole body of the larvae and the adult as a protective layer, has been the subject of many investigations since it was the first animal structure where a cellulose-like polysaccharide, called tunicin, could be demonstrated (for review see : SAINT-HILAIRE, 1931 ; PRUVOT-FOL, 1951).

Although the consistency of the tunic varies remarkably among different species, some structural and biochemical features seem to be characteristic of the ascidian tunic. The adult tunic is a fibrous structure consisting of a thin cuticular layer supported by a large fundamental layer of hydrated ground substance (VAN DAELE, 1989), comprising fibrils and various types of intratunical cells (ENDEAN, 1961 ; STIÉVENART, 1970, 1971 ; DE LEO *et al.,* 1976, 1977 ; DE LEO and PATRICOLO, 1980 ; VAN DAELE and GOFFINET, 1987). In contrast to other invertebrates, eg. the arthropods where the cuticle comprises the whole non-cellular part of the integument, in tunicates the terms cuticle or cuticular layer are reserved only for the external layer of the tunic.

Chemical and histochemical studies of the tunic revealed the presence of cellulose and other polysaccharides (containing galactose, glucose, mannose, xylose and fucose), proteins, glycoproteins, sulfated glycans, as well as acid and neutral mucopolysaccharides (ENDEAN, 1961 ; DECK *et al.*, 1966 ; STIÉVENART, 1970, 1971 ; PATRICOLO and FERRARELLA, 1973 ; D'ANCONA LUNETTA and NUARA, 1975 ; PATRICOLO and DE LEO, 1979 ; ALBANO and MOURAO, 1983, 1986).

The main fraction of fibres consists of tunicin, a cellulose-like polysaccharide with associated proteins and acid mucopolysaccharides (SMITH and DEHNEL, 1970 ; DE LEO *et al.*, 1977, 1981 ; PATRICOLO and DE LEO, 1979 ; VAN DAELE *et al.*, 1988).

Recent investigations paid attention to the origin of the larval tunic, regarding the following species : *Ciona intestinalis* (LINNAEUS, 1767) (DILLY, 1969 ; MANCUSO, 1973, 1974 ; GIANGUZZA and DOLCEMASCOLO, 1980), *Perophora orientalis* ÄRNBÄCK, 1936 (TERAKADO, 1970), *Corella inflata* HUNTSMAN, 1912 and *Ascidia paratropa* (HUNTSMAN, 1912) (CLONEY and CAVEY, 1982) and *Distaplia occidentalis* BANCROFT, 1899 (CAVEY and CLONEY, 1984).

To our knowledge, no study has been done following morphogenesis of the tunic from the very beginning up to the young ascidian.

This paper presents ultrastructural investigations on the whole course of tunic development in *Ascidiella aspersa* (MÜLLER, 1776). Cytochemical studies at the ultrastructural level are in progress and will be presented in a subsequent paper.

MATERIAL AND METHODS

Embryos : breeding

Adult specimens of *Ascidiella aspersa* were collected during their breeding season by divers of the Biologische Anstalt Helgoland (BAH) in the vicinity of Heligoland (North Sea).

Sperm and oocytes of this self-fertile species were obtained separately by puncturing their gonoducts. After artificial insemination, the embryos were raised in millipore filtered seawater at room temperature (22° C). The determinate bilateral cleavage is followed by the blastula, gastrula, neurula and tail-bud stage. About 14 hours later, they hatch as tadpole larvae. The free swimming period, lasting just a few hours, is terminated by their settling on a substrate and the beginning of a radical metamorphosis (for description of the normal development in *Ascidiella aspersa* see : NIERMANN-KERKENBERG and HOFMANN, 1989).

TEM-microscopy

Specimens of the appropriate stages were fixed in 5 % glutaraldehyde buffered in 0.1M s-collidin (pH 7.4) containing 0.5 % alcian blue, and postfixed in osmium tetroxide buffered in 0.1M s-collidin compounded with 0.8 % $K_3(FeCN)_6$. They

were dehydrated in a graded series of ethanol solutions and embedded in Epon 812 (LUFT, 1961).

Ultrathin sections were cut with a Porter-Blum MT2-B ultramicrotome, collected on copper grids, stained with uranyl acetate and lead citrate (REYNOLDS, 1963) and observed with a Jeol JEM-1OO SX electron microscope at 80 kV accelerating voltage.

RESULTS

The tunic of the hatched tadpole larva of *Ascidiella aspersa,* that is occupied by several hundreds of test cells, covers the entire epidermis and solely builds up the larval fins. Hence, it is very important in larval locomotion. The tunic of the trunk is pulled out to form six or seven fins that can reach a height of up to 30 µm. The tail possesses a ventral and a dorsal fin, each of about 80 µm in height, and a caudal fin with a length of approximately 170 µm.

The first signs of the larval tunic are seen during the tail-bud stage. A small discontinous ribbon of fibrous material lies upon the epidermis (Pl. I, 1).

Only a few minutes later, the entire surface of the embryo is covered by a thin tunic layer. It consists of a network of interwoven fibrils and patches of granular material embedded in an electron-transparent ground substance. The outer limit is characterized by condensed granular and fibrillar material. The tunic of the trunk forms many thin protrusions that are perpendicularly oriented to its surface and can reach a height of up to 1,6 µm (Pl. I, 2) ; in the tail, the tunic has a rather wave-like appearance (Pl. I, 3).

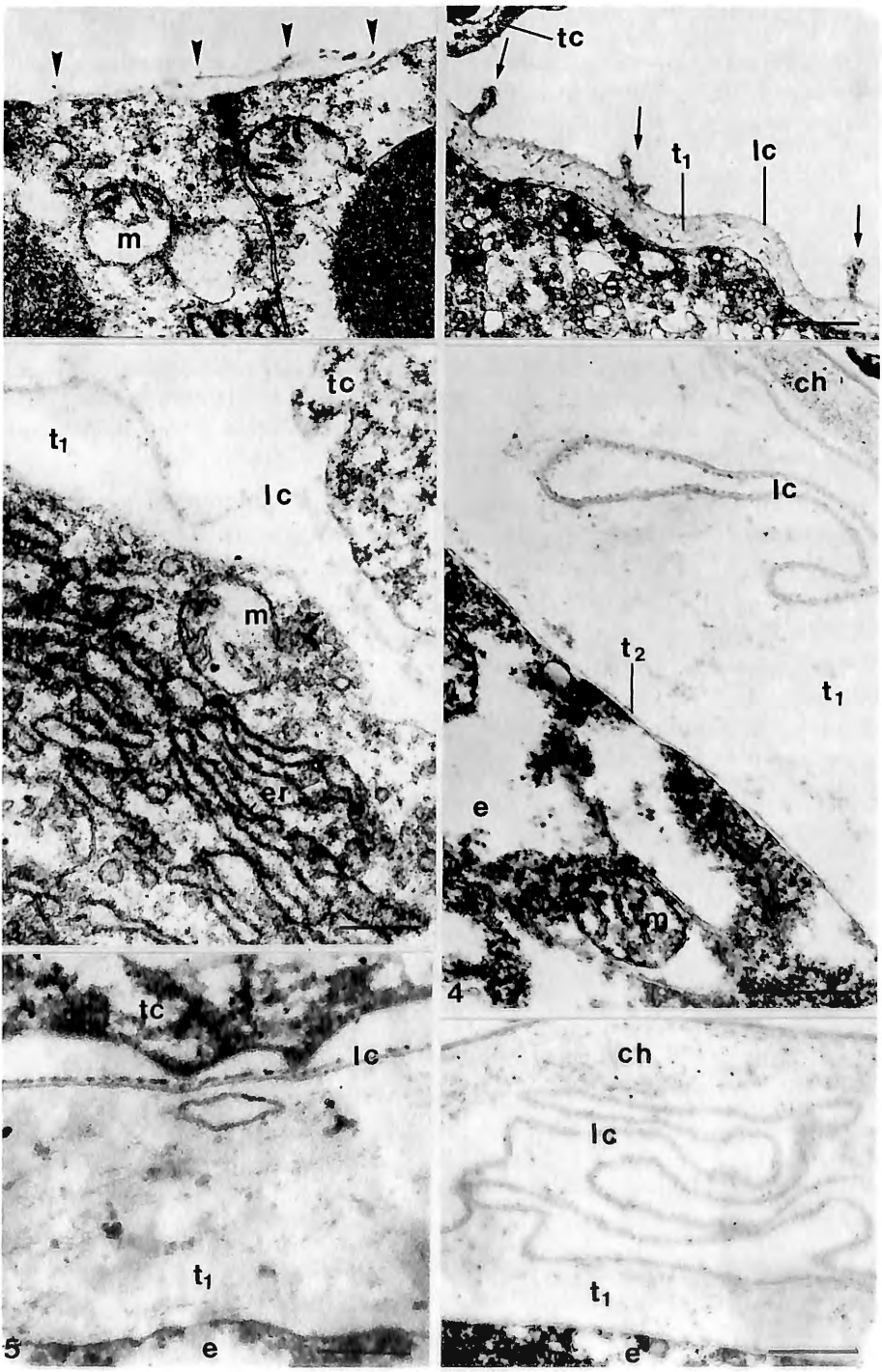
During further progress, an additional fine line of fibrous material appears upon the trunk epidermis (Pl. I, 4). The tunic of the tail enlarges, but there is no further layer visible (Pl I, 5). At the places of the future larval fins, long folded extensions develop. They are made up of the outer dense border and the first tunic layer (Pl. I, 6).

In the trunk, the development of a third tunic layer follows. Pl. II, 7 shows the trunk tunic of a 13h 05min old embryo. The outer dense border, in the following called larval cuticle (according to DILLY, 1969), consists of three sheets. The outer electron-dense one has a dotted appearance in some sections (Pl. I, 4 and 5). The middle sheet is electron-transparent, the inner one an electron-dense continous line.

In the first tunic layer, no special organization of the relatively dense material is recognizable. In contrast, the fibrils of the third tunic layer are arranged almost parallel to the epidermal surface. The second layer, that separates the first and the third from each other, is very thin and resembles the innermost cuticular sheet.

All layers are involved in larval fin formation (Pl. II, 8). At high magnification, it is possible to detect granular material attached to the fibrils of the first tunic layer (Pl. II, 8a).

PLATE I



When the larva hatches, 13 h 40 min after artificial insemination, the larval cuticle is thicker and has lost its stratified arrangement (Pl. II, 9).

The tail tunic still consists of just the larval cuticle and the first tunic layer, that both form the larval fins of the tail (Pl. II, 10).

Some hours later, shortly before the larva attaches to a substrate in order to begin metamorphosis, the tunic of the trunk and the tail looks very different (for comparison see Pl. III, 11 and 12). Now, in the trunk, the larval cuticle and the first tunic layer are thinned out and almost all material is lost. In contrast, the third tunic layer is enlarged. Near the epidermis, the density of fibrils is higher than further outwards. There, they are oriented parallel to the epidermis. In the middle part of the layer, they are arranged reticularly. Near the second tunic layer, they have the propensity to run parallel to it. (Pl. III, 11).

The thickness of the tail tunic hasn't changed, but the arrangement of fibrils is less dense than before. A new, very thin layer limits the tunic interiorly. A small cleft is visible between this layer and the epidermis (Pl. III, 12).

In the course of metamorphosis, the whole tail is resorbed and the entire body is rotated 180° (for description of ascidian metamorphosis see : BARNES, 1980 : p. 1041). The tunic of the tail is shed. The trunk is just surrounded by one tunic layer and a cuticle as soon as metamorphosis is completed. In the tunic, fibrils of random directions are embedded in an electron-lucent ground substance. Near the epidermis, they tend to lie parallel to it. The cuticle is composed of dense granular and fibrillar material which is outwards disaggregated (Pl. III, 13). It is the first time that intratunical cells are seen (Pl. III, 14).

PLATE I

Fig. 1 : First signs of the trunk tunic (arrow heads) on the surface of the epidermis. Tail-bud stage ; age of the embryo : 11 h 10 min. Scale bar : 0.5 µm.

Fig. 2 : The trunk tunic consists of the larval cuticle (lc) and the first tunic layer (t_1). Many protusions (arrows) are formed perpendicularly to the surface of the tunic. Tail-bud stage ; age of the embryo : 11 h 15 min. Scale bar : 1 µm.

Fig. 3 : The tail tunic has a wave-like appearance. The epidermis exhibits a conspicuous rough endoplasmic reticulum (er). Tail-bud stage ; age of the embryo : 11 h 15 min. Scale bar : 0.5 µm.

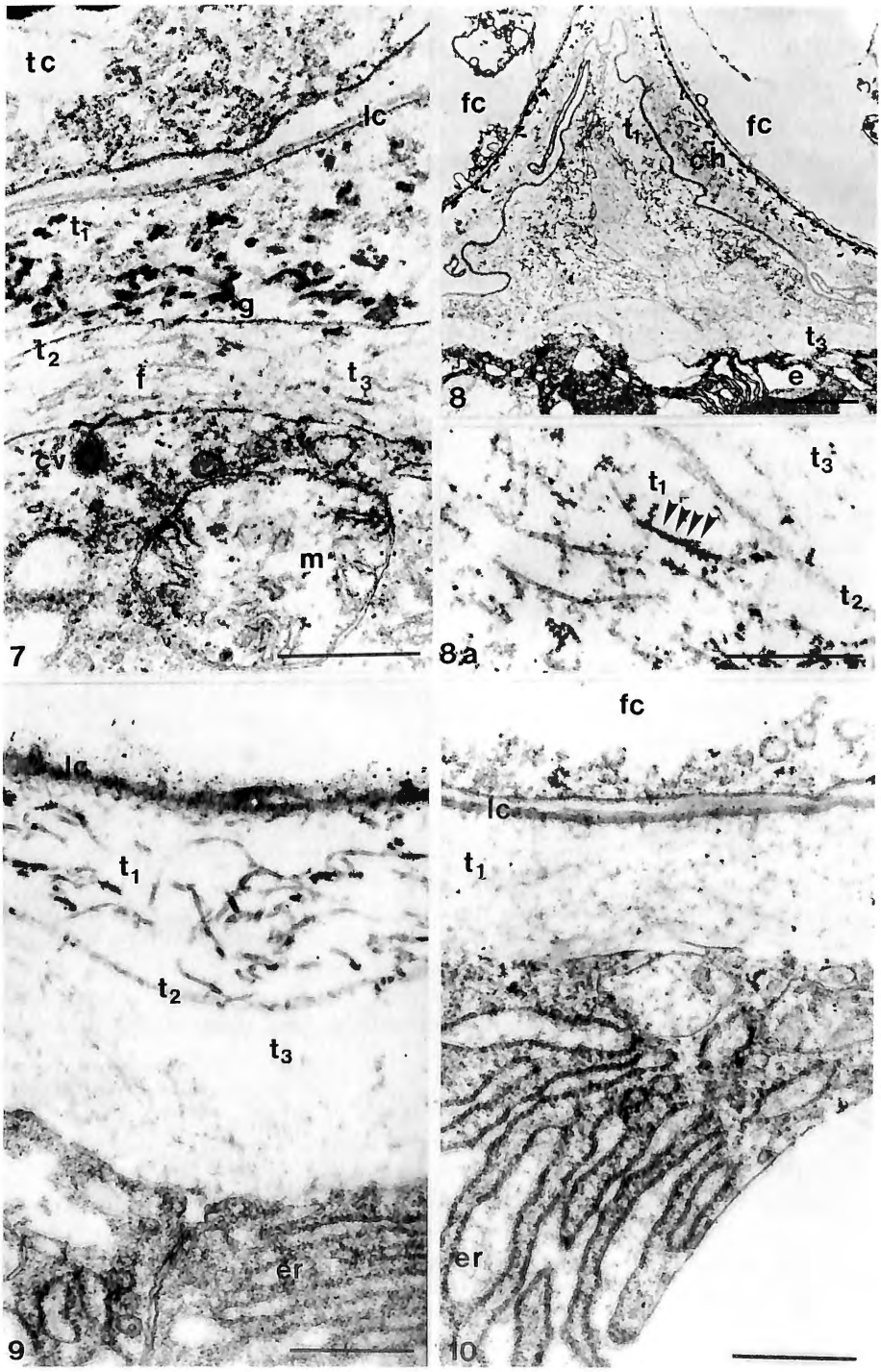
Fig. 4 : In the trunk a second tunic layer (t_2) develops. Tail-bud stage ; age of the embryo : 11 h 20 min. Scale bar : 0.5 µm.

Fig. 5 : No further layer differentiates in the tail. Tail-bud stage ; age of the embryo : 11 h 20 min. Scale bar : 0.2 µm.

Fig. 6 : Long folded extensions mark the places of the future larval fins of the tail. Tail-bud stage ; age of the embryo : 11 h 20 min. Scale bar : 0.5 µm.

ch : chorion ; e : epidermis ; er : endoplasmic reticulum ; lc : larval cuticle ; m : mitochondria ; t_1 : first tunic layer ; t_2 : second tunic layer ; tc : test cell ; v : vesicle ; y : yolk granule.

PLATE II


tc
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10

During the studied period of the embryonic and larval life, the epidermis of the trunk is a columnar (Pl. IV, 15), that of the tail a squamous epithelium (Pl. IV, 16), both resting on a basal lamina. The epidermal cells possess a large nucleus, an extensive rough endoplasmic reticulum (Pl. II, 9 and 10; Pl. III, 11), a Golgi apparatus and many vesicles, ranging in diameter from 0.08 μm to 0.5 μm (Pl. I, 2). These vesicles were never seen fused with the apical plasma membrane. In contrast, coated vesicles are found in the apical region of the epidermal cells, as well as coated pits and coated grooves in the apical plasma membrane (Pl. II, 7).

During the process of metamorphosis, the epidermis of the trunk changes considerably. It becomes a squamous epithelium with a flat, but still prominent nucleus and many mitochondria (Pl. III, 13 and 14). Yolk granules, very common during embryonic and larval life (Pl. I, 1 ; Pl. II, 8 ; Pl. IV, 15 and 16), are now consumed. Cell organelles which characterize a secreting cell are no longer visible. The endoplasmic reticulum has almost disappeared (Pl. III, 13 and 14).

DISCUSSION

Tunic development in the larvae of *Ascidiella aspersa* is executed in several successive phases that are summarized in Fig. 1

Tunic deposition starts during the tail-bud stage. In the trunk, the larval cuticle and three tunic layers arise consecutively. In the tail, the tunic is just built up by the larval cuticle and one tunic layer (Fig. 1, A, B, C, D, E). All layers participate in larval fin formation, in the trunk and the tail respectively.

PLATE II

Fig. 7 : The trunk tunic consists of the larval cuticle (lc) and three tunic layers (t_1, t_2, t_3). The larval cuticle has a tripartite arrangement (see also Figs 4-6). Note the large amount of granular material (g) in the first and the fibrils (f) in the third tunic layer. Tail-bud stage ; age of the embryo : 13h 05min. Scale bar : 0.5 μm.

Fig. 8 : All tunic layers participate in the larval fin formation of the trunk. Tail-bud stage ; age of the embryo : 13 h 05 min. Scale bar : 2 μm.

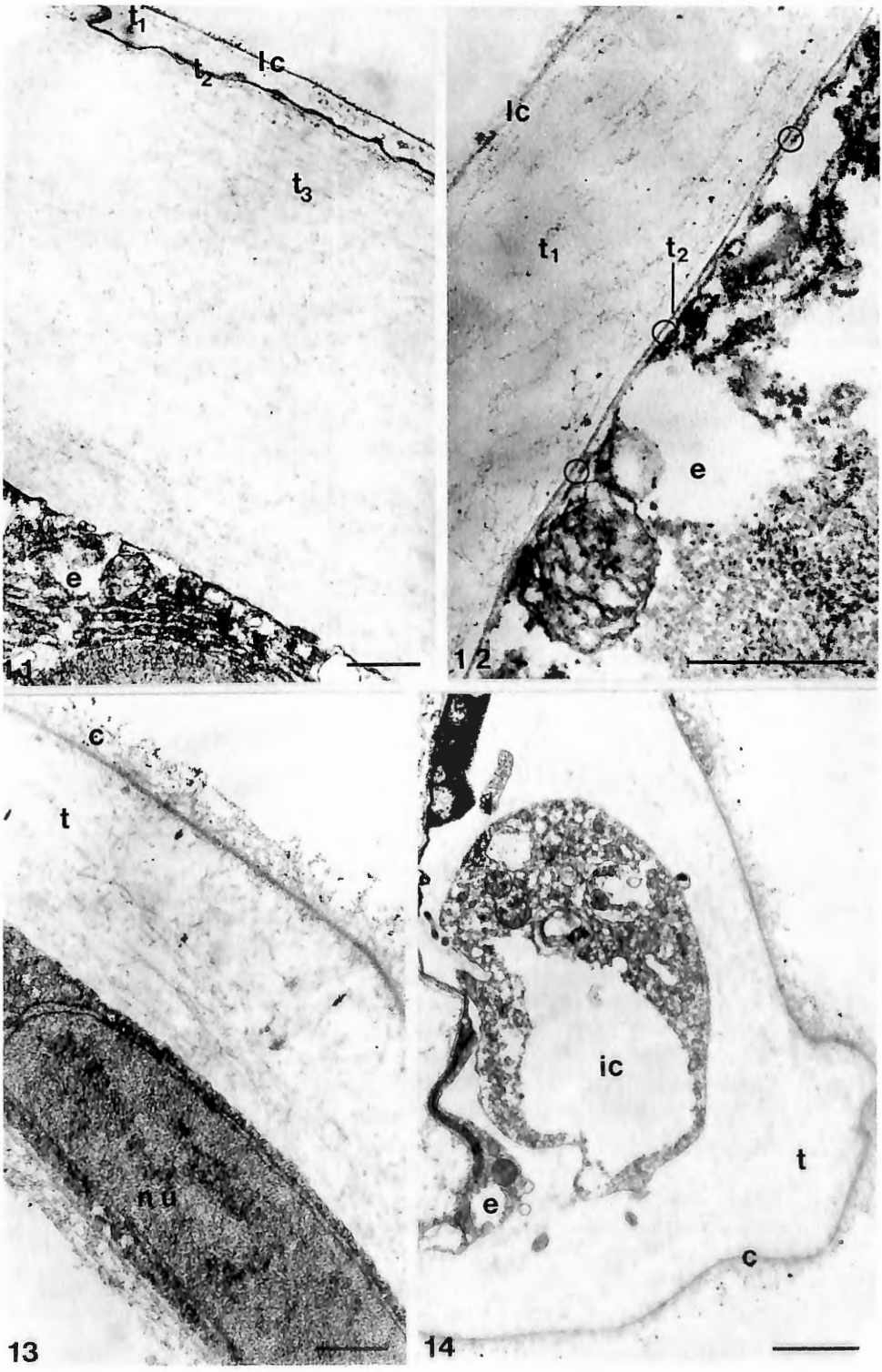
Fig. 8a : Enlargement of Fig. 8. Granular material (arrow heads) is attached to the fibrils (f) of the first tunic layer (t_1). Scale bar : 0.5 μm.

Fig. 9 : The larval cuticle (lc) has lost its tripartite arrangement (see also Fig. 10). Age of the larva : 13 h 40 min, just before hatching. Scale bar : 0.5 μm.

Fig. 10 : The tunic of the tail is still just made up of the larval cuticle (lc) and the first tunic layer (t_1). Age of the larva : 13 h 40 min, just before hatching. Scale bar : 0.5 μm.

ch : chorion ; cv : coated vesicles ; er : endoplasmic reticulum ; f : fibrils ; fc : follicle cell ; g : granular material ; lc : larval cuticle ; m : mitochondria ; t_1 : first tunic layer ; t_2 : second tunic layer ; t_3 : third tunic layer ; tc : test cell.

PLATE III



The larval tunic of *Ascidiella aspersa* consists of a network of interwoven fibrils and patches of granular material embedded in an amorphous ground substance. The larval cuticle and the second tunic layer are distinguished by compact fibrils and a small amount of ground substance.

The onset of tunic formation with respect to the developmental stage of the embryo is comparable in all studied species (*Halocynthia roretzi* (v. DRASCHE, 1884) : NISHIKATA *et al.*, 1987 ; *Ciona intestinalis :* MANCUSO, 1973 ; GIANGUZZA and DOLCEMASCOLO, 1980 ; *Distaplia occidentalis :* CAVEY and CLONEY, 1984 ; *Corella inflata* and *Ascidia paratropa :* CLONEY and CAVEY, 1982).

In contrast, the organization of the larval tunic is distinct among different species. Like in *Ascidiella aspersa* the second and third tunic layers are restricted to the trunk in *Corella inflata, Ascidia callosa* STIMPSON, 1852, *Ascidia paratropa, Clavelina huntsmani* VAN NAME, 1931 and *Molgula occidentalis* TRAUSTEDT, 1883 whereas in *Ciona intestinalis, Distaplia occidentalis, Styela partita* STIMPSON, 1852 and *Boltenia villosa* STIMPSON, 1864 both the trunk and the tail are surrounded by all tunic layers (CLONEY and CAVEY, 1982 ; CAVEY and CLONEY, 1984 ; CLONEY, 1990).

It is also known that in the course of metamorphosis, the tail is retracted by contractile fibres of the epidermis (CLONEY, 1978). The tunic of the tail is left behind.

The tunic of the young metamorphosed *Ascidiella aspersa* is composed of one tunic layer and the cuticle (Fig. 1, F). Ultrastructurally it appears that in addition to the larval tunic of the tail, the larval cuticle and the first tunic layer of the trunk are also lost during metamorphosis. In this respect, probably the second tunic layer of the larva has become the cuticle of the young ascidian.

PLATE III

Fig. 11 : The trunk tunic still consists of the larval cuticle (lc) and three tunic layers (t_1-t_3), but their thickness has changed considerably. The dense granular material is lost. Age of the swimming larva : 20 h 40 min. Scale bar : 0.5 µm.

Fig. 12 : A further layer (t_2) limits the tail tunic interiorly. A small cleft is visible between the tunic and the epidermis (circles). Age of the swimming larva : 20 h 40 min. Scale bar : 0.5 µm.

Fig. 13 : The young ascidian is just surrounded by the cuticle (c) and one tunic layer (t). Age of the ascidian : 4 days after metamorphosis. Scale bar : 0.5 µm.

Fig. 14 : Cells of probably mesenchymal origin have invaded the tunic. Age of the ascidian : 4 days after metamorphosis. Scale bar : 1 µm.

c : cuticle ; e : epidermis ; ic : intratunical cell ; lc : larval cuticle ; nu : nucleus ; t : tunic (after metamorphosis) ; t_1 : first tunic layer ; t_2 : second tunic layer ; t_3 : third tunic layer.

PLATE IV

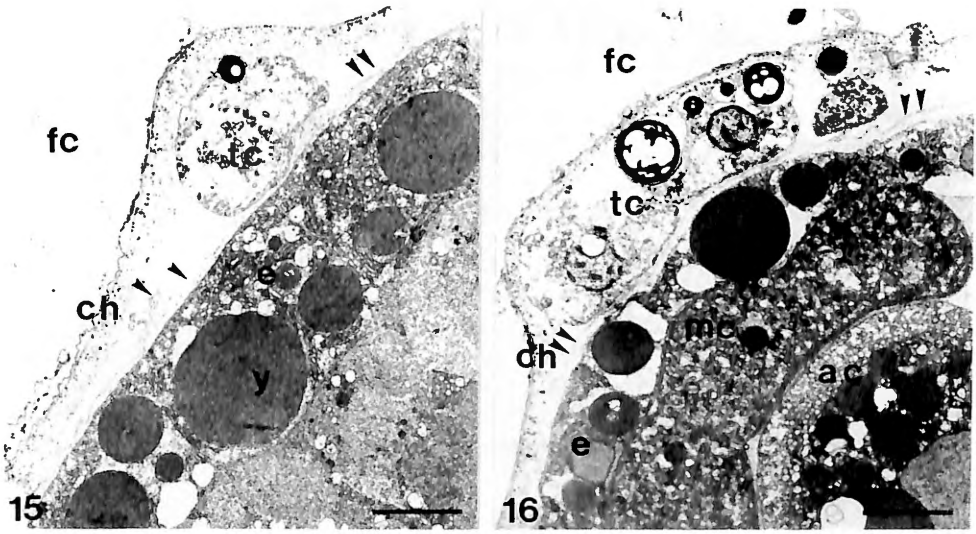


PLATE IV

Fig. 15 : The epidermis (e) of the trunk is a columnar epithelium. Age of the embryo : 11 h 15 min. Scale bar : 5 µm.

Fig. 16 : The epidermis (e) of the tail is formed by a squamous epithelium. Age of the embryo : 11 h 15 min. Scale bar : 5 µm.

ac : axial complex ; arrow heads : larval tunic ; ch : chorion ; e : epidermis ; fc : follicle cell ; mc : muscle cell ; tc : test cell ; y : yolk granule.

The ultrastructure of the epidermis signals secretory activity, both during embryonic and larval stages. The cells possess a vast rough endoplasmic reticulum, a Golgi apparatus, many vesicles and several coated vesicles, especially in the apical cell region, indicating that the epidermis is concerned with the release of components into the larval tunic. This supports the investigations of GIANGUZZA and DOLCEMASCOLO (1980) who studied the ultrastructural changes of the epidermal cells of *Ciona intestinalis* during tunic deposition. They concluded that the larval

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FIGURE 1

A : Tail-bud stage ; age : 11 h 10 min ; B : Tail-bud stage ; age : 11 h 15 min ; C : Tail-bud stage ; age : 11 h 20 min ; D : Larva just before hatching ; age 13 h 40 min ; E : Larva before metamorphosis ; age : 20 h 40 min ; F : Young ascidian ; 4 days after metamorphosis.

e : epidermis ; t : tunic (after metamorphosis) ; t_1 : first tunic layer ; t_2 : second tunic layer ; t_3 : third tunic layer.

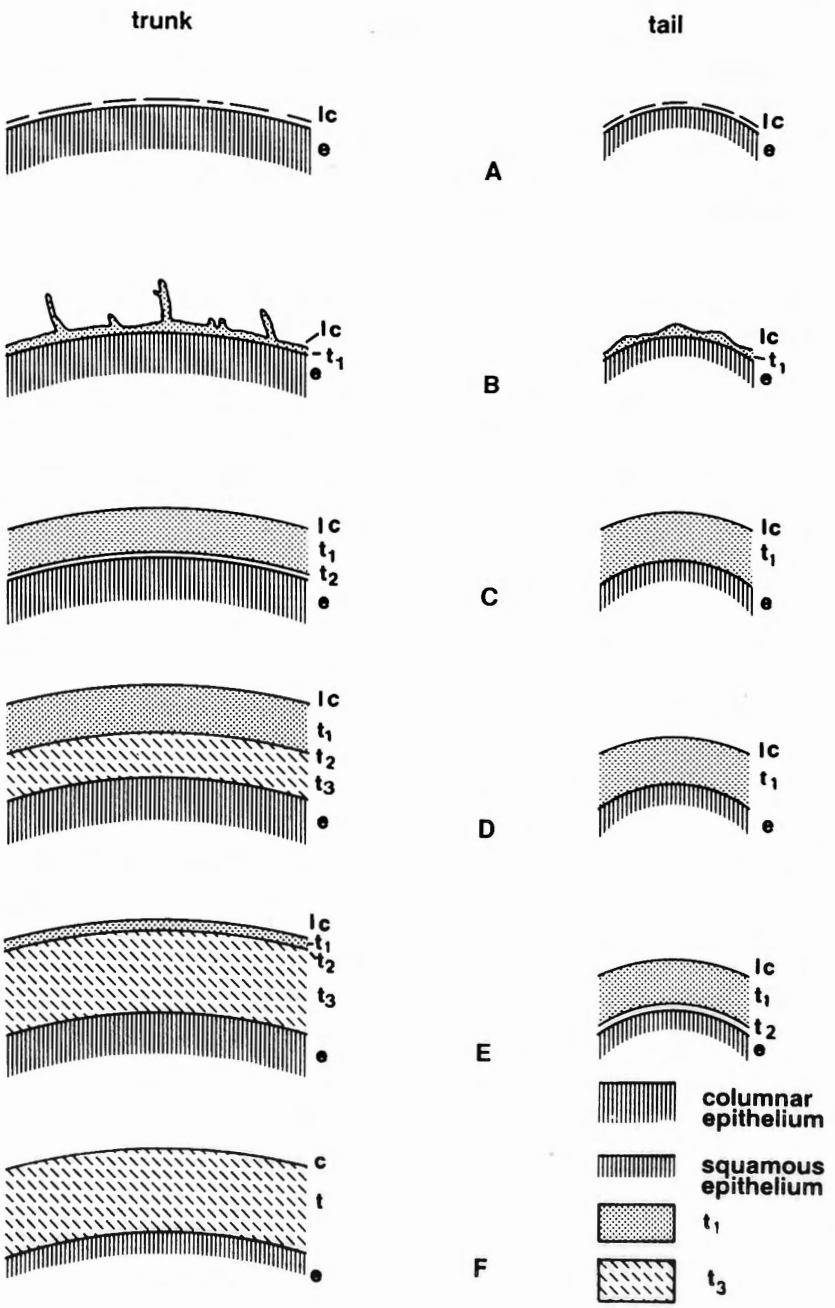

Fig. 1 : Scheme summarizing tunic morphogenesis in *Ascidiella aspersa*.

tunic is secreted by the epidermis. Furthermore, they have shown cytochemically that coated vesicles are probably involved in this process. These vesicles might play a role as a mediator between the Golgi apparatus and the apical plasma membrane (FRIEND and FARQUHAR, 1967).

But ultrastructural observations alone cannot proove whether the epidermis is the only structure responsible for tunic formation. MANCUSO (1974) demonstrated that the epidermis of *Ciona intestinalis* is able to produce the principal elements of the larval tunic by isolating and cultivating the four animal blastomeres of the eight-cell stage, thus the presumptive ectoderm. These developing partial embryos were covered by a cuticular layer and a hydrated matrix with embedded filaments at the instant when the controls had reached the tail-bud stage.

On the other hand CLONEY and CAVEY (1982) claimed that extraembryonic structures modify the larval tunic. In *Corella inflata* and *Ascidia paratropa,* larval fin formation is inhibited when the embryos are dechorionated before they reach the tail-bud stage. Dechorionation at this stage removes the test cells and the chorion simultaneously. Once tunic secretion and larval fin formation has started, dechorionation has no further influence.

Recent investigations demonstrate that test cells are not necessary for larval fin formation but the chorion serves to contain products of the embryo responsible for the morphogenesis of the larval fins (CLONEY, 1990). The author proposed also that the test cells impart a hydrophilic characteristic to the larval tunic.

ROBINSON *et al.* (1986) described an enhancement of fin formation when dechorionated embryos of *Ascidia callosa* were treated with strongly reducing substances.

In the case of *Distaplia occidentalis,* the test cells are responsible for the ornamentation of the larval tunic (CAVEY, 1976), but it is yet not clarified whether the ornaments change any of its properties.

Less is known on the secretion of the adult tunic and the differences among the species seem to be much greater. Intratunical cells might take part in this process. In *Ascidiella aspersa* they invade the tunic during metamorphosis. It is believed that these cells are either blood cells or other mesenchymal cells passing the epidermis during the first minutes after the onset of metamorphosis (BERRILL, 1950 ; CLONEY and GRIMM, 1970 ; CLONEY, 1978, 1982).

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SPATIOTEMPORAL EVOLUTION OF THE TRAIL NETWORK IN *LASIUS FULIGINOSUS* (HYMENOPTERA, FORMICIDAE)

by

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SUMMARY

In *Lasius fuliginosus,* the existence of a permanent trail system, stable during a season and practically unchanged from one year to the next, was confirmed. These trails radiate from the nest and lead the foragers to trees and shrubs colonized by aphids where they collect honeydew. These aphid sites are also stable during a season and from one year to the next.

Other kinds of trails were observed : temporary honeydew collecting trails, temporary hunting trails and ephemeral recruitment trails. These trails, always connected to permanent trails, lead foragers respectively to temporarily exploited aphid sites, to large areas explored by ants and to large prey. Unlike permanent trails, they have a short life-time (several weeks for the temporary trails, some hours for the ephemeral trails) and they are not found in the same position from one year to the next. The temporarily exploited aphid sites are each year new ones originating, at least for a part of them, from the colonization of new sites by winged migrating aphids. They are thus spatially and temporally unpredictable food sources, like prey.

These results show that the ant *L. fuliginosus* combine a predictive foraging strategy (permanent trails) with an opportunist strategy (temporary and ephemeral trails).

Key words : Lasius fuliginosus — Ant-tended aphids — Permanent, temporary and ephemeral trails — Foraging strategy.

INTRODUCTION

Foragers of the ant *Lasius fuliginosus* (LATREILLES, 1798) travel along well defined, heavily frequented and long trails radiating from the nest, leading ants to aphid colonies where they collect honeydew which constitutes a major part of the *L. fuliginosus* diet (DOBRZANSKA, 1966 ; GASPAR, 1967 ; HENNAUT-RICHE *et al.*, 1980). This trail network is remarkably stable : most of the trails persist during the whole season, remaining unchanged in the paths they take and moreover, they are re-established the next year after winter with great accuracy (HENNAUT-RICHE *et al.*, 1980).

L. fuliginosus is also characterized by a territorial specialization : using a mass marking technique, HENNAUT-RICHE *et al.*, (1980) have shown that foragers marked in summer on one of the two branches of a trail retained a high fidelity not only to the trail but also to the branch, for at least 12 days. They also had indications that trail fidelity persist throughout the winter.

The resource structure of the habitat is one of the major factors to which an ant foraging strategy is adapted (HÖLLDOBLER and LUMSDEN, 1980 ; ROSENGREN and SUNDSTRÖM, 1987). Persistency of the trails over long periods and high trail fidelity seem to be well adapted to the exploitation of stable food sources and is the expression of a predictive strategy (CHERIX and ROSENGREN, 1980).

In this paper, we present the results of a 3-years field study on the trail system of a *L. fuliginosus* colony. A detailed description of the trails and how they evolve, seasonally and from one year to the next, in relation to the kinds of food exploited is given so as to disclose patterns which give a better picture of the foraging strategy of these ants.

MATERIALS AND METHODS

Study area description

The *L. fuliginosus* nest was located in a cavity of a living tree, at the base of a cluster of hornbeams situated at the edge of a wood in the commune of Treignes (south-west of Belgium) and consisted of carton pulp (MASCHWITZ and HÖLLDOBLER, 1970). Although *L. fuliginosus* sometimes nest directly in the soil or under stones without making a carton nest, the majority of the nests are made of carton pulp and are located at the base of hollow trees (living or dead ones) (GASPAR, 1965). All the nests of *L. fuliginosus* we observed in the region were of that latter type. The wood was composed of various tree species : mainly hornbeams *(Carpinus betulus)* and oaks *(Quercus robur)* but also field maples *(Acer campestre)* and hazels *(Corylus avellana)*. The south-east edge, where the nest was located, was bordered by a ground strip on which shrubs grew, mainly field maples *(Acer campestre)* and red dogwoods *(Cornus sanguinea)* (Fig. 1). Undergrowth was very scarce in the wood, and on the shrubby strip, rocky spaces were predominant.

Five aphid species provided honeydew for the ants : *Lachnus pallipes* (HARTIG, 1841) and *Stomaphis quercus* (LINNAEUS, 1758) on oaks, *Periphyllus obscurus* MAMONTOVA, 1955 on field maples, *Anoecia corni* (FABRICIUS, 1775) on red dogwoods and *Prociphilus bumeliae* (SCHRANK, 1801) on the single ash *(Fraxinus excelsior)* growing on the shrubby strip.

Field observations

The trail network of the nest was observed from March 1984 to September 1986. In 1984 and 1985, from late March to the end of November, a complete mapping of the trail network was carried out once a week. In 1986, the mappings were

undertaken at two or three weeks intervals. The trails were marked by means of small plastic sticks stuck in the ground every ± 20 cm.

During the seasons 1984, 1985 and 1986, each of the trees or shrubs growing in the territory patrolled by *L. fuliginosus* was searched visually for aphids and ants once a week. A shrub or a tree colonized by aphids and exploited by ants was easily detected either by the direct observation of ants milking the aphids or by observing columns of ants on the trunk of trees (the presence of numerous ants laden with honeydew in these columns was a sure indication of the presence of ant-tended aphid colonies on the trees).

In 1984, 1985 and 1986, the traffic on each of the permanent trails A, B, C, D (Fig. 1) was measured 4 times per week during the whole season (from April to November). The counting time was 5 min for each measurement of both outward and return flow. In the return flow, ants laden with honeydew (replete ants) or carrying prey were distinguished from empty ants (no honeydew, no prey). These measurements were performed between 4 and 5 p.m. Similarly, in 1985, weekly measurements were made of the ants' traffic on the temporary trails.

RESULTS

Every year, 4 types of trails were distinguished : permanent trails, temporary honeydew collecting trails, temporary hunting trails and ephemeral recruitment trails.

Permanent trails

Description and evolution during a season

Fig. 1 shows the permanent trail network as well as the trees and the shrubs colonized by aphids and exploited by *L. fuliginosus* during the three years of observation. Four main permanent trails could be observed every year (trails A, B, C, D — see Fig. 1). These trails led ants to trees and shrubs with aphid populations and they were intensely frequented by ants. From May to November, the traffic on each trail varied from ± 100 to ± 600 ants per 5 min (outward flow + return flows), depending on the trail and the time of the year and some 40 to 80 % of the ants in the return flow were laden with honeydew (replete ants). But, the function of the permanent trails was not only to lead ants to aphids and to bring back to the nest the produce of the harvest of the honeydew. They were also used as main routes by ants going to and coming from hunting in foraging areas both bordering permanent trails and located at their extremities (this point will be developed later). Two features of the permanent trail network distinguish it from the other types of trails : its spatial and temporal stability (permanence) during a season and its similarity from one year to the next.

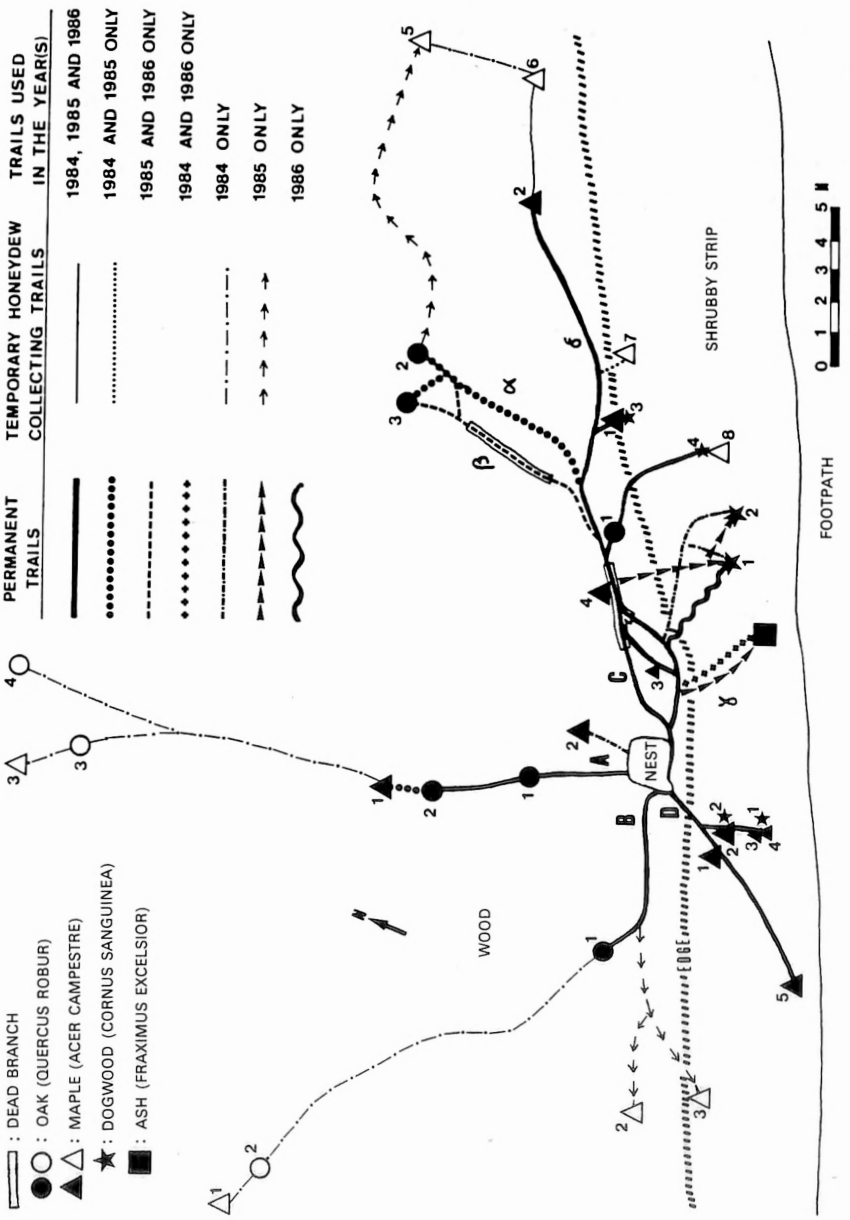

Fig. 1. — Permanent and temporary honeydew collecting trails network in 1984, 1985 and 1986. The capital and the greek letters next to trails designate respectively each of the main trails and sections of trails (see text). The solid symbols on the trails represent the permanent aphid sites and the open ones, the temporary aphid sites. The numbers next to the symbols refer to Table 1.

Permanence during a season

Each year, the first signs of the nest's activity were observed towards the end of March. The permanent network was progressively rebuilt during April reaching its full development at the beginning of May. It remained fully developed and unchanged in the paths taken by the trails and in length for most of the season of activity, shrinking somewhat in August (Fig. 2). The network progressively disappeared in November as the nest entered hibernation.

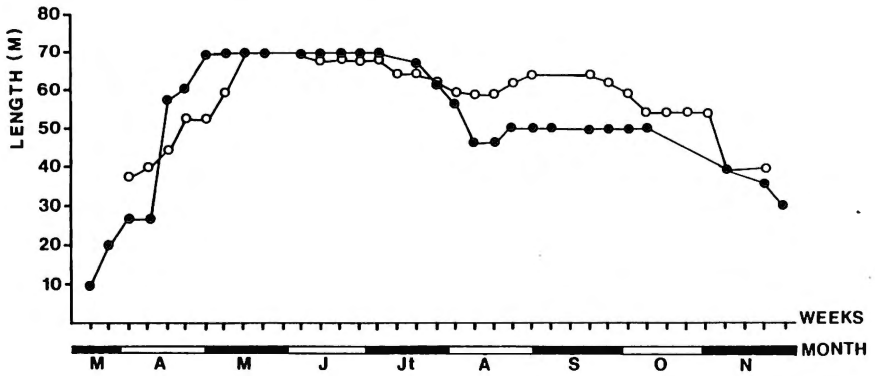

Fig. 2. — The change in the length of the permanent trail network (lengths of all the permanent trails summed) with time in 1984 (●) and in 1985 (○).

In April, the traffic on the permanent trails was low (from ± 30 to ± 150 ants per 5 min — outward + return flows) and neither replete ants nor ants carrying prey were observed. However, as early as the beginning of April, the trees and the shrubs which would be intensely exploited by ants later in the season were already being climbed by columns of ants. At that time, numerous aphid eggs had already hatched on maple and dogwood shrubs and possibly also on trees (oaks, maples), although their height made this difficult to verify. The presence, as early as the beginning of April, of fundatrices on trees and shrubs probably explains why they were already visited by ants, although these fundatrices did not yet produce honeydew (or in negligible amounts), since no replete ants were observed. During April, periods of total inactivity on the trails were frequent during the day, lasting several hours and sometimes all day. This happened when the soil surface temperature was very low (below 10° C) or when the sun warmed up the soil too much (above 28° C). In April, the forest soil is not yet protected by foliage, and thus, on sunny days the soil surface temperature could reach 40° C. During these periods of inactivity, ants were found clustered at the foot of the oaks and in some points on the trails under dead leaves. Trail activity increased during the month of May, when numerous replete ants appeared on the permanent trails, coming from oaks, maples and dogwoods, in concurrence with the development of the second generation of aphids. From that moment, large quantities of honeydew, as well as numerous prey, were carried by ants on permanent trails. At the end of September,

the flow of replete ants gradually decreased until the beginning of December when the ants became completely inactive.

Similarity from one year to the next

Fig. 1 shows the similarity of the permanent trail network during the three years : the foragers followed exactly the same paths every year. At the most we could observe some displacements to the right or left (generaly no more than 10 cm) of small portions of the trails from one year to the next or during a season. There were, however, some minor variations in the paths taken by some lateral branchings of trail C. The main trunk of trail C, following the edge of the wood, was positioned in the same place every year. Its distal part, leading to the oaks labelled 2 and 3 in Fig. 1, deeper in the wood, changed with time. A new trail, β, replaced trail α in the course of the season 1985. This change can be explained by the existence in 1985 of a dead branch fallen on the ground during the winter 1984-85, making path β easier to follow than path α which was exclusively followed by the ants in 1984. Interestingly, this change was not immediate. In 1985, both α and β were followed by the ants and β supplanted α only at the end of August 1985. This suggests that in 1985 the memory of path α was strong enough to prevent some ants from following the easier path β. We speculate that in 1985 mainly naïve foragers followed path β. The next year, these workers would have retained their specialization to path β, progressively acquired the previous year. Some of the smaller lateral branchings leading to the shrubby strip also changed from one year to the next, without any obvious topographical interpretation.

Honeydew rewarding sites of the permanent trail network

Table 1 (see also Fig. 1) records all the trees and shrubs colonized by aphids and exploited by the ants on the permanent trails in 1984, 1985 and 1986 ; it also indicates the time during which they were exploited by ants. One can seen the great stability of these sites during three years, with however some exceptions (see Table 1). Of the 24 honeydew collecting sites observed in 1984, 23 (96 %) were exploited again in 1985 and 20 (83 %) in 1986.

Of all the trees exploited by ants, the oaks were exploited for the longest time, all but one (oak 3 on trail C) being exploited during the whole season. They were colonized by *Lachnus pallipes* and *Stomaphis quercus,* two aphid species which completed their total life cycle, from spring to autumn, on the oak. They were ant-tended without interruption. The majority of the maples and the dogwoods were exploited by ants for periods exceeding three months every year (Table 1, Fig. 1). Maples were colonized by the aphid *Periphyllus obscurus* which lived from spring to autumn on its host and which was ant-tended during all its life cycle. We do not know the reason why some maples were deserted by aphids before autumn while, on others, the aphid populations managed to complete their entire life cycle. From April, the dogwoods were colonized by the aphid *Anoecia corni* whose colonies were invariably ant-tended. In July and August, the dogwoods were deserted by *A. corni*

TABLE 1.

*Aphid sites exploited by the ants in 1984, in 1985 and in 1986. A, B, C, D : permanent trails (see Fig. 1) ; N° : number of the tree or the shrub (see Fig. 1) ; * : exploited during the whole season by ants (in 84 = 32 weeks, in 85 = 33 weeks, in 86 observations ended on 17.09, thus * = 24 weeks) ; -- : no exploitation (not colonized by aphids) ; ● = tree, ○ = shrub. Examples :* Acer campestre, *84, C, N° 1, *, ● : maple (tree) N° 1 on trail C, exploited during the whole season in 1984 ;* Acer campestre, *85, C, N° 3, 25, ○ : maple (shrub) N° 3 on trail C, exploited during 25 weeks in 1985.*

Permanent aphid-sites

Quercus robur

	84	85	86	
A. N°1	*	*	*	●
N°2	*	*	*	●
B. N°1	*	*	*	●
C. N°1	*	*	*	●
N°2	*	*	*	●
N°3	10	28	5	●

Cornus sanguinea

	84	85	86	
C. N°1	27	14	*	○
N°2	25	9	9	○
N°3	14	*	*	○
N°4	25	15	14	○
D. N°1	25	17	19	○
N°2	24	6	--	○

Acer campestre

	84	85	86	
A. N°1	16	10	--	●
N°2	16	--	--	●
C. N°1	*	19	10	●
N°2	14	24	10	●
N°3	*	25	10	○
N°4	9	13	--	●
D. N°1	29	*	17	○
N°2	29	15	17	○
N°3	29	21	21	○
N°4	25	26	*	○
N°5	*	*	*	○

Fraxinus excelsior

	84	85	86	
C.	13	15	10	●

Temporary aphid-sites

Acer campestre

	84	85	86	
A. N°3	3	--	--	●
B. N°1	1	--	--	●
N°2	--	2	--	●
N°3	--	3	--	○
C. N°5	3	3	--	●
N°6	4	2	3	●
N°7	?	8	--	○
N°8	4	2	--	○

Quercus robur

	84	85	86	
A. N°3	7	--	--	●
N°4	6	--	--	●
B. N°2	4	--	--	●

(and of course by the ants) which had migrated to its secondary host. We were not able however to discover the secondary host which can be one of various grass species. In September, *A. corni* was back on the dogwoods where sexuales were produced which were tended by *Lasius fuliginosus*. From the beginning of May, the ash on section γ of trail C was exploited by the ants during three months in the years 1985 and 1985, and during two months in 1986 (Table 1, Fig. 1), the time during which it was colonized by the ant-tended aphid *Prociphilus bumeliae*. At the end of July 1984 and 1985 and the end of June 1986, the ash was deserted by *P. bumeliae* which had migrated to its secondary host. In that case too, we were not able to discover the secondary host which is known to be a fir tree (genus Abies)

otherwise not growing on the study area. In autumn, *P. bumeliae* was back on the ash where it laid sexuales. But unlike the sexuales of the aphid *A. corni,* they were not tended by the ants.

The result of the time-limited exploitation of some aphid sites located at the extremities of permanent trails, and due especially to the life cycle of some aphid species, is that some sections of the network disappeared before the end of the season. For example, section δ of trail C (Fig. 1) disappeared at the beginning of August 1984, the end of October 1985 and the end of June 1986. Section γ of trail C (Fig. 1) disappeared at the end of July 1984 and 1985, and at the end of June 1986 and finally, a small section of trail A disappeared in mid-July 1984 and 1985. The stability of the permanent trail network is thus related to that of the aphid sites.

Temporary honeydew collecting trails

Each year, at the beginning of the season (end of April and May), trails with a short life time (1-8 weeks) were formed by ants towards aphid sites always located outside the zone limited by the permanent aphid sites. These trails, always running on from the extremity of permanent trails (Fig. 1), had a low ant traffic and were poorly honeydew rewarding (the maximum replete ants flow was 15 ants/5 min). Each of these trails, except two of them, was recorded during only one of the three years. The temporary trail network was thus different from one year to the next (Fig. 1).

At least for some temporarily exploited aphid sites (maples n° 2, 3 on trail B and maples n° 5, 6, 7, 8 on trail C — little trees or shrubs whose foliage could be easily surveyed) we could observe how their exploitation by ants began, why they were poorly honeydew rewarding and how their exploitation ended. The beginning of the exploitation of these maples and the formation of the temporary trails leading to them was observed in mid-may and concurred with the arrival on these trees of numerous winged aphids *(Periphyllus obscurus)* which produced generations of apterous aphids. Furthermore, it was also in mid-May that numerous winged aphids *(P. obscursus)* were produced among aphid populations colonizing maples reached by permanent trails. Obviously, in this case, new maples were colonized by migrating winged aphids coming from permanent aphid sites and were then rapidly discovered by ants. Observation has shown that these apterous aphid populations, produced by migrating winged aphids, did not develop extensively on the newly colonized maples and rapidly declined, thus leading to the end of their exploitation by ants. The other temporarily exploited aphid sites (oaks n° 3, 4 and maples n° 3 on trail A, oak n° 2 and maple n° 1 on trail B) were big trees. Due to the difficulties in observing their foliage regulary, we were unable to know the history of the aphids colonizing these trees. But, as the exploitation of these aphid sites by ants also began in May, we can suppose, at least for the maples, that they were also colonized by winged migrating aphids.

From 1984 to 1986, a total of 11 aphid sites reached by temporary trails, and which we describe also as temporary (this is strictly true for at least 6 maples —

see above), were recorded. Only one was recorded each year and 3 during two successive seasons, the others having been recorded during only one of the three years (Table 1). It is interesting to note that maple 5 on trail C, exploited in 1984 and in 1985, was reached by a completely different temporary trail in each of the two years.

In summary, temporary honeydew collecting trails developed early in the season. As with the permanent trails, they led to exploited aphid sites, but, unlike permanent trails, were not similar from one year to the next, owing to the fact that the exploited aphid sites were not the same each year. The ant traffic and the amount of honeydew transported on these temporary trails was low. Some of these trails can be explained by the colonization of new sites by migrating winged aphids.

Temporary hunting trails

Each year, from April to mid-September, trails which never reached any aphid sites were observed. No replete ants were ever seen on these trails. On the other hand, ants carrying prey were frequent. These trails had a short life time (about 6 weeks) and they had a low ant traffic (about 30 ants/5 min, outward + return flows, with, on certain days, maximums of 60 ants/5 min). All along these trails, there was a constant leakage of foragers which explored the surrounding ground. Hunting trails were thus bordered by large areas which were constantly explored by varying numbers of ants according to the weather.

As can be seen from Fig. 3, hunting trails always developed from permanent trails, either from their extremities, which was the most frequent case, or from sections of permanent trails, closer to the nest. They ended either anywhere on the ground or, more frequently, at the foot of hornbeam clusters. A careful weekly examination of the ants' activity on the hornbeam clusters showed that they did not exploit aphids (or other food sources) on these trees. Instead, they scattered at their foot and they never climbed the trunks, except in April when the trail network was rebuilt and the exploratory behaviour was intense. Many hornbeams reached by the developing hunting trails were then climbed by ants and the same observation was made for hornbeams which were in the path of permanent trails. However, the ants climbing a hornbeam trunk were never numerous and they never formed columns like those observed on trunks of trees colonized by aphids. It was difficult to study the ants' activity above two meters in height; nevertheless, observations made on lower parts of these hornbeams gave an indication of what probably happened in higher parts. The ants were observed on many occasions licking hornbeam buds, and, on some occasions, sap exuding from bark. When the collection of sap ended in early May, the hornbeams were no longer climbed by ants but the trails reaching them did not disappear and became hunting trails. Thus, at least some hunting trails seem to have derived from the intense exploratory activity in April, when food is very scarce.

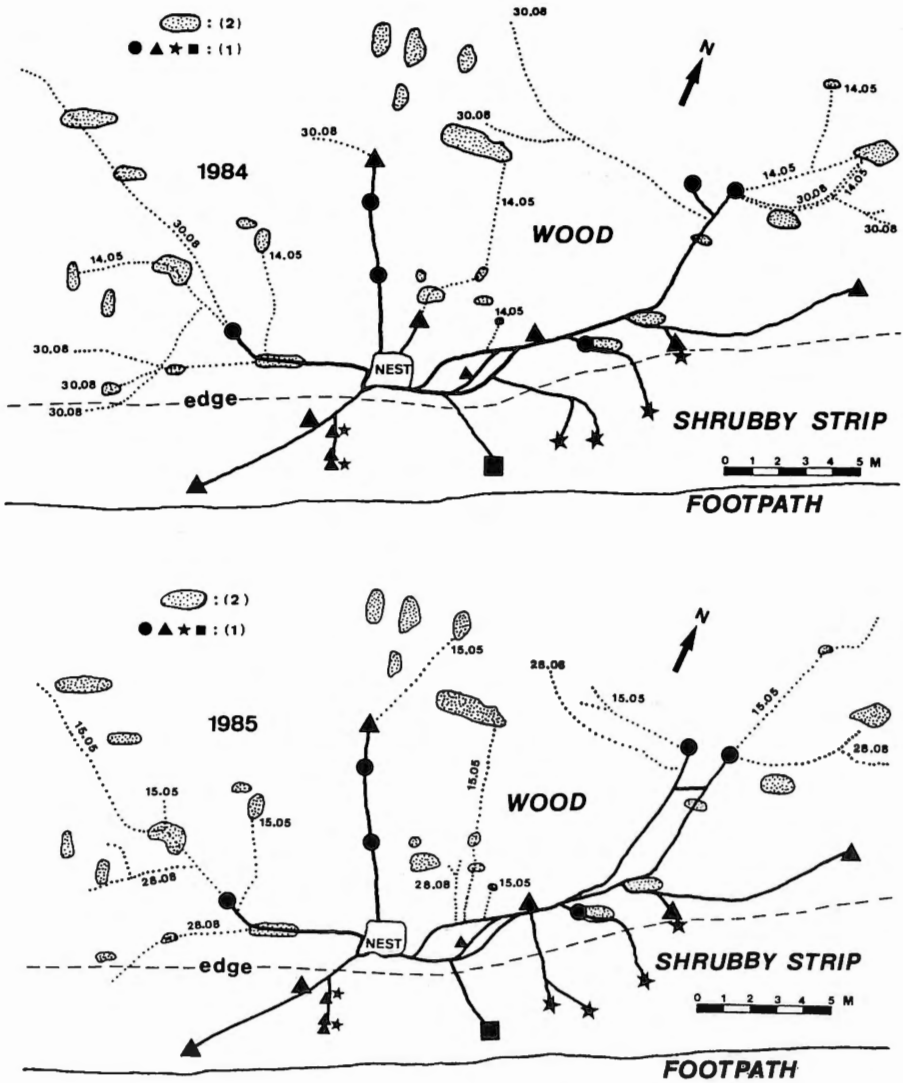

Fig. 3. — Main hunting trails observed between May and September in 1984 and in 1985. The dates on the hunting trails indicate when they were observed. ..... : hunting trails ; ———— : permanent trails. Other symbols : (1) : aphid sites (see Fig. 1) ; (2) : clusters of hornbeams.

Fig. 3 presents the main hunting trails observed in 1984 and 1985, as they were at the height of their development. As can be seen, the hunting trail network was not the same from one year to the next. Moreover, it underwent modifications in the course of a season : trails disappeared after some weeks while others developed and all could vary in shape and length from week to week. The relative instability

of the hunting trail network both in the short and the long term, can be explained by the fact that, unlike the permanent trails which lead foragers to very localized and permanent honeydew sites, hunting trails lead ants to large areas of the territory which are individually explored in search of prey. Nervertheless, it can be seen from Fig. 3 that the general direction of hunting trails and the areas explored by these trails remained the same during a season and from one year to the next.

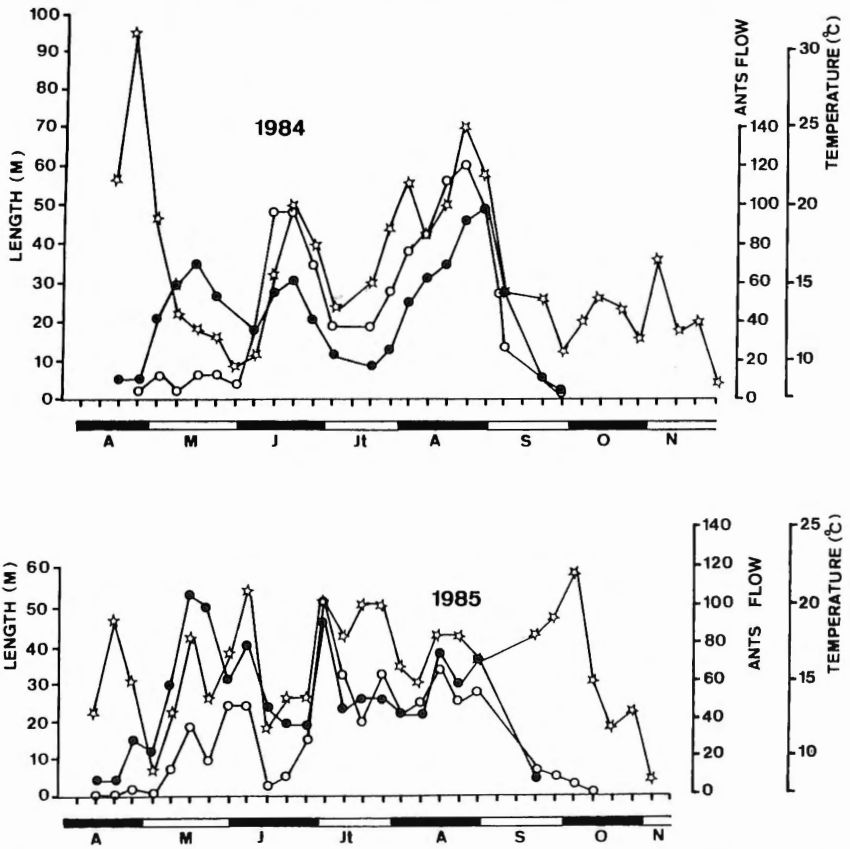

Fig. 4. — The change in the length of the hunting trails network over time (●) compared to the change over time of the weekly mean flow of ants carrying prey (per 5 min) on all the permanent trails (○) and the weekly mean soil temperature (☆).

Fig. 4 compares the changes in the values of three parameters with time : the hunting trail network length, the weekly mean flow of ants carrying prey on the permanent trails and the weekly mean soil temperature. Each year, the first hunting trails appeared in April. After a progressive lengthening, the hunting trail network attained a peak of development by mid-May. Afterwards, from mid-May to the end of August, it underwent periods of regression and extension. In September, it progressively regressed and by the end of September, had completely disappeared.

It was only during the period when hunting trails existed that ants carrying prey were observed on the permanent trails on which they constituted 5 to 15 % of the return flow. It can also be seen from Fig. 4 that there is a clear correlation between temperature and the two variables used to measure hunting activity.

Evolution of the whole trail network

Fig. 5 shows the changes with time in the length of the whole trail network (permanent + temporary, honeydew and hunting trails). It can be seen that it attained a maximum extension in mid-May. At that time, the permanent trail network was completely developed, the temporary honeydew collecting trails were fully developed and the hunting trail network attained a first maximum extension. A marked regression followed. It was largely due to the disappearance of the temporary honeydew collecting trails and to a regression of the hunting trail network, and to a lesser extent, to the disappearance of terminal sections of some permanent trails. Afterwards, the whole trail network underwent the same changes with time as the hunting trail network until the end of September when hunting trails disappeared. At the end of September, only the permanent trail network remained. In November the permanent trail network was progressively reduced from its extremities, and at the beginning of December the nest entered hibernation.

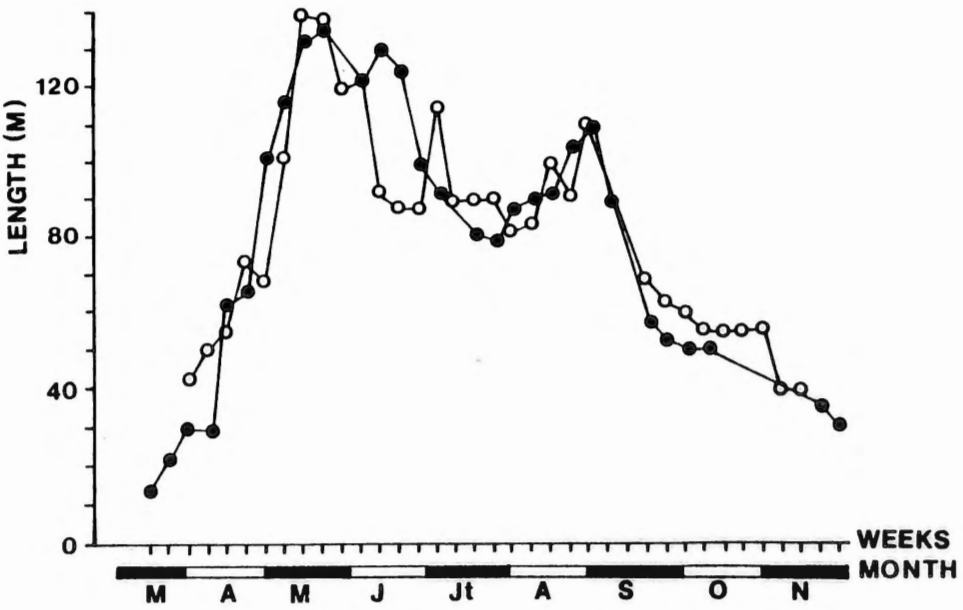

Fig. 5. — The changes in the length of the whole trail network with time in 1984 (●) and in 1985 (○).

Ephemeral recruitment trails

These trails were formed when the ants discovered a prey too large to be carried as such into the nest. Recruited ants cut up the prey and removed it piece by piece. Most of the prey collected in this way were earthworms, intensely hunted by *L. fuliginosus*. From the 90 observed prey which could not be carried by a single ant, 73 (81 %) were earthworms, 7 (7,7 %) large caterpillars and the remainder (11 %) consisted of occasional prey (adult moths, slugs, flies, maggots and centipedes). It was mainly from June to August that the ants heavily preyed upon earthworms (Table 2). In October and November, no ephemeral recruitment trails were observed.

Fig. 6 shows all the recruitment trails observed in 1984 and 1985. 51 % of them (N = 74) joined the permanent network at the level of a tree or shrub which could be used as an orientation cue. They lasted only the time needed to collect the prey (no more than a few hours) and never evolved into temporary hunting trails.

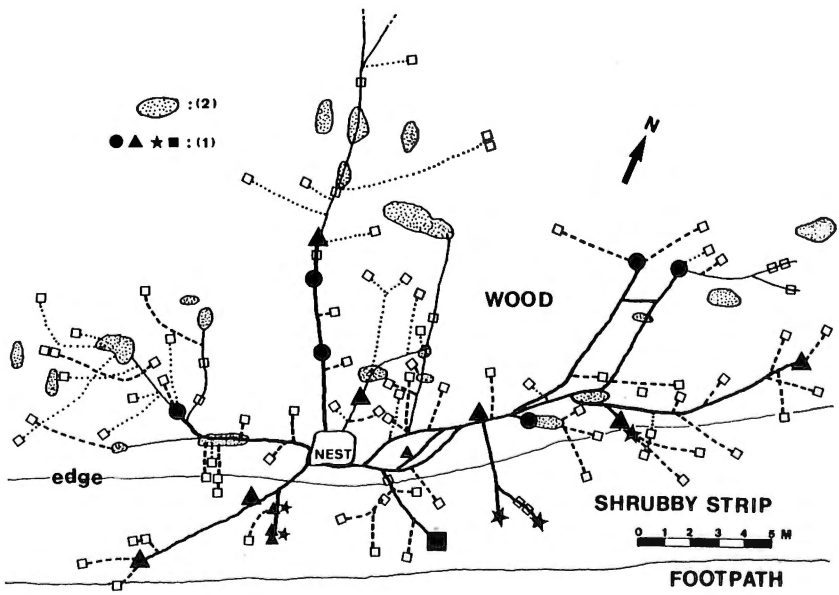

Fig. 6. — Ephemeral recruitment trails observed in 1984 (.....) and in 1985 (------). ———— : long-lasting trails (permanent trails, temporary trails). □ : prey. Other symbols : (1) : aphid sites (see Fig. 1); (2) : clusters of hornbeams.

TABLE 2.

Number of earthworms captured by ants each month in 1984 and in 1985 (one month = 4 observations of the trails network of the colony).

Year	Month								
	M	A	M	J	Jt	A	S	O	N
1984	0	1	0	10	6	5	3	0	0
1985	0	3	2	8	26	8	1	0	0
TOTAL	0	4	2	18	32	13	4	0	0

Every year, we observed one or two raids conducted by *L. fuliginosus* on *Formica fusca* LINNÉ, 1758 colonies. On these occasions, similar recruitment trails were formed. They were heavily frequented, always linked to a permanent trail and led ants to the raiding place. The booty (brood and some sexuals) was brought to the nest along the recruitment trail and the permanent trail. These raiding trails, also observed by DOBRZANSKA (1966) in *L. fuliginosus,* only lasted one day.

DISCUSSION AND CONCLUSION

Confirming other observations (DOBRZANSKA, 1966 ; GASPAR, 1967 ; HENNAUT-RICHE *et al.,* 1980), we observed the existence of a heavily frequented permanent trail system, remaining unchanged over at least three years and leading ants to aphid sites. Furthermore, our observations revealed the existence of other kinds of trails in *L. fuliginosus* : temporary honeydew collecting trails, temporary hunting trails and ephemeral recruitment trails.

The permanent trail system of *L. fuliginosus* is related to very stable food sources ; here aphid colonies providing honeydew. Collection of honeydew through a trail system remaining unchanged for several years (topographic constancy) is found in other ant species like *Dolichoderus taschenbergi* (MAYR, 1866), *Formica obscuripes* FOREL, 1886 (BRADLEY and HINKS, 1968), *Camponotus modoc* WHEELER, 1910 (DAVID and WOOD, 1980) and wood ants of the *Formica rufa* LINNÉ, 1758 group (CHAUVIN, 1962 ; ROSENGREN, 1971 ; MABELIS, 1979). In wood ants, ROSENGREN (1977) has shown that the topographic constancy was most likely determined by the existence of a true topographic tradition. During the season, the wood ant foragers show a high trail fidelity which persists through the winter and which is mainly caused by a long-term individual memory of spatially organized cues (trees, canopy, etc...) (ROSENGREN, 1971 ; ROSENGREN and FORTELIUS, 1986). In spring, the trail system is rebuilt by the old specialized foragers. They recruit and guide on their trails the young, naïve workers which will become themselves specialized for particular trails later in the season (ROSENGREN, 1977). *L. fuliginosus* also shows a high trail fidelity during the season (HENNAUT-RICHE *et al.,* 1980). Short and long-term fidelity to trail will be the subject of a subsequent paper. There is an

obvious convergence in the foraging strategies used by wood ants of the *F. rufa* group and *L. fuliginosus*. That foraging strategy seems to be an adaptation to the exploitation of localized and stable food sources in space and time, such as aphid colonies. This is a predictive strategy which is also indicated by the fact that the permanent trail system was rebuilt in April when honeydew was not yet available (not until May). This latter observation was also made by ROSENGREN and SUNDSTRÖM (1987) and SUDD (1983) in wood ants *(F. rufa* group).

L. fuliginosus has also developed a foraging strategy adapted to the exploitation of unpredictable food sources in space and time. This opportunist strategy is particularly well exemplified by the ephemeral trails exploiting large prey (mainly earthworms in our study) and by the raiding trails. These trails are obviously recruitment trails similar to those experimentally observed by HANGARTNER and BERNSTEIN (1964) and HANGARTNER (1967).

The temporary honeydew collecting trails and the temporary hunting trails are other manifestations of an opportunist foraging strategy in *L. fuliginosus*. These trails exploit respectively aphid colonies established from winged migrating aphids and scattered small prey, two food sources therefore unpredictable in their location.

The reasons why the aphid sites reached by the temporary honeydew collecting trails were exploited during only some weeks and were poorly honeydew rewarding are still unknown. We nevertheless advance an hypothesis based on two observations : firstly, these aphid sites were always located at the extremities of the territory patrolled by the foragers (Fig. 1) and secondly, their appearance and their discovery by ants happened at a period of the season (end of April and May) when the ant flows on the permanent trails underwent a considerable increase following the appearance of the second aphid generation on the permanent aphid sites. It could be, therefore, that the collection of large quantities of honeydew on the permanent aphid sites mobilizes the major part of the forager force. Few ants would then be available for the exploitation of newly appeared food sources, less rich and located far from the nest. These aphid colonies either would be abandonned by the ants or would rapidly decline through lack of ant-attendance. It is well known that aphids derive benefits from ant-attendance : protection against natural enemies, increased reproduction rates, sanitary effects (removal by the ants of the excreted honeydew which otherwise could contaminate them) and some aphid species are unable to survive if they are not tended by ants (WAY, 1963).

Hunting trails have some similarities with the trunk-trails observed in harvester ants of the genus *Pogonomyrmex* and *Pheidole militicida* WHEELER, 1915. The foragers of these ants travel on well established trunk-trails before diverging in individual excursions in search of seeds (HÖLLDOBLER, 1976 ; HÖLLDOBLER and MÖGLICH, 1980). In these harvester ants, a trunk-trail originates from a recruitment process following the discovery of a new rich foraging area. Its persistence varies from some days to several weeks, depending on the amount of seeds available. The trunk-trail is abandonned when the area is depleted of its seed supplies and a new one is established, leading to a new area. Hunting trails of *L. fuliginosus* also lead foragers to areas they individually explore in search of disseminated prey, as is the case with seeds, and are abandonned after some time while others are established.

Therefore, like trunk-trails in harvester ants, hunting trails could originate from recruitment trails and lead to areas where prey are abundant. This would suppose of course that the distribution of the prey is not homogeneous over the foraging area. However we have no information about this.

Another interpretation of hunting trails, not excluding the above hypothesis, is based on the observation that almost all the hunting trails originate from the extremity of permanent trails. Hunting trails would then represent a way of enlarging the hunting territory and of leading foragers to areas located outside those patrolled by permanent trails. This enlargment of the hunting territory could be a consequence of a depletion of prey in the vicinity of the nest or of an increased need in protein. While *L. fuliginosus* is active from early April to late November, the hunting trails and ants carrying prey on the permanent trails were observed only from the end of April to the end of September, with the most intensive periods being in June, July and August. It is interesting to note that it is only during the same limited period that earthworms are captured by ants (see Table 2).

The development of the hunting trail system seems to be, at least partly, regulated by the soil surface temperature, being all the more extensive when temperature is high (see Fig. 4). Soil temperature is known to affect forager activity in *Formica rufa,* either increasing their number (SKINNER, 1980) or their running speed (HOLT, 1955). Temperature could also influence the prey activity, thus increasing the frequency of meetings between foragers and prey. Nevertheless, temperature can not account for all variations in hunting activity. Indeed, in late April and May, the hunting trail network extended while temperature was still low (and even decreased in 1984) and few prey were carried back to the nest. During April and early May, food sources (honeydew and prey) are non-existant or very scarce and the foragers showed an intense exploratory behaviour. The collection of sap in April, as also observed in wood ants (*Formica rufa* group) (HORSTMANN, 1974 ; MABELIS, 1979 ; SUDD and SUDD, 1985 ; ROSENGREN and SUNDSTRÖM, 1987), is the expression of this search of the least food sources, which probably explains the extension of the hunting trail network in April and early May. Also, in October the hunting trails completely disappeared and no more ants carrying prey were observed although the temperature remained relatively high during the daytime, especially in 1985. Other factors must therefore account for the development of the predatory activity of *L. fuliginosus* in the course of a season. Such factors could be prey availability and the colony's need for protein. We know unfortunately nothing about the phenology of *L. fuliginosus* except that the swarming period occurs in June and July (GASPAR, 1967) (our observations have shown that the swarming period was in fact much longer and that it extendend from mid-June to September each year).

It should be pointed out that hunting trails are not the only way whereby the foragers reach areas which they individually explore in search of prey. Indeed, observations have shown that during the hunting period (and mainly in July and August), foragers also explore the surrounding grounds all along the permanent trails. Therefore, it seems that the ants leave the trail system at any point (permanent trails or hunting trails) to explore the surrounding area. This observation is

very similar to that of HOLT (1955) who has shown that *Formica rufa* foragers can leave the trail system at any point along it, at random.

The ephemeral and the temporary trails are all linked up to permanent trails, and almost always to their extremity(ies) in the case of the temporary trails. The permanent trail system seems therefore to be the central axis of the mixed (predictive and opportunist) foraging strategy observed in *L. fuliginosus* : it leads specialized foragers to localized and predictable food sources and it is used as a base for the exploration of the surrounding areas. In this latter case, recruitment processes follow the discovery of food sources (new aphid colonies or prey) and trails are formed which, depending on the kind of food discovered, will be ephemeral or temporary.

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A NEW HYPOTHESIS ON THE AIR FLOW IN AIR BREATHING IN *CLARIAS GARIEPINUS* (TELEOSTEI, SILURIFORMES)

by

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SUMMARY

Clarias gariepinus (BURCHELL, 1822) breathes air at the surface. Aerial breathing requires a flow of air through intercommunicating cavities (buccopharyngeal cavity, opercular and suprabranchial cavities). Cinematographic observations on normal and abnormal catfishes lead to a new hypothesis about air-breathing in *Clarias*. 1) At rest on the bottom, the suprabranchial cavity is filled with air. 2) While coming up to the surface, a part of the air of the suprabranchial cavity passes to the opercular cavities, especially in their anterior part, through slits between the fan-like valves borne on the epibranchials. 3) Somewhat later, opercular adduction results in expulsion of bubbles through the opercular slits. 4) When the snout reaches the surface, the mouth is wide open. Depression of the hyoid bars and opercular abduction produce aspiration of air into the buccopharyngeal cavity. 5) Air is forced into the suprabranchial cavity, through the third branchial slit by mouth closure and buccopharyngeal constriction. Hyoid bars elevation plays a prominent role in that constriction as demonstrated by the partial impossibility for abnormal *Clarias* with locked hyoid bars to push air from the mouth to the suprabranchial cavities.

Key words : Pisces, *Clarias*, air breathing.

INTRODUCTION

Clarias gariepinus (BURCHELL, 1822) (a species of which Clarias lazera CUVIER and VALENCIENNES, 1840, is regarded as a synonym by TEUGELS, 1982) uses two modes of ventilation (POLL, 1959), aquatic and aerial. The latter takes place in two symmetrical and separated suprabranchial cavities which are posterodorsal outgrowths of the opercular cavities. The following description of the suprabranchial cavities and of their anatomical relations is compiled from MARLIER (1938), NAWAR

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(1955), MOUSSA (1956 and 1957), SINGH and HUGHES (1971), COCKSON (1972), HUGHES and MUNSHI (1973), MUNSHI (1976), SINGH (1976), SINGH *et al.* (1982), HELLIN and CHARDON (1983). Each one comprises an anterior region above the branchial region and a posteroventral one surmounting the opercular cavity and extending backward up to behind the level of the pectoral girdle. The communication between opercular and suprabranchial cavities is controlled by downward-bulging fan-like expansions of the first four branchial arches ; these fans work as passive valves allowing one-way upward passage of air from buccal to suprabranchial cavities. They appear as the continuation on the epibranchials of transformed branchial filaments. The transformation consists of loss of vascularization, flattening, longitudinal fusion and greater development of the branchial rays which remain cartilaginous in their distal part. Moreover, the buccal and suprabranchial cavities may communicate through a unique dorsal prolongation of the third branchial slit between the second and third epibranchials. That part of the third branchial slit is separated from its ventral ceratobranchial part by two small fibrous cushions facing each another. In its resting state it seems to remain somewhat open, but the contraction of the obliquus communis branchial muscle (HOLSTVOOGT, 1965) may bring nearer the second and third epibranchials and close it. The suprabranchial cavities contain two highly vascularized arborescent organs which are the sites together with their walls, for gas exchanges to occur. The walls are three-fold. The volume of the cavities may be reduced by contraction of a striated muscle covering its posterior wall. Attempts were made to describe the movement of air-breathing and the path of the air in *Clarias* by MARLIER (1938), MUNSHI (1976), SINGH *et al.* (1982) and HELLIN and CHARDON (1983). Observations on *C. gariepinus* with the hyoid bars locked in their depressed position by a connective tissue thickening and careful observations on normal fish allow us to criticize the previous hypotheses about the path of the air through the cavity and to propose a new one.

MATERIAL AND METHODS

Four normal *Clarias gariepinus* of standard length 27 to 30 cm and one abnormal 28 cm specimen with locked hyoid bars were filmed at 32 and 48 frames/second with a Beaulieu and 1PL Photosonics 16 mm cameras. Twelve scenes of the normal fish and three of the abnormal one were analysed.

For technical reasons it was only possible to observe clearly on films the opening or closing of the mouth and the movements of the hyoid bars and opercles ; the abduction and adduction of the suspensoria and the opening or closing of the opercular membranes could not be observed with certainty.

RESULTS

Observations on the normal specimens (Fig. 1)

Resting *Clarias* perform aquatic respiration by slow movements as it is described for other teleosts (for example BALLINTIJN (1969a and b) in *Cyprinus carpio* LINNE, 1758). At an apparently unexpected time, the fish swims to the surface of the water. In at least 4 cases out of 12, the mouth is clearly open during upward swimming, while according to HELLIN and CHARDON (1983) it is closed during that movement. While swimming up, the fish releases gas bubbles (average diameter at release : 11 mm) through the opercular slits. When the mouth reaches the surface, it is wide open and the opercles start to abduct. A few milliseconds later the mouth partially closes, the fish turns head down and begins to dive. In some cases it releases a small bubble through the opercular slits.

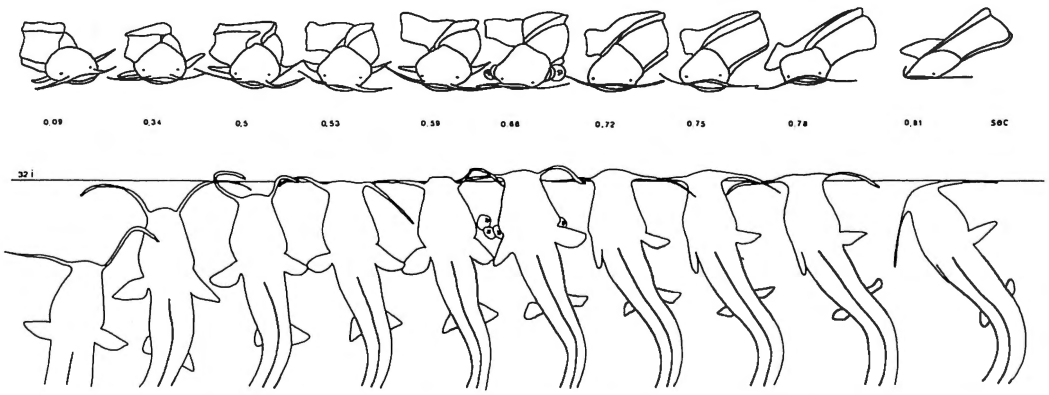

Fig. 1. — Sequence (redrawn after a film at 32 frames/sec) of the air-breathing in *Clarias gariepinus*. Notice that the intervals of time between the schemas are not constant. Bubbles are conspicuous on the sixth drawing. (unpublished drawing of HELLIN).

Observations on the abnormal catfish (Figs 2, 3)

The hyoid bars do not move conspicuously, neither at their articulation on the suspensoria, nor at their medial junction. At rest, slow aquatic respiration consists of movements of the mouth, opercles and branchiostegal membranes only. An air bubble can be observed in the mouth cavity against the palate. This bubble is released through the mouth opening at the beginning of the swimming up. The mouth remains open during the upward movement, and two gas bubbles, smaller (diameter of about 6 mm) than in normal specimens, are released through the gill slits. When reaching the surface, the mouth is open and the opercles begin abduction. Then, the fish dives and releases some air through the opercular slits, while the mouth closes incompletely. As soon as the fish is resting horizontally on the bottom, a bubble is again conspicuous against the palate.

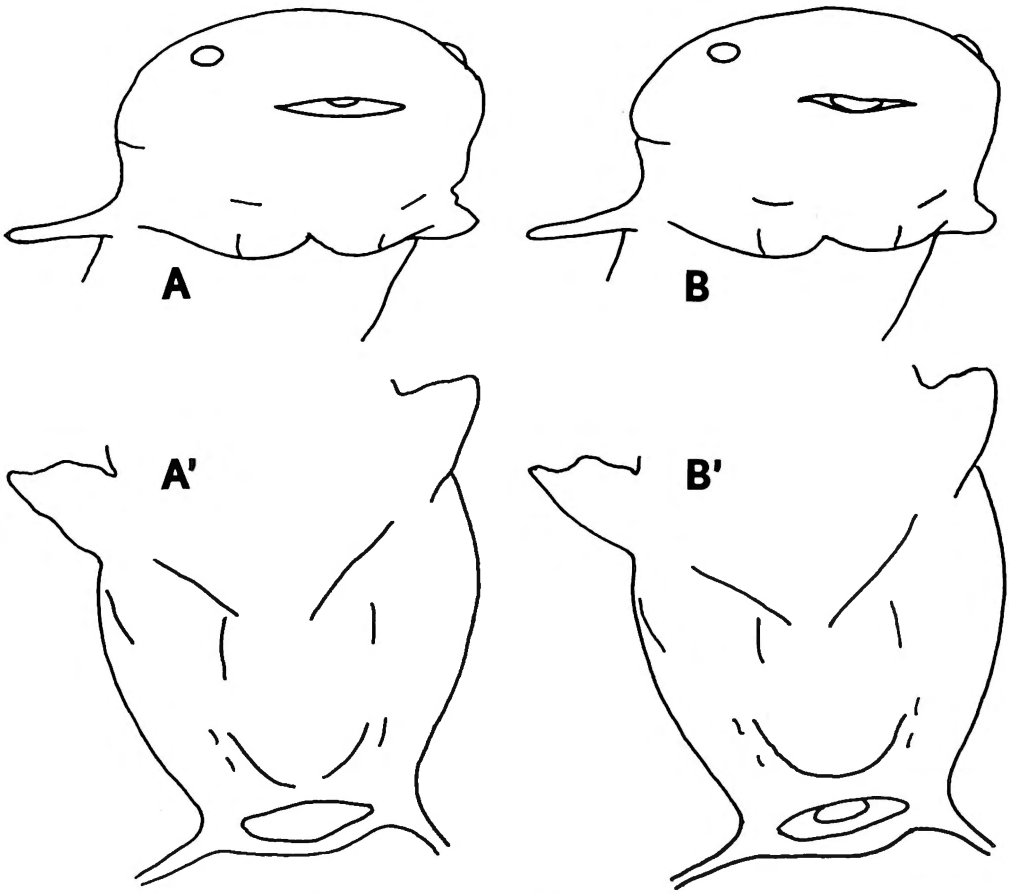

Fig. 2. — A and A'. Frontal and ventral views of the abnormal *Clarias gariepinus* resting on the bottom, showing an air bubble in the open mouth cavity during aquatic inspiration; notice that the bubble is conspicuous in frontal view only. B and B'. Similar views during aquatic expiration with the air bubbles conspicuous in both aspects.

DISCUSSION

The last hypothesis about the path-way of the air in the respiratory cavities of *Clarias* was proposed by HELLIN and CHARDON (1983) on basis of anatomical, optical, cinematographical and low speed cineradiographical observations. According to the latter authors, the air passes from the suprabranchial to the buccal cavity through the epibranchial part of the third branchial slit during up-swimming. As the mouth opening is closed, the air is kept there until it is pushed to the opercular cavities through the normal branchial slits; it is finally brought outside by opercular adduction. The catfish opens its mouth and lowers its hyoid bars when reaching the surface; so it breathes in air. Afterwards, it turns head down, shuts its

mouth and actively swims downward. Then air passes to the opercular cavities by buccal cavity contraction and opercular abduction and also as a consequence of the difference in hydrostatic pressures ; some air bubbles may escape through the opercular slits. Afterwards, the air is pushed from the opercular to the suprabranchial cavities between the fans.

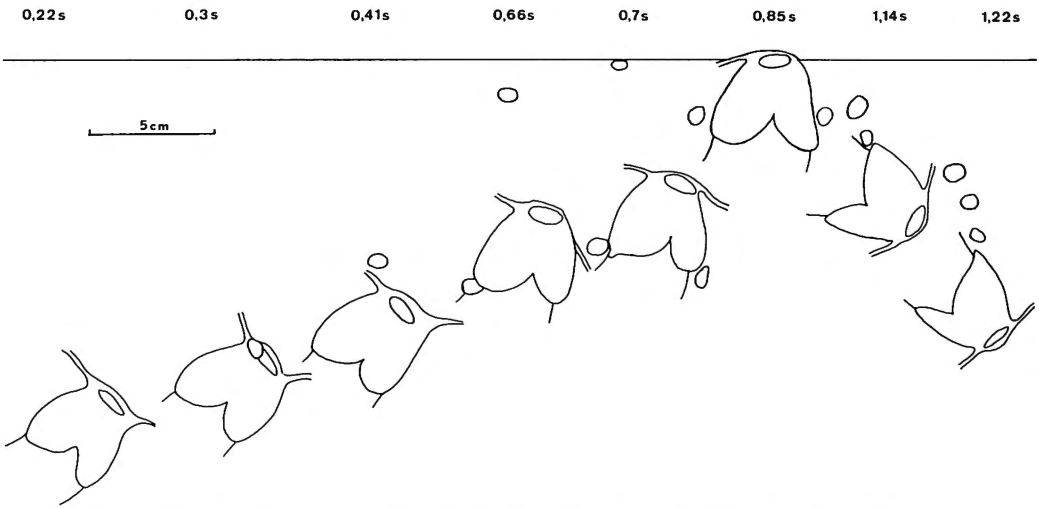

Fig. 3. — Sequence (redrawn after a film, at 100 frames/sec) of air-breathing in an abnormal *Clarias gariepinus*. The time intervals between the schemas are variable. Notice the air bubbles escaping through the mouth and, later, through the opercular slits.

A first objection concerns the possibility of pushing air from the opercular to the suprabranchial cavities ; it is hard to accept that only the dorsal part of the opercular slit opens during opercular adduction, and in any case, it would let escape much air. Further, the above described orientation of the fans prevents air flow from opercular to the suprabranchial cavities. Direct observations of the actual position of the opercular membrane unfortunately proved unsuccessful.

Secondly, our present observations of the mouth opening during up-swimming and careful reexamination of previous unpublished drawings of HELLIN (Fig. 1) clearly show that the mouth is open at that time, at least in most cases. Our observations on the abnormal catfishes are crucial because they demonstrate that gas contained in the buccal cavity cannot but escape by the mouth opening if it is open during the upward movement.

So, we cannot agree any longer with HELLIN and CHARDON's hypothesis. The path-way of the air must be more simple (Fig. 4), as follows. (1) At rest on the bottom, the suprabranchial cavities are filled with air and the other respiratory cavities with water (and maybe a small quantity of air). (2) The fish swims up to the surface, usually with it mouth open ; air is pushed into the opercular cavities by the

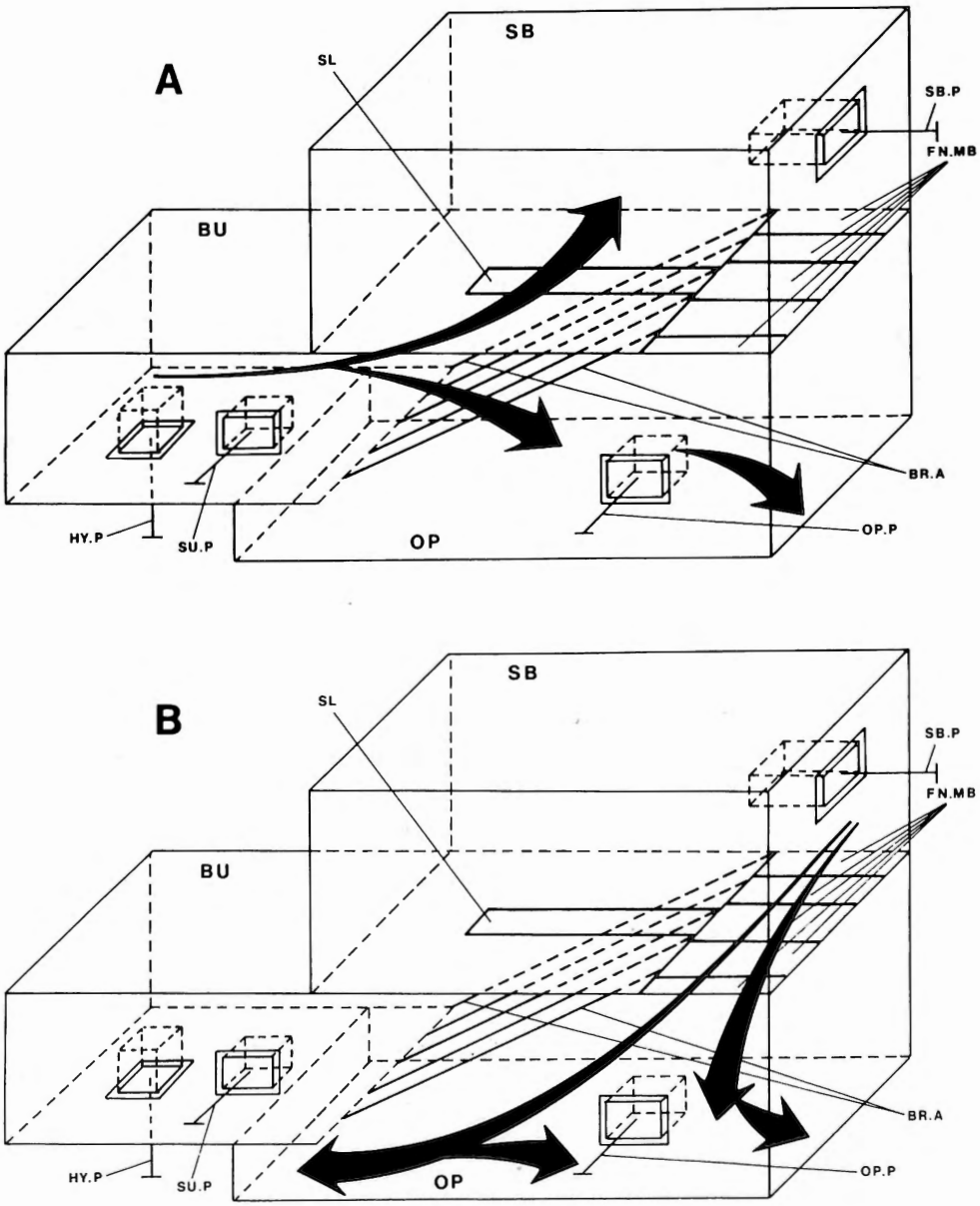

Fig. 4. — Diagram of the path of the air through the respiratory cavities in *Clarias gariepinus*. The proportions and shape of the structures are not respected. Muscular activities resulting in pressure changes in cavities are symbolized by pistons. A. Path of the air during inspiration,. B. Path of the air during expiration (BR. A : branchial arches ; BU : buccal cavity, FN. MB : fans and membranes ; HY.P : hyoidean piston ; OP : opercular cavity ; OP. P : opercular piston ; SB : suprabranchial cavity ; SB. P : suprabranchial piston ; SL : third branchial slit ; SU.P : suspensorial piston).

contraction of the posterior muscles of the suprabranchial cavities concomitent with the dilatation of the opercular cavities ; air flows between the fans which are easily pushed in that sense ; (3) while the fish keeps swimming upward, it adducts its opercles, it lifts and draws closer to each another its hyoïd bars so that air bubbles are emitted through the opercular slits. (4) The snout comes out of water, the mouth is open ; the fish lowers and abducts its hyoïd bars (HELLIN and CHARDON, 1983) so that air enters the mouth cavity ; somewhat later the opercles are abducted, so that the water that was contained in the buccal cavity flows down into the opercular cavities some air may pass to the opercular cavities too. (5) The fish closes its mouth, turns head down and starts diving. (6) Elevating the hyoid bars raises the pressure in the mouth cavity while opening the epibranchial part of the third branchial cleft, and dilatation of the suprabranchial cavities lets the air pass into the suprabranchial cavities ; this is helped by the higher hydrostatic pressure at the level of the buccal cavity. Passage of air into the opercular cavities is now prevented by opercles which explains the small bubbles escaping through the opercular slit ; the epibranchial part of the third branchial slits seems to be slightly open at rest, and to be closed only by the contraction of the obliquus communis muscle ; the dilatation of the suprabranchial cavities results from both the lowering of its ventral wall fastened to the epibranchials by the relaxation of its levatores branchiales muscles, and by the relaxation of its posterior muscles (for description of the branchial muscles, see HOLSTVOOGD, 1965). (7) The catfish reaches the bottom and lies on it ; water ventilation is possible through buccal and opercular cavities.

This interpretation of our data partially agrees with the incomplete hypotheses of MUNSHI (1976) and SINGH *et al.* (1982) but is mainly different about the way the air leaves the suprabranchial cavities.

The abnormal catfish is capable of respiration when resting on the bottom by opercular movements. It swims to the surface like normal fish do, but it seems not to be able to push much air into the suprabranchial cavities. This fact is related to the impossibility of elevating the hyoid bars. It clearly emphasizes the importance of the hyoid bars' movements in air breathing in *Clarias*. Inefficient aquatic and aerial ventilations explain death occurring several months after the disease is observed.

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ALUMINIUM AND pH EFFECTS ON SOME OSMOREGULATORY AND HAEMATOLOGICAL PARAMETERS OF THE ACID-RESISTANT AMERICAN BULLHEAD *ICTALURUS NEBULOSUS* (LE SUEUR)

by

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ABSTRACT

Physiological experiments performed with the acid-resistant fish *Ictalurus nebulosus* gave evidence that :

1) Na^+-influx at high H^+- and Al-concentrations increased, in contrast to acid-sensitive fishes, which decrease their Na^+-influx in such conditions ;

2) high external Ca^{++}-concentrations have no beneficial effect on low pH and Al toxicity ;

3) differences in populations from acid compared to circumneutral lakes disappeared when acclimated to the same soft water of low ionic content (pH 6.8).

Key words : acidification, aluminium, bullhead, acid-resistance, calcium.

INTRODUCTION

Atmospheric emissions of SO_2 and NO_x are responsible for an acid deposition which can cause acidification of poorly buffered fresh waters (IRWIN and WILLIAMS, 1988). As a consequence of the H^+-ion input in the soil, especially leaching of Al can take place. This can result in elevated Al-concentrations in streams and surface waters (DICKSON, 1980 ; VANGENECHTEN, 1983).

Freshwater fishes exhibit physiological disturbances e.g. in ion balance, at conditions of high H^+- and Al-concentrations (MCDONALD and WOOD, 1981 ; MCDONALD *et al.*, 1983 ; DALZIEL *et al.*, 1985 ; WITTERS, 1986 ; GONZALEZ and

DUNSON, 1987 ; WITTERS *et al.*, 1987 ; FREDA and MCDONALD, 1988 ; HÔBE and MCMAHON, 1988). Elevated Ca^{++}-concentrations in the water sometimes can exercise an ameliorating effect on pH- and Al-stress (MCDONALD *et al.*, 1980, 1983 ; FREDA and MCDONALD, 1988). Most studies have been made on acid-sensitive fishes, especially Salmonids (MCDONALD and WOOD, 1981 ; MCDONALD *et al.*, 1983 ; DALZIEL *et al.*, 1985 ; WITTERS, 1986 ; WITTERS *et al.*, 1987 ; FREDA and MCDONALD, 1988).

American bullheads are found in neutral as well as in acid lakes. As far as is known, only the effect of low pH has been investigated on bullheads of a circumneutral water (VANGENECHTEN *et al.*, 1987). The latter study revealed that the physiological response to pH 4.0 was qualitatively similar but less pronounced compared to the reaction of acid-sensitive fishes.

In order to get more information on the effects of high H^+- and Al-concentrations on the American bullhead *I. nebulosus* (LE SUEUR), experiments focussed on the following questions :

1) What is the effect of low pH and elevated Al-concentrations on *I. nebulosus* ?

2) Do elevated external Ca^{++}-concentrations influence pH- and Al-sensitivity of *I. nebulosus* ?

3) Are *I. nebulosus* from an acid and a non-acid water physiologically different ?

MATERIALS AND METHODS

Bullheads were obtained from three lakes in the Campine region of Belgium (Table 1). Several experiments were conducted when the fishes remained on their natural water, while in some experiments an artificially made water was used (Table 1).

TABLE 1.

pH and ion concentrations (mmol/l) of the natural and artificially prepared waters.

	pH	Na^+	Ca^{++}	Al_{total}
Kooldries	6.1	0.26	0.13	0.007
Zwart Water	4.5	0.75	0.26	0.03
Zegge	7.0	0.53	0.51	B.D.
Artificially prepared water	6.8	0.44	0.03	B.D.

(B.D. = below detection limit of Al = < 0.001 mmol Al/l)

The methods used in our experiments to measure physiological parameters are given in detail in WITTERS (1986) and VANGENECHTEN *et al.* (1987). The measurements of Na^+ fluxes were performed over 4 hours. In the figures, results of Na^+-fluxes are presented at the start of the measurement period.

RESULTS AND DISCUSSION

(1) The influence of low pH (pH 4.0) and several Al-concentrations (7 ; 40 ; 110 µmol/l) was examined on the NaCl-balance, the haematocrit value and the plasma glucose and protein levels of the bullhead. Experimental animals were caught in the « Kooldries » lake (pH 6.1). They remained in their natural water for about 5 days and at t = 0, the water was acidified to pH 4.0 (Figure 1). After 84 hours at pH 4.0, two experimental groups were treated with elevated Al levels (40 and 110 µmol/l) while one group remained at pH 4.0 with the natural Al-concentration (7 µmol/l).

About 2 hours after acidification of the water to pH 4.0 a net whole body loss of Na^+ (Fig. 1 A)was established, which was entirely caused by a strongly increased efflux (Fig. 1 C). Most acid-sensitive fishes show both an increased Na^+-efflux and a decreased Na^+-influx at acute pH decreases (McDONALD and WOOD, 1981 ; McDONALD *et al.*, 1983 ; GONZALEZ and DUNSON, 1987 ; FREDA and McDONALD, 1988). Our results on the contrary showed an increase of the Na^+-influx (Fig. 1 B) after 84 hours exposure to pH 4.0 with low (7 µmol/l) Al levels. In this way, the bullhead seemed to be able to restore its Na^+-net flux. When Al levels were acutely raised up to 40 µmol/l or 110 µmol/l at 84 hours of acid exposure, Na^+-influx remained significantly higher than control values at pH 6.1 and significantly higher than acid exposure values at t = 2 hours. The negative net whole body Na^+-balance at 110 µmol Al/l was the result of a strongly increased Na^+-efflux (Fig. 1 C).

(2) In a second experiment, we investigated whether high external Ca^{++}-concentrations have an ameliorating influence on the physiology of *I. nebulosus* at low pH and high Al-concentrations.

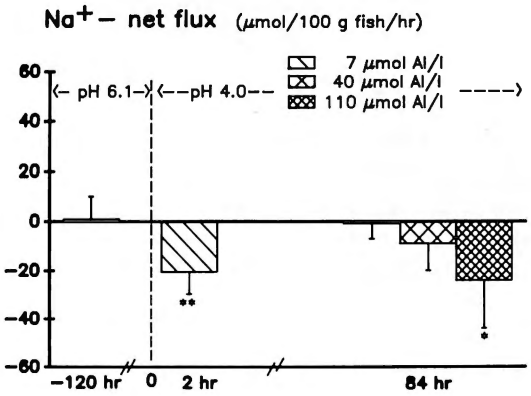
Fishes of the « Kooldries » lake which remained 3 to 4 months in artificially prepared water (Table 1) were used. After a pre-exposure to pH 4.0 for 4 days some ionoregulatory and haematological parameters were measured. Then, the physiological effects of 30 and 1000 µmol Ca^{++}/l, with and without addition of 40 µmol Al/l, were examined on the bullheads after 3 days exposure to these conditions. The exposure to low pH and to low pH with Al caused minor changes. The elevated Ca^{++}-level (1000 µmol/l) had no effect, neither at low pH, nor at low pH with Al.

(3) Finally, it was investigated whether pH-differences of natural waters can be an initiating factor in forming physiological strains of *I. nebulosus*. It was questioned whether bullheads from an acid lake are physiologically adapted to low pH and therefore exhibit some physiological differences with bullheads from a less acid lake.

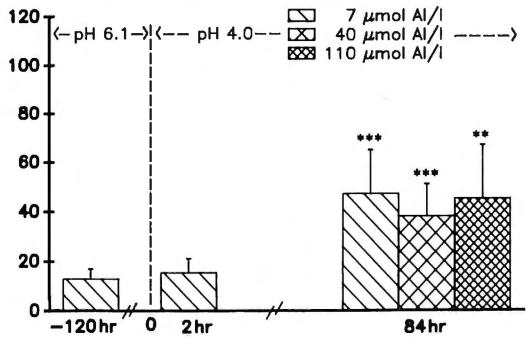
Samples of two populations of bullheads (Kooldries : pH 6.1 and Zwart Water : pH 4.5) were kept in their natural waters. Significant differences in plasma ion concentrations, haematocrit value and plasma glucose concentration were measured.

After an acclimation of 2 populations of bullheads (Zegge : pH 7.0 and Zwart Water : pH 4.5) to pH 6.8 for 5 weeks, these physiological differences disappeared,

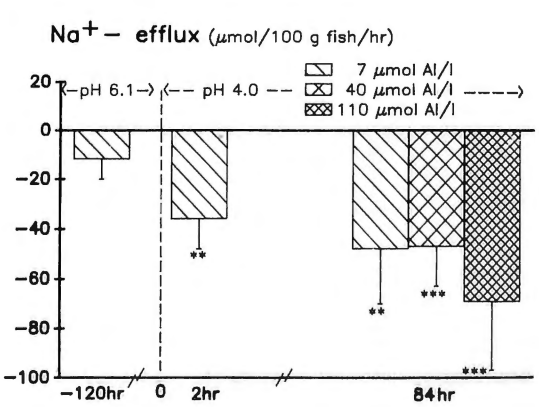
A


Na+ – net flux (μmol/100 g fish/hr)
7 μmol Al/l
40 μmol Al/l
110 μmol Al/l
<– pH 6.1–><––pH 4.0––––>
60
40
20
0
–20
–40
–60
**
*
–120 hr
0
2 hr
84 hr

B


Na+ – influx (μmol/100g fish /hr)
7 μmol Al/l
40 μmol Al/l
110 μmol Al/l
<– pH 6.1–><–– pH 4.0––––>
120
100
80
60
40
20
0
***
***
**
–120hr
0
2hr
84hr

C


Na+ – efflux (μmol/100 g fish/hr)
7 μmol Al/l
40 μmol Al/l
110 μmol Al/l
<–pH 6.1–> <–– pH 4.0 ––––>
20
0
–20
–40
–60
–80
–100
**
**
***
***
–120hr
0
2hr
84hr

except for the haematocrit value. The physiological response of both populations to a subsequent acidification to pH 4.3 remained comparable during the whole course of the experiment (14 days). This is in contrast with the results of Na^+-influx measurements in the waterbug *Corixa punctata* (ILLIGER). Animals from a non-acid water exhibited a decreased Na^+-influx at low pH in contrast to animals from an acid water (VANGENECHTEN *et al.*, 1989).

CONCLUSIONS

Extremely high H^+- and Al-concentrations had a relatively small effect on the examined physiological parameters of the American bullhead. The increased Na^+-influx was a striking result. It can be an adaptive response to resist pH- and Al-stress by compensating Na+ losses.

Our second experiment indicated that *I. nebulosus* experienced no beneficial effect of high external Ca^{++}-concentrations. Some experimental evidence was obtained arguing that pH differences of natural waters have not yet given rise to physiological strains of *I. nebulosus*.

ACKNOWLEDGEMENTS

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Figure 1. — Na^+-netflux(A), Na^+-influx(B), and Na^+-efflux(C) (mean value ± 95% confidence limits) in *I. nebulosus* at several conditions of pH and Al. Significant differences were tested by a two-tailed Student 't'-test by comparison with the control value (pH 6.1) and are indicated by asterisks * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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CONSIDÉRATIONS SUR LA CROISSANCE OVOCYTAIRE CHEZ LES POISSONS À OVOGENÈSE SYNCHRONE ET ASYNCHRONE

par

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RÉSUMÉ

Chez les poissons, trois modèles de base du développement ovocytaire ont été identifiés en se basant sur une approche dynamique de l'ovogenèse : les ovaires synchrones, groupe-synchrones et asynchrones. Dans le premier groupe se trouvent les poissons à ponte unique chez qui l'ovaire ne contient qu'un seul stade ovocytaire en début de recrudescence gonadale ; en période de reproduction, l'histogramme de répartition des tailles d'ovocytes ne présente que deux modes bien distincts : les ovocytes protoplasmiques et les ovocytes en fin de vitellogenèse. Le second groupe est caractérisé par la présence de trois modes dans l'histogramme de répartition des tailles ovocytaires : une classe d'ovocytes protoplasmiques, une classe d'ovocytes en prévitellogenèse avancée mais qui ne participent pas à la ponte de l'année et une classe d'ovocytes en fin de vitellogenèse et qui seront pondus prochainement. Le troisième groupe comprend les poissons à pontes multiples et se caractérise par la présence simultanée de tous les stades ovocytaires sans prédominance d'une classe particulière.

Cet article décrit succinctement l'évolution de la maturité sexuelle chez différentes espèces appartenant à ces trois groupes, en termes de variations de l'indice gonadosomatique, de composition de l'ovaire et d'évolution de la taille des ovocytes.

Mots-clef : poisson, reproduction, indice gonado-somatique, ovogenèse.

Patterns of oocyte growth in fish with synchronous and asynchronous ovogenesis

SUMMARY

In fish, three patterns of oocyte growth were determined by a dynamic approach of ovogenesis : the synchronous, group-synchronous and asynchronous ovary. The first group includes the monospawner fishes in which the ovary contains only vacuole free oocyte during the start of recrudescence. During the spawning season, the histogram of oocyte size contains only two classes corresponding to the vacuole free and globule stage oocytes. The second group is characterized by the presence of three classes in the histogram of oocyte size : vacuole free, yolk vesicle and yolk globule stage oocytes. The third group includes the multi spawner fishes in which ovaries contain different oocyte stages without predominance of one class. This paper briefly describes the maturity of different species from these three groups in terms of variations of gonadosomatic index, composition of ovary and characteristic of oocyte growth during sexual cycle.

Keywords : fish, reproduction, gonado-somatic index, ovogenesis.

INTRODUCTION

La survie de toute espèce dans un environnement saisonnier fluctuant est dépendante de mécanismes qui lui permettent d'ajuster ses fonctions physiologiques aux changements du milieu. La compréhension des mécanismes régissant le succès de la production des jeunes (recrutement) est un objectif important de l'écologie animale et de l'halieutique. Différentes stratégies de reproduction ont été identifiées en se basant sur des critères tels que :

1) Les caractéristiques démographiques de l'espèce : ce sont les stratégies r et K, établies d'après l'équation logistique dN/dt = rN(K-N/K) (Mac Arthur et Wilson, 1967 ; Stearns, 1976). La stratégie K apparaît chez des espèces vivant dans un environnement stable ; elle favorise une maturité tardive, un effort de reproduction faible, mais une progéniture de grande taille et une longévité élevée. A l'inverse, la stratégie r apparaît chez des espèces vivant dans un environnement moins prévisible et favorise une maturité précoce, un effort de reproduction ainsi qu'une fécondité élevés et une vie courte.

2) L'influence des facteurs externes proximaux (température et photopériode) : la maturation des gonades est principalement induite par une photopériode décroissante chez les salmonidés alors que la température constitue le facteur primordial chez les cyprinidés (De Vlaming, 1974 ; Billard *et al,* 1978).

3) La taille de la larve et son importance par rapport à l'alimentation et à la résistance à l'inanition, à l'activité et à l'aptitude à chercher la nourriture et au risque de prédation (Miller *et al,* 1988).

Une analyse de la reproduction des poissons en milieu naturel révèle que ceux-ci, indépendamment de la période à laquelle ils se reproduisent, présentent deux stratégies de reproduction bien distinctes : 1) celle des poissons qui ne pondent

qu'une seule fois durant la saison de reproduction qui est généralement annuelle dans les régions tempérées et 2) celle des poissons qui pondent à plusieurs reprises durant une même saison de reproduction. Cette différence de stratégie reproductive peut avoir des conséquences importantes sur le recrutement annuel en alevins et donc sur la dynamique des populations, puisqu'on est en présence d'une part, de poissons concentrant leur effort de reproduction sur une seule ponte et de l'autre, de poissons qui répartissent leur potentiel reproductif sur plusieurs pontes et qui sont donc moins soumis aux aléas de l'environnement.

MATÉRIEL ET MÉTHODES

Les poissons utilisés pour cette étude (tableau 1) ont été collectés lors de pêches scientifiques (électricité, filet, vidange d'échelles à poissons) réalisées dans le cadre des travaux de Philippart sur la dynamique des populations dans l'Ourthe et de Kestemont sur la biologie de la reproduction du goujon. Les poissons ont été prélevés à différents moments de leur cycle sexuel et leur maturité a été évaluée par des critères tels que :

1) l'indice gonadosomatique (IGS) défini comme le rapport (en pour cent) entre le poids des gonades et le poids corporel du poisson,

TABLEAU 1.

Nombre, origine et mode de capture des poissons femelles utilisés dans cette étude.

Espèce	Nombre	Rivière	Capture	Source
Hotu	144	Ourthe	PE	Philippart, 1980
Vandoise	147	Ourthe	PE	Philippart, 1981
Chevaine	59	Ourthe	PE	Philippart, 1977
Barbeau	116	Ourthe	PE	Philippart, 1977
Ombre	101	Ourthe	PE	Philippart, non publié
Gardon	84	Ourthe	PE	Philippart, non publié
Goujon	75	Eau d'Heure	PE	Kestemont, 1988

PE = pêche à l'électricité.

2) la taille des ovocytes et leur proportion relative dans l'ovaire, établies par fixation des ovaires dans du liquide de Gilson et comptage des différentes classes de tailles d'ovocytes,

3) le stade de maturité ovocytaire, déterminé par préparation histologique et coloration topographique des gonades (KESTEMONT, 1987). Bien qu'étant un processus continu, l'ovogenèse a toutefois été scindée en plusieurs stades à des fins de quantification :

— les ovogonies, très semblables aux spermatogonies, qui se multiplient puis se transforment en ovocytes primaires non vacuolisés (stade 1) (Pl. I, 1),

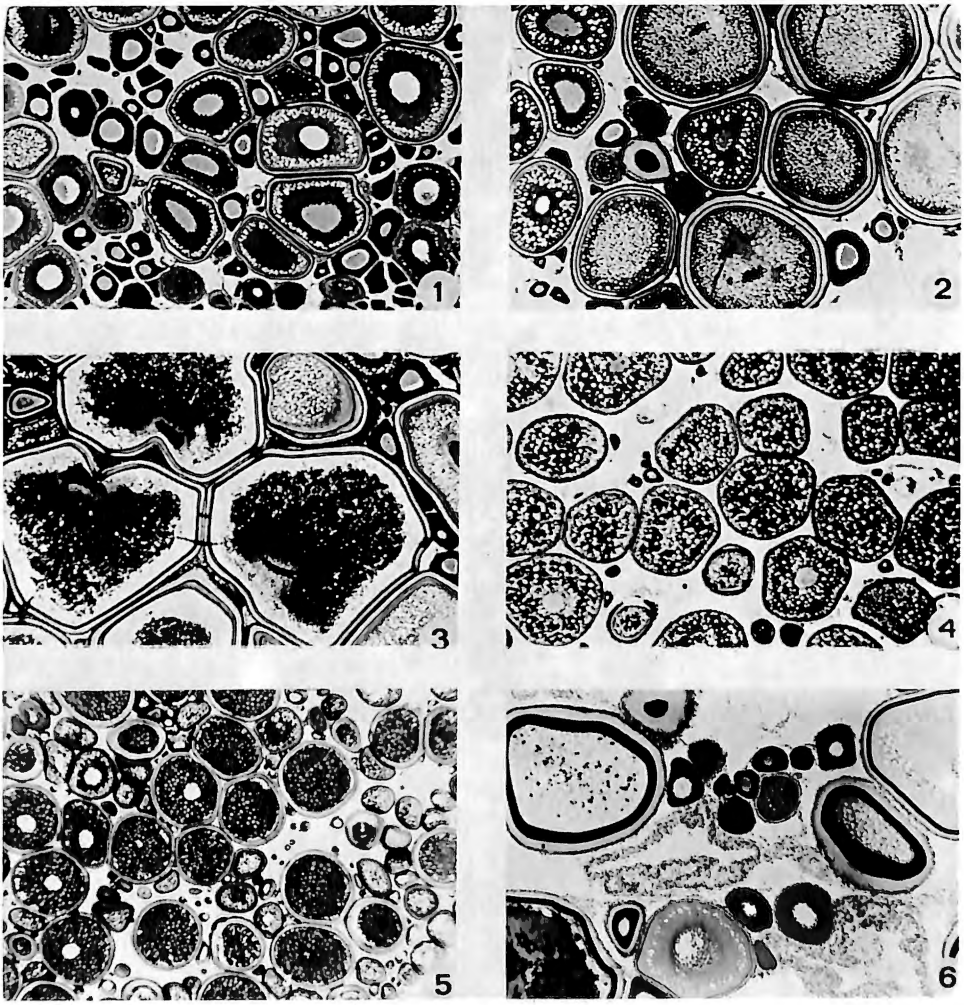

PLANCHE I

1. Ovaire en début de recrudescence. Présence d'ovocytes protoplasmiques (stade 1) et d'ovocytes en début de prévitellogenèse (stade 2).
2. Ovaire en fin de prévitellogenèse (stade 3). Cytoplasme envahi par les vésicules vitellines.
3. Ovaire en fin de vitellogenèse. Cytoplasme envahi par les globules de vitellus. Formation du micropyle et migration de la vésicule germinative en périphérie du cytoplasme.
4. Ovaire synchrone. Présence uniquement d'ovocytes vitellogéniques.
5. Ovaire groupe-synchrone. Présence d'ovocytes vitellogéniques et d'ovocytes en fin de prévitellogenèse. (photo Cl. Remacle).
6. Ovaire asynchrone après ponte. Présence simultanée d'un follicule vide et d'un ovocyte proche de l'ovulation.

— les ovocytes prévitellogéniques (ou en vitellogenèse endogène) dont la taille cellulaire augmente suite à l'accumulation dans le cytoplasme de vésicules à résidus glycoprotéiques. La prévitellogenèse débutante (stade 2, Pl. I, 1) se distingue de la prévitellogenèse avancée (stade 3, Pl. I, 2) par divers critères tels que l'abondance des vésicules, la basophilie du cytoplasme ou la taille de l'ovocyte ;

— les ovocytes vitellogéniques (ou en vitellogenèse exogène = stade 4) caractérisés par l'accumulation de globules de vitellus de nature lipoprotéique refoulant les vésicules vers la périphérie du cytoplasme où elles constitueront les alvéoles corticaux destinés à la protection de l'embryon dans l'oeuf (Pl. I, 3).

RÉSULTATS ET DISCUSSION

L'examen des variations saisonnières de l'indice gonadosomatique montre que chez les poissons à ponte unique, comme le salmonidé *Thymallus thymallus* (ombre commun) et le Cyprinidé *Chondrostoma nasus* (hotu), l'IGS s'élève fortement au moment de la saison de reproduction puis décroît brutalement dès que la ponte a eu lieu (Fig. 1a). De plus, pour un milieu donné, cette ponte se produit de façon synchronisée chez la plupart des poissons de l'espèce et le frai de l'ensemble de la population se concentre sur quelques jours seulement (PHILIPPART, 1977).

Au contraire, chez un poisson à ponte multiple comme le Cyprinidé *Gobio gobio* (goujon), l'IGS augmente nettement dès le retour des conditions favorables, après l'hiver, mais le pic d'IGS décroît très progressivement (Fig. 1b) et des pontes se produisent durant plusieurs mois et de façon répétée chez une même femelle (KESTEMONT, 1987).

En étudiant plus spécifiquement la structure ovarienne et la dynamique de l'ovogenèse, on peut également distinguer deux types de stratégie reproductive en fonction du mode de développement ovocytaire, à savoir : les poissons à ovogenèse synchrone et ceux à ovogenèse asynchrone. Une troisième classe, intermédiaire des deux premières, est constituée par les poissons à ovogenèse groupe-synchrone.

Ovogenèse synchrone

Dans le premier groupe se trouvent les poissons à ponte unique, comme les Salmonidés, les Esocidés et Percidés ainsi que certains Cyprinidés tels que *Chondrostoma nasus* (hotu), *Rutilus rutilus* (gardon) et *Leuciscus leuciscus* (tabl. 2). Chez *Stizostedion lucioperca* (sandre) par exemple, l'ovaire ne comporte que des ovocytes protoplasmiques en début de recrudescence. De ces ovocytes va se détacher une seule cohorte de cellules qui entameront une phase de croissance trophoplasmique pour atteindre le stade vitellogénique en période de reproduction (Pl. I, 4). Sur le plan quantitatif, l'histogramme de répartition des tailles d'ovocytes ne comprend que deux modes comme le montre la Fig. 2 décrivant la répartition des tailles d'ovocytes chez *Thymallus thymallus*.

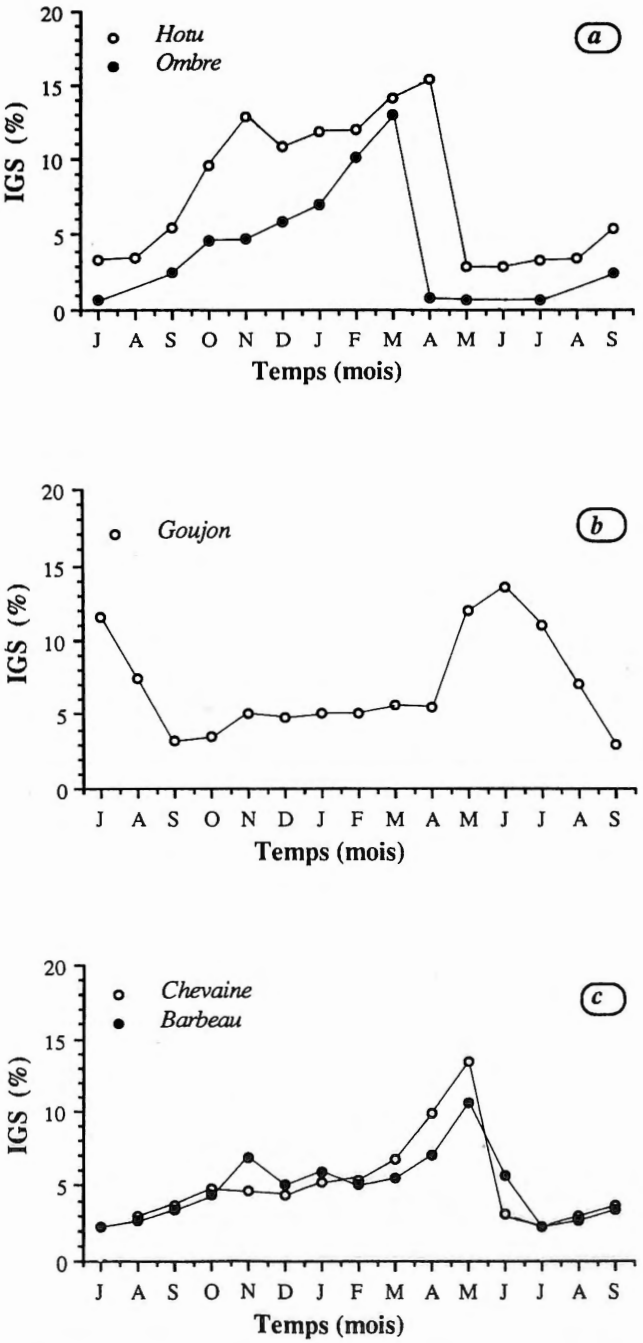

Fig. 1. — Variations saisonnières de l'index gonadosomatique (IGS) chez des poissons à ovogenèse synchrone (a), asynchrone (b) et groupe-synchrone (c).

TABLEAU 2.

Classement, selon leur mode de développement ovocytaire, des principales espèces de poissons d'eau douce se reproduisant dans les cours d'eau de Belgique.
(?) : classement incertain.

Poissons à ovogenèse synchrone	
Salmonidae	
Truite de rivière	*Salmo trutta trutta fario* LINNAEUS, 1958
Truite arc-en-ciel	*Oncorhynchus mykiss* WALBAUM, 1792
Omble chevalier	*Salvelinus alpinus* (LINNAEUS, 1758)
Saumon de fontaine	*Salvelinus fontinalis* (MITCHELL, 1815)
Thymallidae	
Ombre commun	*Thymallus thymallus* (LINNAEUS, 1758)
Esocidae	
Brochet	*Esox lucius* LINNAEUS, 1758
Percidae	
Perche	*Perca fluviatilis* LINNAEUS, 1758
Sandre	*Stizostedion lucioperca* (LINNAEUS, 1758)
Cyprinidae	
Hotu	*Chondrostoma nasus* (LINNAEUS, 1758)
Vandoise	*Leuciscus leuciscus* (LINNAEUS, 1758)
Gardon	*Rutilus rutilus* (LINNAEUS, 1758)
Gasterosteidae	
Epinoche (?)	*Gasterosteus aculeatus* LINNAEUS, 1758
Epinochette (?)	*Pungitius pungitius* LINNAEUS, 1758
Poissons à ovogenèse groupe-synchrone	
Cyprinidae	
Carpe commune	*Cyprinus carpio* LINNAEUS, 1758
Brême commune	*Abramis brama* (LINNAEUS, 1758)
Barbeau fluviatile	*Barbus barbus* (LINNAEUS, 1758)
Chevaine	*Leuciscus cephalus* (LINNAEUS, 1758)
Poissons à ovogenèse asynchrone	
Cyprinidae	
Goujon	*Gobio gobio* (LINNAEUS, 1758)
Vairon	*Phoxinus phoxinus* (LINNAEUS, 1758)
Loche de rivière	*Cobitis taenia* LINNAEUS, 1758
Loche d'étang	*Misgurnus fossilis* (LINNAEUS, 1758)
Brême bordelière	*Blicca bjoerkna* (LINNAEUS, 1758)
Ablette commune	*Alburnus alburnus* (LINNAEUS, 1758)
Ablette de rivière	*Alburnoides bipunctatus* (BLOCH, 1782)
Rotengle	*Scardinius erythrophthalmus* (LINNAEUS,1758)
Carassin	*Carassius carassius* (LINNAEUS, 1758)
Tanche	*Tinca tinca* (LINNAEUS, 1758)

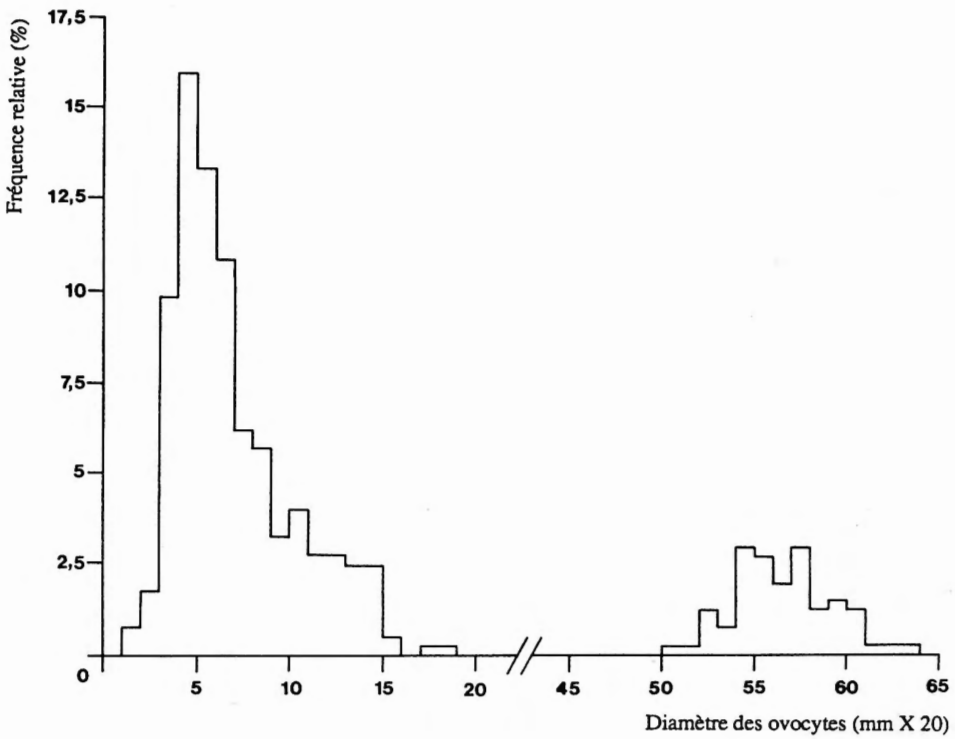

Fig. 2. — Structure ovarienne de l'ombre commun *Thymallus thymallus* avant la ponte.

Ovogenèse groupe synchrone

Le groupe des poissons à ovogenèse groupe-synchrone (tabl. 2) se caractérise par la présence simultanée d'au moins deux populations d'ovocytes bien distinctes (Pl. I, 5). C'est le cas de *Barbus barbus* (barbeau) dont l'histogramme de répartition des tailles d'ovocytes est représenté à la Fig. 3. Un peu avant la période de reproduction, on distingue trois classes d'ovocytes : une classe dominante constituée par les ovocytes de petite taille (stade 1 et 2), une classe d'ovocytes en croissance trophoplasmique avancée (stade 3) mais qui ne participent pas à la ponte de l'année, et enfin une classe d'ovocytes mûrs qui seront pondus très prochainement (stade 4). Les variations de l'IGS de poissons appartenant à ce groupe (barbeau et chevaine *Leuciscus cephalus*) sont décrites à la Fig. 1c.

Ovogenèse asynchrone

Enfin, le groupe des poissons à ovogenèse asynchrone (tabl. 2) se caractérise par le fait que tous les stades ovocytaires sont présents sans prédominance d'une classe

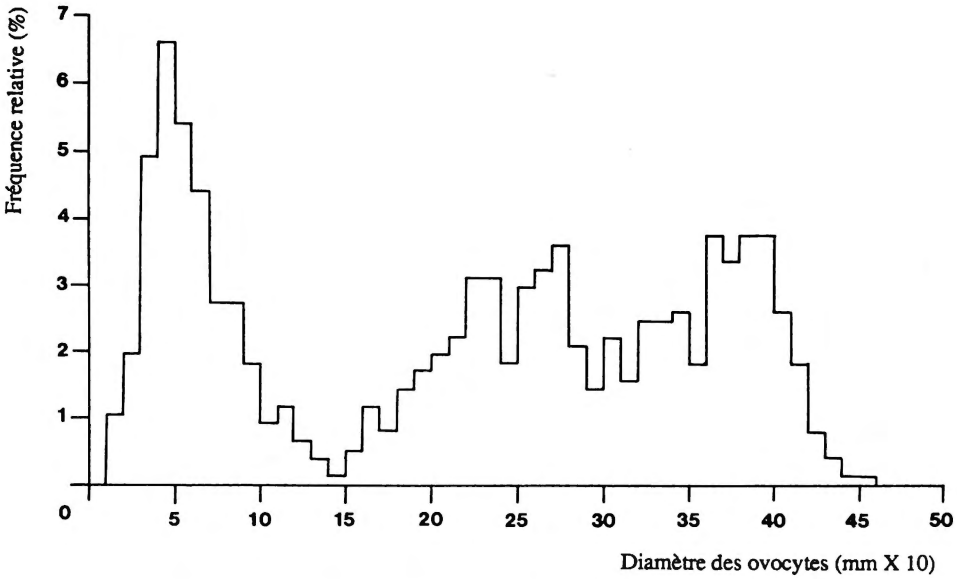

Fig. 3. — Structure ovarienne du barbeau fluviatile *Barbus barbus* avant la ponte.

particulière, excepté la classe des ovocytes de réserve constituée par les ovocytes protoplasmiques qui peuvent être très abondamment représentés comme l'indique l'ensemble des figures décrivant les ovaires du goujon à différents moments du cycle gamétogénétique (Fig. 4). On remarque une prédominance très nette des ovocytes de stade 1 et un étalement de la fréquence des autres classes. Cette prédominance marquée des ovocytes de stade 1 est d'ailleurs associée à une absence quasi totale d'ovogonies, laissant supposer que le stock d'ovocytes de réserve est constitué très tôt et semble suffire pour plusieurs années sans qu'il y ait transformation continue des ovogonies en ovocytes.

Les variations saisonnières du diamètre moyen des différents stades ovocytaires indiquent que le recrutement en jeunes ovocytes s'accentue un peu avant la période de reproduction ce qui entraîne une élévation du diamètre moyen de ce stade (Fig. 5). La même observation s'applique aux ovocytes en prévitellogenèse avancée (stade 3) et cette croissance continue durant toute la période de ponte. En période de reproduction, des follicules vides, signe d'une ponte récente, sont d'ailleurs présents dans l'ovaire de même que des ovocytes proches de l'ovulation (Pl. I, 6).

Cet aspect asynchrone de l'ovogenèse et les pontes répétées qui en résultent sont provoquées, soit par l'entrée des ovocytes protoplasmiques en prévitellogenèse à des moments différents, soit par une différence dans la vitesse de croissance de ces ovocytes.

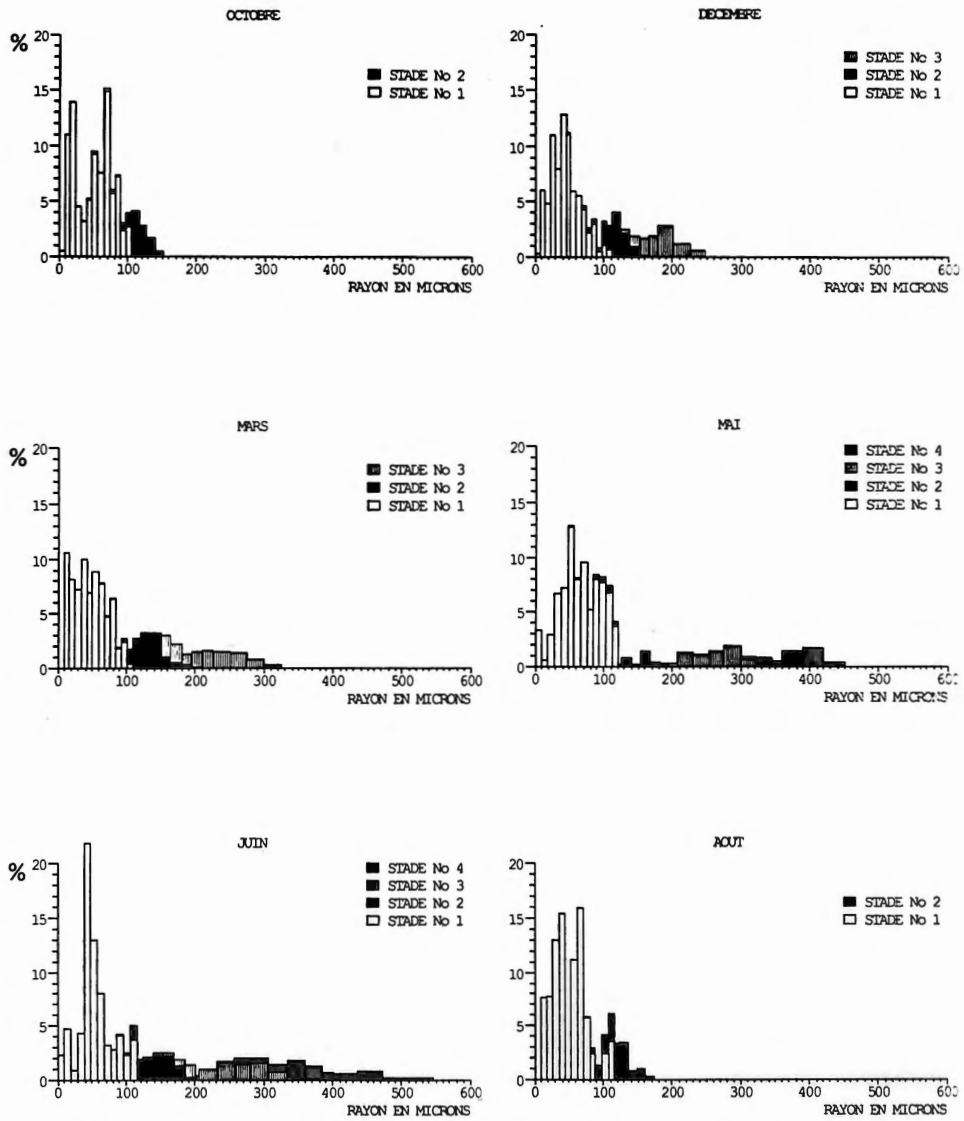

Fig. 4. — Structure ovarienne du goujon *Gobio gobio* à différents moments de son cycle gamétogénétique.

Dans l'analyse du problème du développement ovocytaire, il faut noter enfin qu'une espèce placée dans des conditions particulières peut modifier sa stratégie de reproduction. Ainsi, le barbeau, *Barbus barbus* peut passer d'un développement ovocytaire groupe-synchrone en milieu naturel à une ovogenèse totalement asynchrone en captivité lorsque les femelles sont élevées en conditions optimales de

température, photopériode et alimentation. Il en résulte un recrutement continu des ovocytes qui entraine des cycles de pontes répétées pour la même femelle durant une année (PONCIN, 1988).

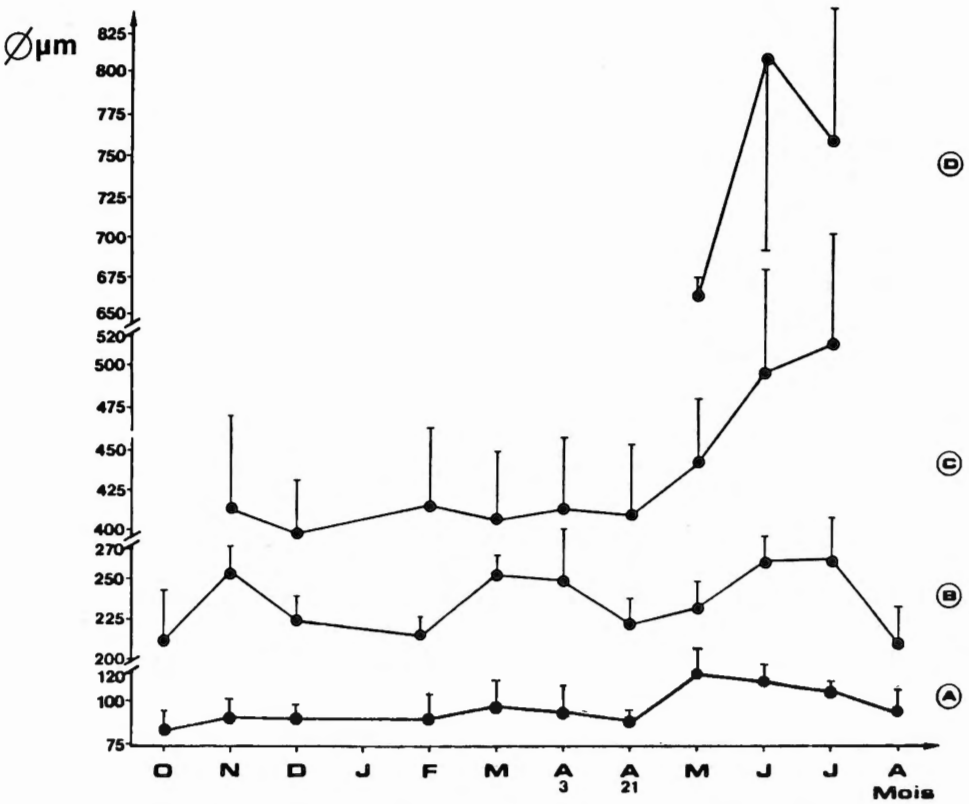

Fig. 5. — Variations du diamètre moyen des différents stades ovocytaires au cours du cycle sexuel du goujon *Gobio gobio*. A stade 1, B stade 2, C stade 3, D stade 4.

Dans l'état actuel des connaissances sur la croissance ovocytaire chez les différents groupes de poissons étudiés, deux axes de recherche s'avèrent intéressants à développer : d'une part, une analyse approfondie des mécanismes hormonaux contrôlant la maturité des gonades et d'autre part, une caractérisation de la valeur de survie pour les espèces des différentes stratégies de ponte mises en oeuvre.

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SOME PECULARITIES OF LIZARD DENTAL SYSTEMS

by

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SUMMARY

On the basis of complex data (studies of morphology and teeth replacement *in vivo*, dissections of stomachs, observations of feeding behavior with recordings on cine-film) particularities of the dental systems are established for 65 lizard species from 9 families. The obtained data allows us to apply a universal approach to this system in connection with taxonomy, problems concerning variability of teeth, feeding mechanisms and ecology of lizards. Among the components of the jaw apparatus the dental system of these animals demonstrates the highest degree of plasticity and diversity. Its features display interrelations of compensatory nature, and adaptations to similar kinds of food can be achieved on the basis of different specializations of teeth. Pecularities of taxonomic significance are outlined for some of the species.

Key-words : Lizards, dental system, teeth.

INTRODUCTION

Numerous contributions on the dental system of lizards fail to produce a complete impression and are often incompatible. This is true even of the most voluminous account by EDMUND (1969). Currently this information is steadily being enriched, mostly by morphological and histological descriptions of teeth in separate species.

The latest classification of squamata elaborated by RIEPPEL (1988) almost entirely lacks information on dental morphology. At the same time such problems of dental morphology as growth and development of teeth, correlations with types of feeding, and interrelations with other parts of the jaw apparatus remain unsolved.

We attempted a comparative morpho-functional analysis of the dental system in different lizard species, with the goal of unification of previous and newly acquired data and further application of these to morphological, ecological, taxonomical and phylogenetical interpretations.

Six morphotypes were defined (VOROBYEVA and KRASNOV, 1979) in the dental system from the study of 33 lizard species, representing 20 genera and 8 families. Their evolutionary history involved adaptations of feeding mechanisms and adaptations to certain food items. Later these investigations were developed (VOROBYEVA and CHUGUNOVA, 1986a) through introduction of new species and new methods (filming, dissections of stomachs, staining of teeth in the process of their replacement). This confirmed the definition of six morphotypes and considerably added to their descriptions. We were also able to separate the more stable features from variable ones in dental morphology and replacement. The compensatory and correlative trends were traced. The age and sex-dependencies of dental differentiation and their relations to wearing and replacement of teeth were studied in greater detail. In our research we employed a view, presuming that the dental system always remains a part of the jaw apparatus as a whole (in the sense of an intricate morpho-functional adaptive complex).

MATERIAL AND METHODS

More than 400 lizards specimens belonging to 65 species, 29 genera and 9 families (Gekkonidae, Scincidae, Lacertidae, Xantusiidae, Agamidae, Chamaeleontidae, Iguanidae, Anguidae, Varanidae) were studied, predominantly from the USSR fauna. Dental system structure was studied on macerated skulls totally devoid of soft tissues and on those with soft tissues partially removed from the dental area.

The analysis of teeth morphology included the following parameters : 1) shape of the teeth (in lingual, labial views, in transverse section and form of the dental apex) ; 2) number and differentiation of teeth ; 3) interdentary spaces ; 4) dental recline as related to the maxillary bone ; 5) type of teeth attachment to the jaw-bone and features of their resorption ; 6) dental differentiation along the jaw bones ; 7) teeth height above the jaw-bone ; 8) nature of dental wear, acquired defects ; 9) sexual dimorphism and age-associated changes ; 10) features of teeth replacement and intensity of this process.

An extensive observation on teeth replacement (3-5 months) was carried out for two lizard species : *Tenuidactylus caspius* (EICHWALD, 1831), and *Lacerta armeniaca* (MEH, 1909). A new method of *in vivo* staining was established for the study of this process. This method includes a single injection into the body cavity of a concentrated solution of alisarine red « C » (1, 5-4 ml). It was established that differentiation in the staining of teeth depends on their age : the junior teeth have a more red colour (Fig. 1).

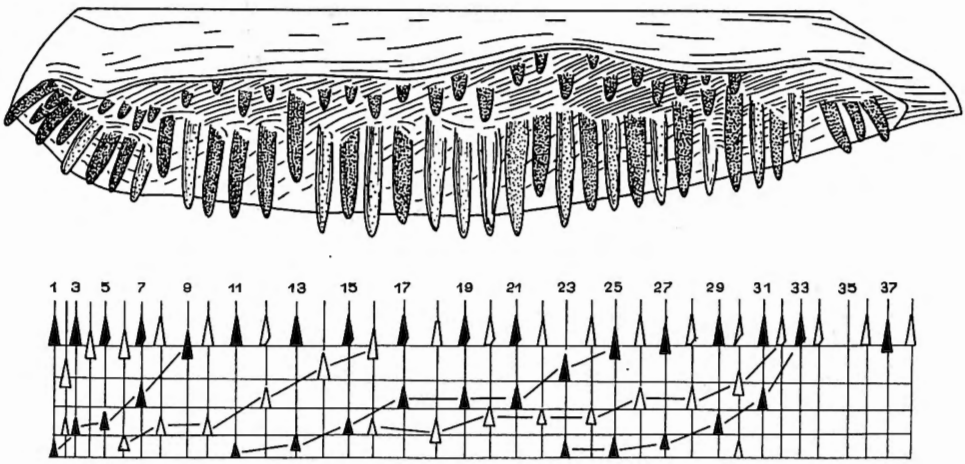

Fig. 1. — *Tenuidactylus caspius*. Right maxillary in lingual view.
A diagram of teeth replacement (after Edmund) is based on the results of alizarine red « C » injections. The youngest teeth feature the deepest staining.

A resection of stomachs was made in 54 specimens from 8 different families and diet components were studied. The nature of food treatment in the mouth cavity, the degree of its morcelment were defined according to teeth markings on the prey and to the different patterns of bitesk and breaks of the hard sclerotised parts of invertebrates. The use of various types of dentition in this process was thus defined (Fig. 2).

The feeding behavior of lizards, i.e. pecularities of capture, treatment of prey in the mouth cavity, transportation of the particles to the pharynx was observed in laboratory and in Zoo. In some species movements of the head and of the jaws were recorded on cine-film (VOROBYEVA and CHUGUNOVA, 1986a). This material was used to describe the feeding mechanisms of some species : *Trapelus sanguinolentus* (PALLAS, 1913), *Stellio lehmanni* (NIKOLSKY, 1896), *Phrynocephalus mystaceus* (PALLAS, 1776), *Lacerta viridis* (LAURENTI, 1768), *Eremias velox* (PALLAS, 1771), *Pseudopus apodus* (PALLAS, 1775), and *Varanus griseus* (DAUDIN, 1903). The film was shot by « Krasnogorsk-3 » 16 mm camera with the use of negative material A-2 and positive OCh 180. The speed was 32 frms/sec.

RESULTS

The completed comparative-anatomical analysis of the teeth system in lizards revealed that its diversity presents on the one hand a result of certain adaptive trends and on the other hand a result of initial morpho-phylogenetical features. And while in lizards the skeletal-muscular jaw apparatus is relatively uniform in structure and functions (FRAZETTA, 1962 ; DE VREE and GANS, 1987 ; IORDANSKY, 1970), the dental system presents the most variable of its components, providing

access to the wide spectrum of food items and opening possibilities of adaptive radiation.

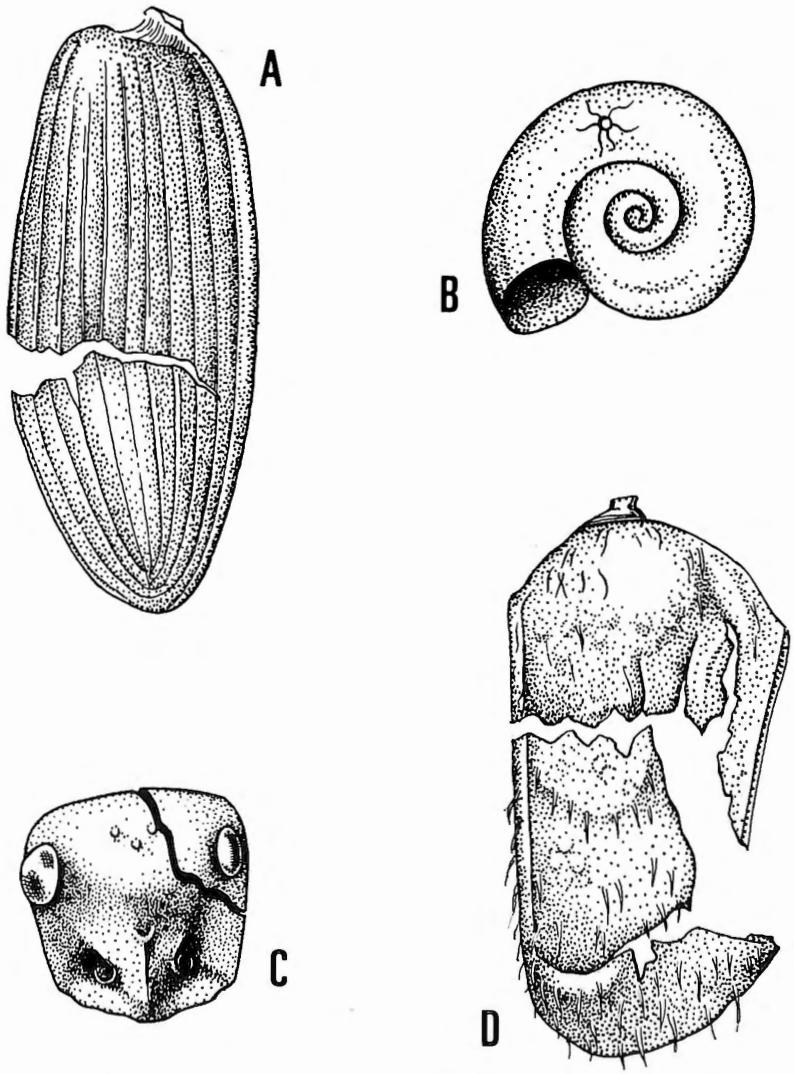

Fig. 2. — Traces of bites on the prey in different lizards : A : *Eremias velox* ; B : *Pseudopus apodus* ; C, D : *Phrynocephalus* sp.

Dental morphology.

The dental system in different taxonomic groups of lizards and in separate representatives differs by a number of morphological parameters : type of attachment,

replacement, shape of the crown, number of teeth etc. All these parameters are subjected to individual, sexual and age variability and can be met in different combinations in different lizard groups. They often display difference in phylogenetically close groups and similarity in distant groups (convergences) (VOROBYEVA and KRASNOV, 1979 ; VOROBYEVA and CHUGUNOVA, 1986b). Thus a set of parameters must be used when a certain group is discussed.

From the positions of compensatority and correlations we assumed that the subdivision of lizards into « acrodont » and « pleurodont » types is valid, if not only the type of dental attachment is taken into consideration (OSBORN, 1984), but also other features related to this. So, pleurodont teeth system correlates with such features as occurrence of dental replacement, high teeth crown, extensive number of teeth and their weak differentiation. Acrodont teeth system features irreplaceability, teeth few in number and well differentiated. The most constant feature for relation of the lizards to one of the two these groups is the presence or absence of tooth replacement.

Our study revealed that in lizards with the iguanid pattern of teeth replacement variations of this process may occur. In *Corucia zebrata* (GRAY, 1855), *Eremias grammica* (LICHENSTEIN, 1823), *E. arguta* (PALLAS, 1773) and *Lacerta viridis* the successive tooth in the posterior part of the row arises somewhat posteriorly from its predecessor. In *Trachydosaurus rugosus* (GRAY, 1845) the successive tooth arises behind the predecessor : thus in forms of larger size, feeding on larger and harder prey (among *Eremias, Lacerta*) or in durophagous forms (*Trachydosaurus rugosus, Pseudopus apodus*) the successive tooth does not arise exactly underlying the functional tooth, but a little to one side. Probably, such a shift of the tooth bud occurred in connection with the growing pressure applied to the functional tooth.

In all pleurodont lizards the anterior teeth are similar in form and fulfil similar functions. Further posteriorly the teeth somewhat differ — to a different extent in different families. The most posterior teeth display generic tendency — widening of crowns and complication of their form. The more massive posterior teeth in part are due to different mechanical influence in the anterior and posterior part of jaw. In the majority of geckos the difference between anterior and posterior teeth is minor. In skinks it is more strongly expressed. In some lizards (iguanids, ophisaurids and varanids) the difference can be rather significant. Diversity of crown form in posterior teeth permits the definition of several stages with regard to their increasing complexity : sharp elongated crown with round basis in geckos, somewhat transversely widened in skinks, with an additional cusp before the main cusp in racerunners, and tricuspid crown in lizards and some iguanids (Fig. 3). Complexation of crown shape, and the enlargement of tooth surface through formation of additional cusps undoubtedly simplifies and improves treatment of food in the mouth cavity. Thus this increase in complexity must be regarded as progressive in the evolution of the lizard dental system.

In acrodont lizards all the particularities of attachment, shape and number of teeth are probably connected with a certain mechanism of jaw interaction, which

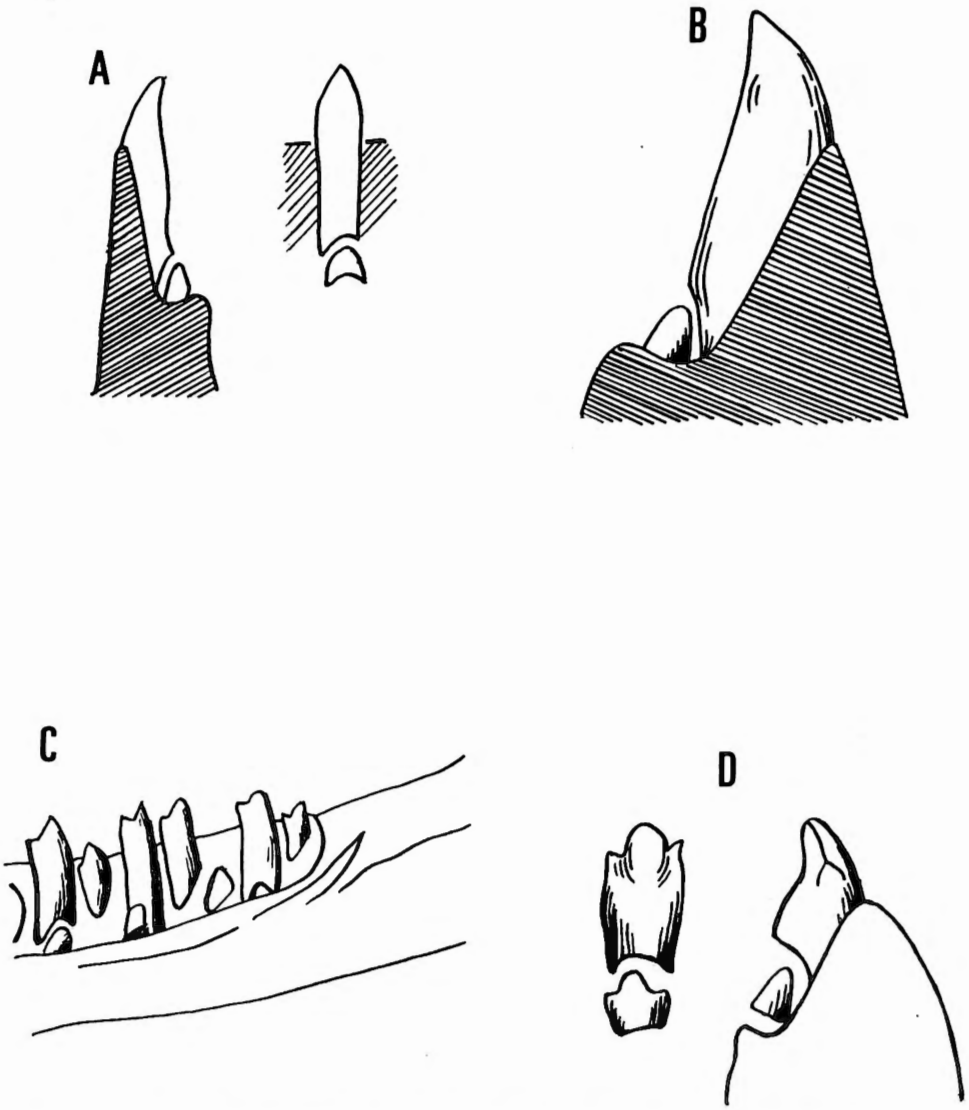

Fig. 3. — Crown form in posterior teeth. Side and lingual views : Geckos (A), skinks (B), racerunners (C), lizards (D).

differs from that in pleurodont lizards. The acrodont dental system was primarily adapted to « precision — shear bite » as in *Uromastyx* (ROBINSON, 1976) and other agamids ; when the teeth of the upper jaw covering the teeth of the lower jaw, on closing of the mouth, establish a close contact.

Teeth of the upper jaw leave traces on dental bone. With the growth of the animal there appear new acrodont « molars » in the posterior part of the tooth row

and each of them is larger than it's predecessor. The difference in « premolars » and « molars » is well defined in *Agama* and *Phrynocephalus*. According to acrodont teeth differentiation 11 species of *Phrynocephalus* were classified into four groups (Table 1). In these groups some correlation between degree of teeth differentiation and size of the lizards was achieved. The larger species feature the stronger differentiation of dental system (CHUGUNOVA *et al.*, 1987).

DISCUSSION

Feeding mechanism.

The feeding mechanism is treated as a certain cycle of actions with a number of successive phases : (1) prey capture, (2) treatment of prey in the mouth cavity, (3) transportation into the pharynx, (4) cleaning (THROCKMORTON, 1976, 1980 ; SMITH, 1982 ; BRAMBLE and WAKE, 1985).

Treatment of prey and the « chewing » phase (TROCKMORTON, 1980) presents a series of repeated openings and closures of mouth, accompanied by movements of tongue and head. We ascribe a species specific meaning to this phase and in our studies of some species dedicate special attention to the nature of head and jaw movements, tongue activity, also to the role of tongue in prey capture and to the time of treatment in the mouth cavity.

The filming of the feeding mechanisms enabled us to describe this phase in detail for seven species of lizards (see above).

In geckos the prey almost escapes dental treatment in the mouth cavity. This is confirmed also by dissections of stomachs. In smaller species of geckos (*Hemidactylus frenatus* DUMERIL and BIBRON, 1836 ; *Tenuidactylus caspius, T. russowi* STRAUCH, 1887 ; *Crossobamon eversmanni* WIEGMANN, 1834) the number of cycles in the « chewing » phase during consumption of the flour worm amounts to 3-4. In these lizards during the 1 and 2 phases sudden head movements and secondary prey captures might be observed.

The observations on the feeding of *Eumeces schneideri* (DANDIN, 1862), *Lacerta viridis,* and *Eremias velox* revealed that the phase of prey treatment of the flour worm persists from 5-6 up to 12 (in *Eremias velox*) cycles. One cycle of the « chewing » phase in *Eremias velox* takes about 0,25 seconds. During capture and treatment of prey in lizards the sudden head movements and tongue movements are employed. The recapturing of prey by the jaws occurs with the active participation of the tongue. The principle disintegration of food in these forms is achieved with the use of teeth from the middle area of the dental series. Most apical damage was recorded in this area.

The filming of feeding mechanism in *Eremias velox* enabled us to observe the propalinaric move-ability of the lower jaw is rarely used by the lizard and serves usually for orientation of prey in the mouth.

The tongue actively participates in prey treatment in the feeding mechanism of *Pseudopus apodus*. Thus the lizard employs it's tongue to dispose of broken par-

ticles of snail shell. During swallowing of a large prey the lizard places its tongue under the prey, and then, simultaneously with recapturing via the jaws, the tongue is pulled inside the mouth cavity with the prey.

In the varanids (monitors) the tongue is, on the contrary, excluded from the feeding process (CONDON, 1989). The food is transported to the pharynx with the help of inertial thrust (RIEPPEL, 1979) and with participation of cranial kinesis (FRAZETTA, 1962).

SMITH (1984) demonstrated that the difference in the activity of the specialized tongue during feeding of the northern tegu from that of the unspecialized hervivorous iguana *Ctenosaura similis* (GRAY, 1983) is slight. At the same time MAC LEAN (1974) noted the activity of the specialized tongue in feeding of the crocodile-tegu.

Possibly, specialization of the tongue in lizards (often combined with it's sensory functions) does not influence it's activity in feeding processes.

TABLE 1

Differentiation of acrodont teeth in Phrynocephalus *(evaluated in points from 1 to 4)*

Species	Degree of acrodont teeth differentiation (upper/lower jaw)
1. Phrynocephalus mystaceus (PALLAS, 1776)	4/4
2. Ph. versicolor STRAUCH, 1876	3/3
Ph. melanurus EICHWALD, 1831	3/3
Ph. maculatus ANDERSON, 1872	3/3
3. Ph. raddei BOETTGER, 1890	3/2
Ph. rossikowi NIKOSKY, 1899	3/2
Ph. reticulatus EICHWALD, 1831	2/2
Ph. helioscopus (PALLAS, 1771)	2/1
Ph. guttatus GMELIN, 1787	
Ph. moltschanovi NIKOLSKY, 1913	2/1
4. Ph. interscapularis LICHTENSTEIN, 1856	1/1
Ph. sogdianus ČERNOV, 1959	1/1

Our observations revealed that all of the studied species of agamas and most toad agamas (Table 1) capture minute prey with the aid of their tongue. The relatively large prey is captured by jaws. The phase of flour worm treatment in agamas includes 7-20 cycles. One cycle of the « chewing » phase takes about 0,27 seconds. The treatment of a solid large object, for instance, of a bug, takes 20-30 cycles and is carried out mainly by the hindmost teeth. The jaws are opened to about 50 ° for this. For comparison, the maximal opening of the jaws in *Eremias*

velox gives about 30,5 °. An inconsiderable forward displacement of the lower jaw during mouth opening was registered in *Trapelus sanguinolentus*.

Thus, the time of one cycle of the « chewing » phase is similar in agamas, *Lacerta viridis, Eremias velox* and as in *Amphibolurus barbatus* (CUVIER, 1929) (THROCKMORTON, 1980) amounts to 0,25-0,30 seconds. However, the number of cycles is different, and that depends upon the carnivore-prey size relations and upon the hardness of the prey. Apart from that, the intensity of prey treatment increases in a hungry carnivore.

Thus the particularities of feeding mechanisms in different lizards are explained not only by the morphological features of dental apparatus, but also by variations in prey (size, hardness) and also by the state of the carnivore at the moment of feeding.

Pleurodont and acrodont teeth systems evidently provide different functions and both have a long history behind them. In comparison with acrodont teeth, the pleurodont ones display a wider spectrum of diversity of dental parameters, connected with adaptations to different types of prey.

Diet correlation.

The specialized forms feature the most vivid correlation between dental structure and diet (GREENE, 1982). The specialization of the teeth system in durophags, predators swallowing large prey in a single piece, phytophages and myrmecophags is expressed in a number of parameters. In durophags and predators most of the parameters are subjected to changes (attachement, crown shape, type of substitution) ; in phytophages only the shape of the crown apex is modified ; in myrmecophags the adaptation of the dental system followed different trends and no certain single type of specialization is observed (Table 2).

Specialization towards duraphagy can be observed in almost all of the families. Among geckos this happens in the case of *Teratoscincus,* among skinks — *Trachydosaurus rugosus ;* true lizards — *Lacerta viridis* and *L. agilis* (LINNÉ, 1758), iguanids — *Chamaeleolis chamaeleontides* (DUMERIL and BIBRON, 1837), anguids — *Pseudopus apodus.* In the diets of these animals insects with hard shells are common, together with large and hard objects. Adaptation to feeding on hard objects involves modification of posterior teeth crowns, however there is a suggestion that many lizards with molariform teeth may be functioning as everyphages rather than specializing in durophagy (ESTES and WILLIAMS, 1984).

In specialized predators, swallowing their prey in a single piece — *Varanus* and *Anguis* — we also find subpleurodont attachment of functional teeth and their low number. Still, the crown shape, number and distribution of teeth on the jaws and type of their replacement are rather specific : the teeth are high, sharp, posteriorly curved, widely spaced, and their number is low.

TABLE 2

Correlation of specialization with different changes (t) *of various parts of dental system*

Kind of adaptation / Some parameters of dentition	Tooth shape	Nature of thooth attachment	Type of tooth replacement	Number of teeth
durophagy	+[+)]	+	+	+
predators, swallowing, large prey in a single piece	+	+	+	−
phytophagy	+	−	−	−
myrmecophagy	+/−	−	−	−

[+)] The parameter subjected to changes.

Adaptation towards plant eating involves mostly changes in the form of the crown apex. All of the other family — specific features remain unchanged.

The acrodont lizards feature a diet which is also quite diversified. However, they display fewer adaptative modifications of the dental system. Probably, the acrodont dental system is more universal. It is adapted to animal foods of different hardness and size, as well as to plant food. Still, among Agamidae, for example, there are also forms with food specializations. Thus *Moloch horridus* (GRAY, 1841) is highly specialized towards myrmecophagy. *Phrynocephalus* and North American iguanids feature a diet based largely on ants, but including some other types of food as well.

The differences in dental morphology in different taxonomic groups of myrmecophagous lizards (blunt crowns in horned lizards, flattened and triangular in toad agamas ; of more complicated form in moloch) reveal that myrmecophagous specialization is achieved by different means and does not provide a single morphological result.

Morphotypes.

Six morphotypes of lizards were defined in the course of unified morphofunctional evaluation of teeth systems and of their feeding mechanisms (VOROBYEVA and KRASNOV, 1979 ; VOROBYEVA and CHUGUNOVA, 1986a). These morphotypes correlate partially with the taxonomical subdivision and in part unite the specialized forms from different families : I — geckos, II — skinks + true lizards, III — iguanids, IV — agamids + chameleons, V — durophags, VI — predators, swallowing their prey as a whole;

Each morphotype demonstrates certain interrelationship between morphological components of the dental system (skeletal-muscle jaw system, tongue, teeth) and compensatory phenomena in prey capture or its treatment and further transportation into the pharynx.

Taxonomical conclusions.

The use of dental morphology in taxonomy of many recent lizards is limited by wide distribution of convergencies, caused by specific adaptations, and by considerable variability of features. The complexes of such features provide a possibility for establishing the status of lizards at the family level and in some cases at the level of subfamilies and genera. In his family diagnoses CAMP used particularities of the dental system as early as in 1923. CAREVSKIJ (1929) tried to employ such features as teeth number and degree of dental differentiation in *Phrynocephalus* taxonomy. RIEPPEL (1978) distinguished Platynota and Anguinoidea on the basis of difference in tooth replacement (with resorbtion of the changed tooth base and without such resorbtion).

The literary data provide examples of dental number used for separation of a species or for distinguishing between two similar species. Thus, two species of venomous lizards possess a different number of teeth (6-7 or 8-9), apart from that one species lacks palatal teeth (BOGERT and CAMPO, 1956). Thus, the number of teeth was used by GEER (1970, 1974) in a subfamiliar classification of scincid lizards, and also by BICHOFF (1986) for the separation of *Podarcis filfolensis* (BEDRIAGA, 1876). EREMTCHENKO and TSCHERBAK (1986) use the number of premaxillary teeth in their classification of lidless skinks.

Fig. 4. — *Eremias grammica*. Diastema between maxillary and premaxillary teeth.

We defined some features useful for separation of genera and species. Thus, in racerunners, unlike that in some lizards, the « diastema » is always found between premaxillar and maxillar teeth in the upper jaw (Fig. 4). In *Prynocephalus* the crown features a simpler triangular form and is more strongly compressed laterally, than in *Agama* (Fig. 5). Such a feature as number of premaxillar teeth turned out to be rather constant and less liable to change in the majority of pleurodont forms. This feature is diagnostically valid for two allied species of geckos — *Teratoscincus scincus* SCHLEGEL, 1858 and *T. przjewalskii* STRAUCH, 1887 (the first one has 9, the second one possesses 11) (VOROBYEVA and CHUGUNOVA, 1989).

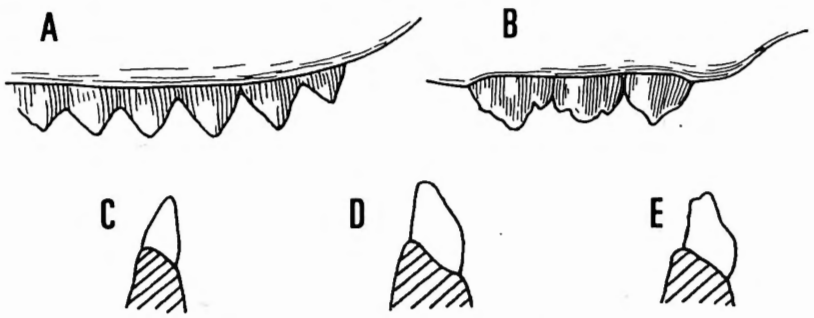

Fig. 5. — Acrodont teeth in *Phrynocephalus* (A), *Agama* (B) labial view. Transverse section through posterior tooth in *Phrynocephalus* sp. (C) and *Stellio caucasicus* (D, E).

On the whole, we come to a conclusion that the main components of jaw apparatus are closely connected by their participation in the feeding process. Often the compensatory phenomena occur when development of one adaptive particularity compensates the unadaptedness of the other.

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THE ANURAN MIDDLE EAR : DEVELOPMENTAL HETEROCHRONIES AND ADULT MORPHOLOGY DIVERSIFICATION

by

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SUMMARY

Many anuran related species with similar modes of life and similar mating calls display the occurrence of extreme differences in their middle ear morphology (for example, middle ear present in *Microhyla ornata* but absent in *M. heymonsi* ; stapes present in *Bombina orientalis* but absent in *B. bombina* ; the tympanic membrane is thin and transparent in *Bufo melanostictus,* but covered by thich unmodified skin in *B. bufo*). As loss or reduction of this system has a profound negative effect on hearing sensitivity, these patterns of middle ear diversification cannot be explained in terms of functional adaptivity. The formation of the anuran middle ear is greatly delayed and retarded, it begins at relatively late premetamorphic stages and progresses through postmetamorphic development. Because of the delayed and retarded morphogeny this system is subjected to paedomorphic underdevelopment through : progenesis (*M. heymonsi*), neoteny (*Bombina* species), and post-displacement (*B. bufo*). The late onset of the middle ear development is predetermined ; because of the peculiar anuran pattern of its morphogeny, it can be formed only at or after the end of metamorphosis. The author thinks that the occurrence of non-tympanic routes for airborne hearing decreases the pressure of functional requirements and allows developmental heterochronies to be the main factor in the diversification of the anuran middle ear.

Key words : Anura, middle ear, morphogeny, heterochrony, paedomorphosis.

INTRODUCTION

The anuran middle ear usually includes the middle-ear cavity, tympanic membrane, and plectrum, or stapes (Fig. 1). The plectrum consists of a medial cartilaginous pars interna plectri, ossified pars media plectri, and cartilaginous pars externa plectri. The latter distal element articulates with the tympanic membrane, which is stretched over the cartilaginous, ring-shaped tympanic annulus. A cartilaginous processus ascendens plectri extends from the pars externa plectri to the crista parotica.

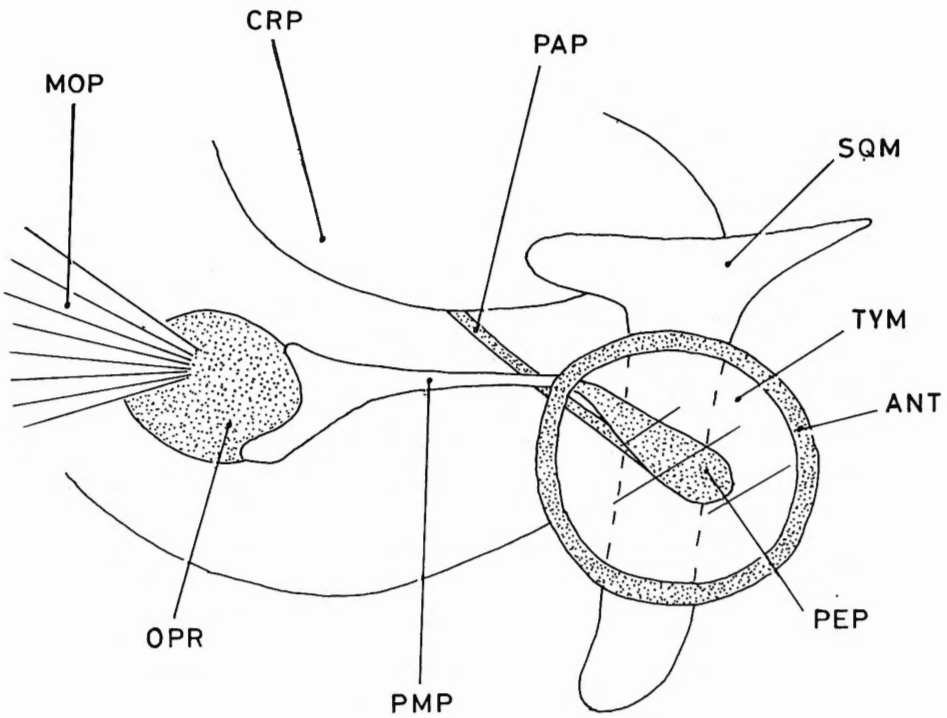

Fig. 1. — Scheme of the generalized anuran middle ear pattern.
ANT - annulus tympanicus ; CRP - crista parotica ; MOP - musculus opercularis ; OPR - operculum ; PAP - processus ascendens plectri ; PEP - pars externa plectri ; PMP - pars media plectri ; SQM - squamosum ; TYM - membrana tympani.

The generalized condition of the middle ear described here typifies most anurans, but many species display derived features down to the reduction or loss of the middle ear (SMIRNOV, 1984).

The main function of the anuran middle ear is the reception of high frequency air-borne sounds involved in mating calls (LOMBARD and STRAUGHAN, 1974). As acoustic communication plays an important role in the anuran life, one could suggest that selective forces would tend to improve the middle ear morphology to serve as an effective transducer of air-borne sounds. Sometimes this seems to be the case (SMIRNOV, 1984), but most patterns of the derived middle-ear morphology cannot be explained in terms of functional adaptivity. For example, two congeneric *Microhyla* species (Microhylidae) with similar modes of life and similar mating calls (the frequency constitution and wave form are essentially identical in both species — KURAMOTO, 1987) differ in their middle-ear morphology : *M. ornata* (DUMERIL and BIBRON, 1841) possesses a tympanic ear, whereas *M. heymonsi* VOGT, 1911 lacks it (Fig. 2A, B).

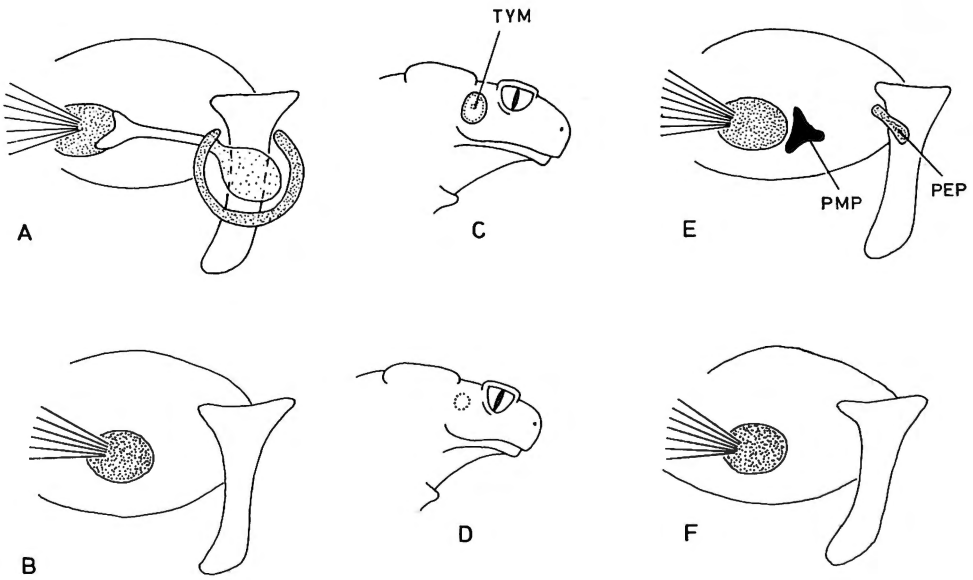

Fig. 2. — Diversification of the anuran middle ear patterns.
A - Microhyla ornata ; B - Microhyla heymonsi ; C - Bufo melanostictus ; D - Bufo bufo ; E - Bombina orientalis ; F - Bombina bombina ; PEP - pars externa plectri ; PMP - pars media plectri ; TYM - membrana tympani.

The occurrence of differences in the middle-ear morphology is displayed by many related congeneric anuran species with similar modes of life and similar mating calls : 1) *Bufo melanostictus* SCHNEIDER, 1799 (Bufonidae) has the middle ear with a large thin transparent tympanic membrane, whereas in *B. bufo* (LINNÉ, 1758) the tympanic membrane is covered by thick almost unmodified skin (Fig. 2C, D) ; 2) *Bombina orientalis* (BOULENGER, 1890) (Discoglossidae) displays the occurrence of bony stapes, whereas *B. bombina* (LINNÉ, 1761) lacks it (Fig. 2E, F).

As the loss or reduction of the middle ear has a profound negative effect on anuran air-borne hearing sensitivity, it is obvious that these patterns of diversification are absurd in terms of orthodox adaptivity related to selection on hearing sensitivity and cannot be explained on functional grounds.

However, numerous attempts to find functional explanations for reduction or loss of the anuran middle ear continue to be made (see review by JASLOW *et al.*, 1988 for details). Concerning the background of general adaptationist's enthusiasms, surprisingly little attention was paid to PARKER's (1940) hypothesis that heterochronic shifts account for the loss or reduction of the anuran middle ear. Only recently this heterochronic explanation was rejected all over again by TRUEB and ALBERCH (1985) who proposed the loss of the anuran stapes to be a conse-

quence of paedomorphic trend in miniature anuran species. While this hypothesis is rather intriguing, it is scarcely well founded without proper evidence supporting it. However, three points indicate the validity of the heterochronic explanation for reduction and loss of the anuran middle ear : 1) heterochronic shifts in the sexual or somatic development resulting in paedomorphosis are common events among anurans (TRUEB and ALBERCH, 1985 ; SMIRNOV, 1989), 2) paedomorphosis is accompanied by the reduction or loss of morphological features that develop later (TRUEB and ALBERCH, 1985), and 3) in anurans the formation of the middle ear is one of the latest ontogenetical events ; it begins at relatively late metamorphic stages and usually progresses through postmetamorphic development (VOROBYEVA and SMIRNOV, 1987 ; HETHERINGTON, 1987).

The aim of the current study is to provide evidence supporting the heterochronic explanation for diversification of the anuran middle ear. For this purpose the mechanism of the middle ear diversification was studied in three pairs of related congeneric anuran species displaying the similar modes of life and similar mating calls, but differing greatly in their middle-ear morphology : 1) *Microhyla ornata* - *M. heymonsi* ; 2) *Bombina orientalis* - *B. bombina* ; 3) *Bufo melanostictus* - *B. bufo*.

MATERIALS AND METHODS

The main idea of the current study is based on two mutually complementary working hypotheses : 1) it is paedomorphosis resulting from developmental heterochronies, that may account for the middle ear reduction, and 2) in species under study the middle ear formation is delayed and retarded, and that is why this system may be subjected to paedomorphic reduction. To prove them one needs information about the rate of the middle ear development as well as information about the rate of somatic development (evaluated here on grounds of anuran cranial ontogeny). For these purposes information about sequence and timing of the middle ear development as well as of cranial development was gathered from ontogenic series stained with Alizarin Red S. These series included newly metamorphosed individuals, juveniles of various ages, half-grown individuals, and adult specimens of the following taxa : *Microhyla ornata, M. heymonsi, M. pulchra* (HALLOWELL, 1860), and *Kaloula pulchra* GRAY, 1831 (Microhylidae), *Alytes obstetricans* (LAURENTI, 1768), *Discoglossus pictus* OTTH, 1837, *Bombina orientalis,* and *B. bombina* (Discoglossidae), *Bufo melanostictus* and *B. bufo* (Bufonidae). Additional information was obtained from histological sections of newly metamorphosed anurans — *Microhyla ornata* and *M. pulchra* (Microhylidae), *Discoglossus pictus* (Discoglossidae), and *Bufo bufo* (Bufonidae). These species were sectioned transversely at a thickness of 10 μm, and the sections Mallory stained. All information was derived from specimens used in earlier studies (SMIRNOV, 1986, 1989, 1991) and readers interested in details are referred to these papers.

RESULTS

Microhyla ornata - M. heymonsi

Microhyla species display the retarded rate of the middle-ear morphogeny as may be seen from the underdevelopment of this system in the postmetamorphic animals. For example, in newly metamorphosed *M. pulchra* only the pars interna plectri achieves its definitive state of histological differentiation and is chondrified, whereas all other middle ear elements are represented by mesenchymatous primordia. In somewhat older *M. ornata* froglets (several days after metamorphosis is complete) the middle ear morphogeny is more advanced and one can record the first signs of chondrification in pars media plectri and annulus tympanicus, but the pars externa plectri is still mesenchymatous and the anlage of the tympanic cavity is discontinuous from the Eustachian tube. On the whole, one may conclude that *Microhyla* species metamorphose when the middle ear development is far beyond the end.

On the other hand, *Microhyla* species constitute a group of small-sized frogs which display some features of cranial underdevelopment (reduced sphenethmoids and quadrato-jugals, reduced or lost palatines) if compared with larger generalized related species (for example, *Kaloula pulchra*). These bones (the sphenethmoids, quadrato-jugals, and palatines) are among the last cranial elements to appear in the sequence of anuran skull ossification (TRUEB, 1985), and their failure to develop as well as small body size characteristic for *Microhyla* species indicate the possibility of the paedomorphic origin of the *Microhyla* genus.

Paedomorphosis, which can be defined as the occurrence of ancestral juvenile morphology in a descendent adult (GOULD, 1977), can occur in three principle ways : progenesis, neoteny, and post-displacement (MCNAMARA, 1986). Progenesis results from acceleration in rate of sexual development. As precocious sexual maturation produces the overall truncation of the somatic growth and development, progenesis is accompanied by small body size, reduced somatic morphology, and loss of features that develop later (ALBERCH and ALBERCH, 1981 ; TRUEB and ALBERCH, 1985). As *Microhyla* species meet all these requirements, one may conclude that they are progenetic animals. Earlier it was shown that the middle ear development is retarded in *Microhyla* species and is one of the latest ontogenetical events. Then one could suggest that if *Microhyla* species are progenetic, their middle ear would also be subjected to paedomorphic underdevelopment and, moreover, the smallest species would display the more pronounced morphological reduction of this system. This seems to be the case : if compared with the middle ear morphology of the generalized larger related *Kaloula pulchra,* the adult middle ear of *Microhyla* species displays some features of underdevelopment and resembles the immature condition in *K. pulchra* (VOROBYEVA and SMIRNOV, 1987) and, moreover, in the smallest *Microhyla* species — *M. heymonsi* — the middle ear is completely lost.

Bombina orientalis* - *B. bombina

The middle ear may be lost another paedomorphic way-by neoteny. Neoteny occurs by retardation in the rate of somatic development (GOULD, 1977; MCNAMARA, 1986) and is accompanied by reduced morphological development as only juvenile morphology is achieved to the onset of sexual maturity which causes the cessation of somatic development.

Bombina, aquatic anurans, have a highly derived morphology. In comparison with generalized anurans, the main derived features of *Bombina* are as follows : retention of the lateral line system in adult animals (FRITZSCH *et al.,* 1987), juvenilized teeth structure (CLEMEN and GREVEN, 1980), absence or reduction of some cranial ossifications (for example, palatines), and reduced middle ear. These features are larval or juvenile for generalized anurans.

If compared with generalized discoglossids (*Alytes obstetricans* and *Discoglossus pictus*), *Bombina orientalis* displays the retarded rate of development of different systems (the hyoid apparatus, cranial ossifications, and middle ear).

For example, in *B. orientalis* the first appearence of the os parahyoideum (bone attached to the ventral surface of the corpus of the hyoid apparatus) is recorded in 2-years-old specimens (mean SVL = 33,3 mm), whereas in *A. obstetricans* and *D. pictus* this bone appears as an ossification in even recently metamorphosed animals (mean SVL = 18,0 and 19,0 mm respectively).

The similar trend to retarded developments is displayed by cranial ossifications in *B. orientalis* : whereas in *A. obstetricans* adult cranial state is attained prior to sexual maturation (SMIRNOV, 1991), in *B. orientalis* cranial development is greatly prolonged in time and sexual maturation is achieved before osteogenesis of the cranium is complete (SMIRNOV, 1989).

In *A. obstetricans* the middle ear elements complete histological differentiation within a short time after the end of metamorphosis and in recently metamorphosed juveniles (mean SVL = 18 mm) the pars media plectri is ossified and the annulus tympanicus and pars externa plectri are chondrified. In *D. pictus* the ossification of the pars media plectri and chondrification of the annulus tympanicus and pars externa plectri begin even before the metamorphosis is complete, as was exemplified by the tadpole of 45 GOSNER stage, and in recently metamorphosed animals (mean SVL = 19 mm) the process of histological differentiation of the middle ear elements is already completed. However, in *B. orientalis* the first appearence of the bony pars media plectri is recorded only in a 2-years-old animal (SVL = 33,9 mm).

The occurrence of retardation in the development of different systems (the hyoid apparatus, cranial ossifications, and middle ear) argues for the coordinated overall retardation of the *Bombina*'s somatic development rather than independent retardations in the morphogeny of these systems. This conclusion is supported by the retarded rate of the hemoglobin change in metamorphosing *Bombina* (CARDELLINI and SALA, 1979) and by the occurrence of the highest DNA level (genome size) among anuran species, indicating the low rate of the *Bombina*'s development (OLMO *et al.,* 1982).

Then from the foregoing data it can be gathered that : *a*) *Bombina* displays the retarded rate of somatic development if compared with the generalized relatives, *b*) *Bombina* displays the occurrence of paedomorphic features in the morphology of different systems. These two points indicate that in the *Bombina* genus retention of the paedomorphic morphology was attained through the retardation of the somatic development, in other words, through neoteny.

Retardation of the middle ear morphogeny accompanying the overall retardation of the somatic development leads to the severe reduction of the middle ear in *B. orientalis* : only chondrified pars interna plectri and bony pars media plectri, appearing earlier in the anuran ontogeny, achieve their definitive state of histological differentiation, while the annulus tympanicus and pars externa plectri normally appearing later are lacking in *B. orientalis* (1).

If compared with *B. orientalis,* the development of *B. bombina* proceeds slower (UTESHEV and VASILIEV, 1986). Then one could suggest that more retarded somatic development would result in more pronounced morphological reduction in *B. bombina.* This seems to be the case. *B. bombina* displays much more juvenilized morphology if compared with *B. orientalis* (SMIRNOV, 1989) and lacks any signs of the pars media plectri.

Bufo melanostictus* - *Bufo bufo

In the previous two examples heterochronies occurring in sexual (*M. heymonsi*) or in overall somatic development (*Bombina* species) result in the diversification of the middle ear morphology. However, the middle ear itself may display developmental heterochronies which produce differences in the adult middle ear morphology.

Two *Bufo* species under study greatly differ in the rate of their middle ear morphogeny. Thus, in a recently metamorphosed *B. bufo* toadlet (SVL = 11 mm) all components of the middle ear are still mesenchymatous except for the pars interna plectri which is chondrified. In a juvenile specimen (SVL = 18,5 mm) the tympanic membrane is absent, the pars media plectri still unossified, and the annulus tympanicus and the pars externa plectri mesenchymatous. In even larger specimens (SVL = 40-45 mm) the annulus tympanicus is not yet fully chondrified, and the pars media plectri is ossified only partionally. Only in sexually matured animals did the middle ear complete its development, but the tympanic membrane remains covered with thick skin. In contrast, in *B. melanostictus* the middle ear morphogeny is greatly accelerated if compared with *B. bufo.* Even in a juvenile specimen (SVL = 17 mm) (2) the histological differentiation of the middle ear components is complete, and a thin tympanic membrane is already formed (VOROBYEVA and SMIRNOV, 1987).

(1) The morphogenetic basis for their development retains and in some old animals the rudimentary cartilaginous pars externa plectri may appear (Fig. 2 E).

(2) We can use size as an indirect indicator of age as both species metamorphose at similar sizes and are related by their adult size and age of sexual maturation.

The formation of the tympanic membrane is the last event in anuran middle ear morphogeny (SMIRNOV, 1986). On the other hand, a tympanic membrane development is a rather complicated process itself : it proceeds through several histological changes of integument overlying the annulus tympanicus, and ends in the transformation of this integument into a thin tympanic membrane (HELFF, 1928). Then one may conclude that the occurrence of a thick skin covering the tympanic membrane in *B. bufo,* indicates the failure to complete the histological tympanic membrane changes. Moreover, it indicates the truncated middle ear development in *B. bufo.*

Then if compared with *B. melanostictus,* two features are characteristic for the middle ear in *B. bufo* : 1) its truncated development and 2) delayed onset of its morphogeny. These two points indicate the post-displacement (the third principal way of paedomorphosis occurring by a change in timing of the onset of development of certain structures — MCNAMARA, 1986) to account for the derived middle ear condition exemplified by *B. bufo.*

However, it should be said in all fairness that the middle ear underdevelopment in *B. bufo* might be attained through another heterochronic mechanism — through changes in the time of inductive tissue interaction. Earlier the experiments of HELLF (1928) have indicated quite conclusively that in anurans the annulus tympanicus constitutes the immediate influence responsible for the skin transformation which results in the formation of the tympanic membrane. As the annulus tympanicus and skin constitute a system of inductive tissue interactions, the period of development during which they are physiologically active is an important point. If the appearence of the annulus tympanicus is delayed, it may appear when the skin has already lost its ability to transform into the typical tympanic membrane. This seems to be the case in *B. bufo.* Late appearing of the chondrified annulus tympanicus is accompanied by only imperfect modification of skin covering the tympanic membrane. As a result, in *B. bufo* the tympanic membrane is covered by thick almost unmodified skin, whereas in *B. melanostictus* displaying the accelerated development of the annulus tympanicus, the tympanic membrane is thin and transparent.

As one may conclude, the exact mechanism of the middle ear underdevelopment (through post-displacement or through changes in the time of inductive tissue interaction) in *B. bufo* is obscure, but whatever it is, it is obvious that the derived middle ear state in *B. bufo* is a consequence of heterochronic shifts in its middle ear morphogeny.

DISCUSSION

All these examples indicate that diversification seen in the anuran middle-ear morphology is often accompanied by the reduction of the middle ear as a transducer of air-borne sounds. This diversification is not a result of selection for middle-ear morphology but is a consequence of developmental heterochronies as well as of the late onset of the middle ear morphogeny. Then there may be a question : is there any possibility for acceleration of the onset of middle-ear development ? The answer is no. There are strict developmental constraints precluding this

acceleration. The anuran middle ear develops from two sets of elements : one set including the pars interna plectri and pars media plectri appears near the otic capsule, and another one including the annulus tympanicus and pars externa plectri develops near the rostral end of the larval palato-quadrate cartilage (according to GAUPP (1893) the annulus tympanicus forms as an outgrowth of this cartilage). The middle ear may be formed only when these two sets of elements meet each other. However, this event is related to the posterior drift of the palato-quadrate cartilage — the event that determines a change in the jaw mechanism from larval state to the adult one (Fig. 3). This change means the end of metamorphosis, so the middle ear may be formed only at the end of metamorphosis, and the late appearence of the anuran middle ear is predetermined.

But the middle ear is not the only structure that develops late. Many anuran features appear after the end of metamorphosis, but in spite of their delayed and retarded development they do occur, whereas the middle ear is absent. The second question is why the anuran middle ear may be omitted from the ontogeny and why it is greatly subjected to underdevelopment ? It seems that the occurrence of other channels for sound conduction that can participate in perception of acoustic reproductive information decreases the pressure of functional requirements and allows the developmental heterochronies to be the main factor in generating diversification in the anuran middle ear morphology. The main candidate for such an alternative sound conducting channel is an opercular system.

The operculum is an oval or round cartilage that fits into the oval window and provides an insertion for the opercular muscle — a muscle that originates from the suprascapula of the pectoral girdle (Fig. 1). The main function of the amphibian opercular system is the providing of seismic sensitivity to ground vibrations (KINGSBURY and REED, 1909 ; HETHERINGTON, 1985), but there are certain data suggesting the possible functioning of the opercular system in airborne hearing (LOMBARD and STRAUGHAN, 1974). Another possible extratympanal pathway for airborne sounds was recently proposed by NARINS *et al.* (1988). This route includes the lateral body wall, lungs and endolymphatic sacs. The exact mechanism of the extratympanal airborne hearing is obscure, yet whatever it is, it is obvious that frogs can use non-tympanic routes of transmission of airborne sounds. As WILCZYNSKI *et al.* (1987) has shown for *Rana pipiens* SCHREBER 1782, frogs may be equally sensitive to sounds below 1000 Hz through either the tympanic ear or through an alternative route.

As both presumed non-tympanic routes appear before the end of metamorphosis, the middle ear morphogeny occurs when they are already formed and functional. As these alternative routes of hearing can provide the function of the middle ear (airborne hearing), the tympanic middle ear may be subjected to loss or reduction as a by-product of a selection for small body size or early sexual maturation (in progenetic species), a selection for retaining larval features in adult morphology (as seems to be the case in aquatic neotenic *Bombina* which retain the lateral line system), or as a by-product of a selection for other yet unknown adaptive features.

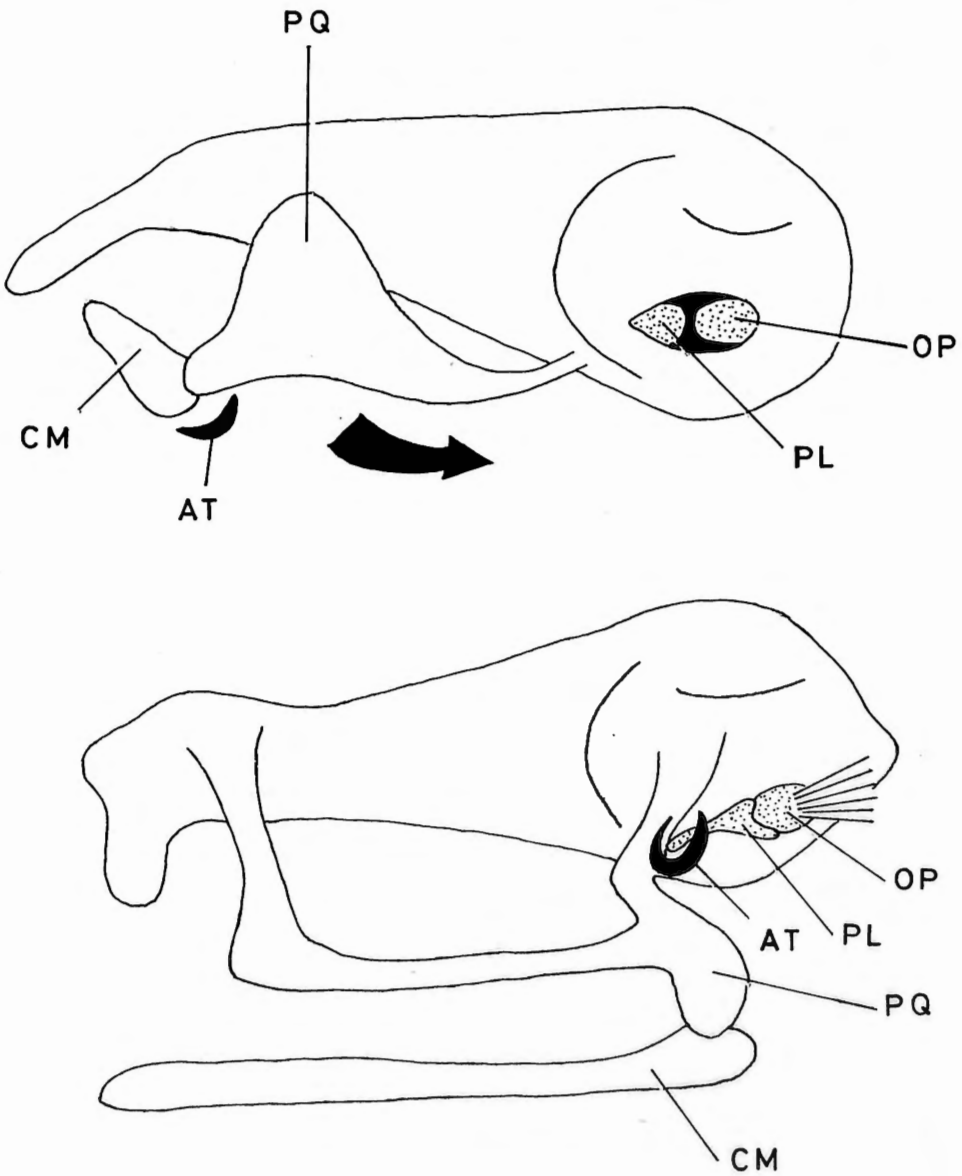

Fig. 3. — Metamorphic changes in the anuran middle ear and jaw mechanism (Modified from BOLT and LOMBARD, 1985).

A - late larva ; B - postmetamorphic juvenile ; AT - annulus tympanicus ; CM - cartilago Meckeli ; OP - operculum ; PL - plectri ; PQ - palato-quadratum.

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ABSTRACTS

SECOND SYMPOSIUM ON THE AMPHIBIA : ENDOCRINOLOGY OF AMPHIBIANS WITH FOCUS ON CELLULAR AND MOLECULAR ASPECTS (*)

Leuven, Belgium, 14-17th September 1990

ULTRASTRUCTURE AND ARRANGEMENT OF CHROMAFFIN CELLS IN THE ADRENAL GLAND OF URODELES, F. Accordi, Dipartimento di Biologia Animale e dell'Uomo, Università « la Sapienza », viale dell'Università, 32, 00185 Roma (Italy).

Different conditions in the arrangement of the adrenal gland are observed in Amphibians : in Gymnophiona and Urodeles the gland consists of islets scattered on the ventral surface of the kidneys, in Anurans the islets are grouped to form a streak. In Urodeles the amount, size and position of the islets vary consistently within different families and even within a genus (1). In order to investigate whether the infraordinal variation is also extended to the fine structure of the gland, further observations were performed in Urodeles. The adrenal glands of 13 species belonging to 6 different families were studied, the ultrastructural characteristics of chromaffin cells and their relationships with interrenal cells were examined. In all the observed species the interrenal cells are always grouped, whereas the chromaffin cells appear either grouped or isolated ; as regards the relationships between chromaffin and steroidogenic cells, various degrees of aggregation are observed in different species. In primitive Urodeles (Sirenidae and Proteidae) the chromaffin cells are isolated or in small groups, mostly separated from interrenal cells and often in contact with renal cells. In Neourodeles the chromaffin cells may be found either grouped or isolated, and located generally at the periphery of the groups of steroidogenic cells, but they may also be found isolated near renal tubules or blood vessels. This double localization usually occurs in Amphiumidae and Ambystomidae, whereas in Salamandridae and Plethodontidae the chromaffin cells appear generally grouped and intermingled with steroidogenic cells.

The different relationships between chromaffin and steroidogenic cells in Urodele families may be related to their phyletic position : conditions of great dispersion occur in primitive urodeles (Sirenidae and Proteidae). An intermediate condition is observed in those families (Amphiumidae and Ambistomidae) which are considered (2, 3) ; at a lower level of organization within the Neourodeles, whereas the gland of advanced Urodeles (Salamandridae and Plethodontidae) shows a higher degree of aggregation.

As regards the cytological characteristics of the chromaffin cells of Urodeles, variable features were observed in the shape and electrondensity of chromaffin granules. In Anurans and higher Vertebrates these characteristics are homogeneous within the same cell and permit the distinction between adrenaline — and noradrenaline — cells ; only in Neourodeles can

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two types of chromaffin cells be clearly identified, whereas in primitive Urodeles this distinction is slight, since shape and electrondensity of granules vary even within the same cell.

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STAGE DEPENDENT REGULATION OF INTERRENAL ACTIVITY IN *XENOPUS LAEVIS* TADPOLES, S. ALBRECHT, W. KLOAS and W. HANKE, Zoological Institute II, University of Karlsruhe, Kaiserstr. 12, D-7500 Karlsruhe (F.R.G.).

The importance of the interrenal gland for osmoregulation and metamorphosis in Amphibia is well established. The cytological and biochemical pattern shows a peak in interrenal cell activity of *Xenopus laevis* tadpoles during the climax of metamorphosis (1, 2). However, data of the mainly occuring steroids coticosterone and aldosterone in the earliest larval stages are not available. The aim of the present study was to investigate the occurrence and the changes of corticosterone and aldosterone levels from egg to juvenile animals. Furthermore, we determined *in vivo* the regulatory influences of ANF, A II, AVT and ACTH on the interrenal secretion at stage 50/51 (according to the Normal Table by NIEUWKOOP and FABER). In comparison to a possible regulatory influence of ANF and A II we studied the localization of binding sites for ANF and A II in the kidneys of *Xenopus laevis* tadpoles at stages 49/50, 51/52 and 55/56 by *in vitro* autoradiography (ARG).

Adult males and females of *Xenopus laevis* twice received an injection of 6 mg human chorionic gonadotropin within two days, which is necessary for breeding. For determinations of stage-dependent corticosterone and aldosterone levels, we sampled eggs and tadpoles. Tadpoles were taken one day after hatching (stage 43/44) and in two-stage steps from 45/46 up to 65/66. The regulatory influences of ANF, A II, AVT and ACTH were studied in normal and hypophysectomized tadpoles (stage 50/51), which received a single injection of 0.1 nM hormone in 10 µl frog Ringer's solution. Control animals were treated with pure frog Ringer. The tadpoles were killed one hour after injection. The contents of corticosterone and aldosterone in homogenized eggs and larvae were determined by radioimmunoassay after extraction by dichloromethane. Eggs and larvae up to stage 47/48 had to be pooled, the steroid contents were determined (in ng steroid/g body weight). ARG for ANF and A II binding sites was performed as described elsewhere (3). The steroid determinations during development show neither corticosterone nor aldosterone in eggs and tadpoles until stage 43. In animals of stage 43/44, a low corticosterone content (1.58 ng/g body weight) was measured, but still no aldosterone. A fast increase of both steroid levels could be observed between stage 43/44 to stage 47/48 (7.70 ng corticosterone and 1.70 ng aldosterone/g body weight) which remain high for aldosterone until stage 51/52 and for corticosterone until stage 57/58. Both aldosterone and corticosterone decreased gradually from stage 57/58 onward but showed a peak at stage 61/62.

The *in vivo* treatment of *Xenopus laevis* tadpoles with ACTH and AVT resulted in an increase of both steroids in normal and hypophysectomized tadpoles. The aldosterone level was relatively more elevated than corticosterone. Val^5-A II had no effect, while ANF caused an insignificant decrease of both steroids in hypophysectomized animals.

ARG results indicate that binding sites for ANF and A II exist in glomeruli and interrenals of the kidneys in all investigated stages, while A II binding sites could be found only in glomeruli and not in interrenal tissue.

Our results demonstrate that interrenal activity develops fast, about one day after hatching (43/44). The high increase of both corticosteroids within a day (stages 43/44 to 45/46) and relatively high constant levels during the period of stages 47/48 to 53/54 suggests that corticosteroids are very important for development and osmoregulation. The gradual decrease of corticosterone and aldosterone, except the peak of both corticosteroids during mid-climax (stages 61/62), to normal levels of juveniles demonstrate the involvement in metamorphosis as observed by LELOUP-HATEY *et al.* (2).

The results of *in vivo* treatment of larvae agree with findings in adult animals (KLOAS, unpublished) where only ACTH and AVT cause an increase of both steroids, while A II is ineffective, and ANF effects may be covered by endogenous factors.

The ANF- and A II binding sites in lavae show the same pattern as in adult animals. Therefore, it can be concluded that in larvae and in adults of *Xenopus laevis* the regulation of interrenal activity is quite similar.

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INFLUENCE OF PHOTOPERIOD AND MELATONIN ON DAILY OSCILLATION OF T_4 ACTION ON GROWTH AND METAMORPHOSIS OF *RANA PEREZI* TADPOLES, A.L. ALONSO-GÓMEZ, M.J. DELGADO, J.L. DOMINGUEZ, B. GANCEDO and M. ALONSO-BEDATE, Dept. Biología Animal II (Fisiología Animal), Fac. Biología Univ. Complutense, 28040 Madrid (Spain).

A circadian rhythm of T_4 action on anuran (*Rana pipiens, Xenopus laevis*) metamorphosis has been described, thus T_4 is more efficient as metamorphic inducer during the light than during the dark phase of the photocycle. Melatonin production is mainly regulated by photoperiod with maximal values appearing at nighttime in almost all of the vertebrate species studied.

The present study was undertaken to investigate the influence of melatonin on day-night changes in tissue responsiveness to T_4 in pre- and prometamorphic larvae of *Rana perezi*. Tadpoles were maintained under two different photoperiodic conditions (24L and 12L:12D), T_4 (1×10^{-7} M) and melatonin (5×10^{-4} M) treatments were carried out by immersion of the tadpoles for one hour twice during the daily photocycle : in the middle of the light phase and early in the dark phase (in 24L group, the time of hormone administration corresponds to subjective day and night). At the end of the experimental period (two weeks) total lenght and metamorphic stage of development were determined. Photoperiodic conditions used in this study do not affect rate growth and metamorphosis in premetamorphic larvae, T_4 stimulated metamorphic development, independently of time of administration and light cycle. In tadpoles maintained under 12L:12D photoperiod, T_4 during the light phase delayed prometamorphic growth compared to dark administration. Constant light did not allow this effect. Melatonin treatment did not interfere with T_4 action in any case.

Results obtained in the present study support a different responsiveness of tadpole tissues to T_4 administration. This response could be due to an activity change of the enzymes implied

in T4 metabolism, or to a circadian oscillation of the thyroid hormone receptors appearance. In this sense, a circadian rhythm in the type II thyroxine 5'-deiodinase activity in mammalian pineal gland has been described, together with a parallelism in melatonin production. To our knowledge there is no study concerning this possible circadian rhythm in Amphibia. If this cyclic activity existed, a high T4-T3 conversion during the light period would explain the different T4 action, depending on the time of administration, and the lack of effect observed under 24L conditions would be a consequence of the hypothetical disappearance of the deiodinase rhythm. Many more experiments are necessary to demonstrate this possibility.

OSMOREGULATORY DISTURBANCES ASSOCIATED WITH STRESS IN *XENOPUS LAEVIS* : EFFECTS ON PLASMA CORTICOSTEROIDS, P.H.M. BALM, B.G. JENKS and F. LEBOULENGER (*), Dept. of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen (The Netherlands), and (*) Lab. Endocrinol. Mol. UA CNRS 650, University of Rouen, 76134 Mont-Saint-Aignan (France).

In all vertebrate classes, corticosteroids regulate a wide variety of adaptational processes associated with stress. Peptides derived from the prohormone POMC have been established as important regulatory factors in the control of adrenal function. ACTH, secreted by POMC cells in the pituitary pars distalis, has by far recieved most of the attention, and relatively few experiments have focused on the possibility that products from the second pituitary cell type, the intermediate lobe MSH cell, could also be involved in the regulation of corticosteroidogenesis. This is surprising because, especially in the lower vertebrates, the pars intermedia is relatively large and circulating levels of αMSH are usually much higher than those of ACTH. Moreover, previous studies in our laboratory have shown that the pars intermedia of teleosts is involved in the regulation of the adrenals during stress. This was based on the finding that the teleostean pars intermedia releases factor(s) with substantial corticotropic activity.

The present study investigates the acclimatization to stressors, and the possible involvement of intermediate lobe POMC products therein, by the aquatic amphibian *Xenopus laevis*. This species provides an appropriate model for these studies, since, the activity of the pituitary MSH cells can be experimentally manipulated by changing the background color. The cells become highly activated when the background becomes black, which results in severalfold higher plasma αMSH levels. To test the effects of this modulation of MSH cell activity on plasma corticosteroids and the capacity of the animals to adapt to stressors, we challenged both black- and white adapted *Xenopus* either with a handling protocol or by lowering the environmental pH. The results show that black adapted animals, which possess high plasma αMSH levels, are more effective in coping with the experimental challenges, as indicated by measured values for several blood constituents. The role of corticosteroids, and the possible involvement of POMC derived factors, during acclimatization to the stressors in *Xenopus laevis* will be discussed.

HORMONAL CONTROL OF *XENOPUS* OOCYTE MATURATION : ROLE OF NON-STEROIDAL FACTOR, D. BOUJARD, F. CHESNEL, A. BOURRY, G. BONNEC and J. JOLY, Laboratoire de Biologie de la Reproduction URA 256 CNRS, Université de Rennes I, 35042 Rennes Cedex (France).

Resumption of meiosis in *Xenopus* oocytes, blocked at the diplotene stage of prophase I, can be induced by steroids (a great variety of non estrogenic steroid) or by proteins (IGF ; insulin ...). These two kinds of inducers act, at least partially, *via* different pathways since insulin-induced maturation can be specifically inhibited (See review of SMITH (1989), *Dev.* **107**). Whatever the inducer is, there is a great variation in the timing of Germinal Vesicle Break Down (GVBD) according to the females. This results, in part, from physiological conditions, since injection into females of low dose of gonodotrophins (« priming » dose) before oocytes denudation reduces dramatically the time of progesterone induced maturation (1). Addition of insulin may also reduce the time of progesterone-induced maturation (2), but great variations are observed according to the experiments.

To understand the cause of these differences, we have studied the effect of insulin, progesterone and both together on maturation of oocytes issued from various unstimulated females. Our results demonstrate the existence of at least two kinds of oocytes : the first category is characterized by a significant synergistic action of insulin on progesterone-induced maturation. In this case, the GVBD 50 (time at which 50 % of oocytes exhibit GVBD) is of 528 mn ± 30 with progesterone alone, of 533 mn ± 26 with insulin alone and of 438 mn ± 25 with progesterone and insulin. In the second category, where no significant synergistic action is observed, progesterone and insulin alone induce a GVBD 50 as short as both together in the first category (respectively 446 mn ± 43 ; 453 mn ± 28 ; 423 mn ± 41). Taken together, these results seem to indicate that in the oocytes of the second category some events have already occured prior to treatment. Such events should be responsible for obtaining a GVBD short time after exposure, whatever the kind of inducer used. In contrast, this « short » pathway leading to GVBD, requires the presence of the two inducers in the first category.

In addition, using the first category of oocytes, we have undertaken to demonstrate the existence, in post vitellogenic follicles of a peptidic substance which can, *in vivo*, act on oocytes as insulin does *in vitro*. Our results showed that a non steroidal factor, secreted by follicular cells, can significantly reduce the appearance time of progesterone-induced maturation. This factor is inactivated by high temperature, suggesting its proteic nature. Moreover, treatments of follicles with cyanoketone (an inhibitor of steroidogenesis), suppress the synthethis and/or secretion of this factor. All thse results strongly suggest that the different pathways leading to GVBD and meiosis have physiological importance. They also indicate that, according to the physiological stage of the female, there is a great variability in the pathway leading to MPF activation.

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***IN VITRO* HORMONAL CONTROL OF VITELLOGENIN SYNTHESIS IN *RANA ESCULENTA* LIVER**, O. CARNEVALI [1], G. MOSCONI [1], K. YAMAMOTO [2], T. KOBAYASHI [2], S. KIKUYAMA [2] and A. POLZONETTI-MAGNI [1], 1. Dept. of Cellular Biology, Camerino (Italy); 2. Dept. of Biology School of Education Nishi Waseda, Tokyo (Japan).

Vitellogenin (VTG), a very complex protein, is known as a precursor of yolk proteins : phosvitin and lipovitellin, in all oviparous vertebrates so far studied.

In amphibians as in other oviparous vertebrates, vitellogenin synthesis is a hormone-dependent process and 17β-estradiol (E_2) seems to be the factor mainly responsible for the synthesis and release in the blood circulation.

Previous results evidenced the pituitary involvement in liver VTG synthesis and secretion in *Rana esculenta.*

In vitro experiments carried out on male and female liver, homologous pituitary (HP) and E_2, both stimulated VTG synthesis although the pattern of VTG secretion, assayed by ELISA, showed some differences concerning the induction time and the rate of VTG secretion. The estradiol response was obtained after 3 days incubation, while maximal HP response came after 4-5 incubation days, and the HP response rate was higher in comparison with E_2 response.

In addition, during the refractory period (July) VTG synthesis in male and female liver was induced by HP only.

In order to identify the pituitary hormones involved in this process, mammalian FSH, LH, PRL and GH were used.

In October experiments, the incubation of *Rana esculenta* male and female liver with FSH and LH failed to induce VTG synthesis and release.

In November experiments (winter stasis), the liver in both sexes was unresponsive to incubation with PRL, while GH was able to stimulate VTG synthesis in the liver.

Moreover, *Rana esculenta* liver positively responded to *Rana catesbeiana* pituitary homogenate; in fact *Rana catesbeiana* pituitary induced the same effects as homologous pituitary on both male and female *Rana esculenta* liver.

Since at the moment *Rana esculenta* pituitary hormones are not available, we are going to experiment using hormones purified from the *Rana catesbeiana* pituitary.

HORMONAL CONTROL OF THE HARDERIAN GLAND OF *RANA ESCULENTA*, G. CHIEFFI, G. CHIEFFI BACCARI, L. DI MATTEO, M. D'ISTRIA, C. MARMORINO, S. MINUCCI and B. VARRIALE, Napels (Italy).

The Harderian gland (H.G.) is the only orbital gland in anuran amphibia whose function is to lubricate the eyeball. It is located at the medial corner of the orbita and in *Rana esculenta* is an oval acinar gland. The seromucoid secretion of this essentially nonmitotic secretory tissue is either merocrine or apocrine and varies during the year.

Little is known about the regulation of the secretory activity of the H.G. in amphibians. In our laboratory we designed some experiments with the green frog, *Rana esculenta*, keeping in mind that the H.G. displays a seasonal activity : the secretory activity is highest during July-August, drops to the lowest in September, and from October onwards resumes slowly.

Both exogenous and endogenous factors have been investigated as possible regulators of the H.G. secretory activity. With regard to endogenous factors, the following endocrine manipulations were carried out.

1) *Pinealectomy*. The presence of melatonin in H.G. and pineal tissue extracts and in plasma was radioimmunologically demonstrated. However, the melatonin content in H.G. and pineal was ten times lower than in plasma (80pg/ml), suggesting that the major source of melatonin in this frog is very likely the retina, as show in *R. perezi* and *R. tigrina regulosa*. A more detailed analysis carried out in *Rana esculenta* kept in continuous darkness for at least two weeks, showed, in a six time point assay, a daily rhythm in plasma melatonin but not in pineal or H.G. Whether H.G. melatonin is synthesized in situ is still to be investigated. Two month pinealectomized frogs (spring animals) were kept for two weeks under different photoperiods (24L:OD ; OL:24D ; 12L:12D). No effect was observed in the H.G. morphology. these data seem to exclude either a regulatory function of the pineal on the H.G. or a possible role of melatonin within the frog H.G. because of its low concentration in the gland which is unaffected by photoperiod manipulations.

2) *Hypophysectomy*. Hypophysectomy carried out during February, when the acinar cells start to elongate and accumulate secretory material, induces a further increase in the height of glandular cells which appear full of secretory granules. Hypophysectomy affected also the electrophoretic pattern of water soluble proteins preserving a protein fraction in the range of 480 kD, which disappears in the captive sham-operated controls. Replacement therapy with crude homogenate of homologous pituitary restored the morphological and biochemical pattern of intact animals.

Among pituitary hormones tested in hypophysectomized animals, bTSH and oxytocin show an inhibitory effect, while prolactin does not modify the stimulatory effect of hypophysectomy on the secretion of the glandular cells in both male and female frogs. oACTH and oLH administration provoke a reduction of secretion in the female, while they have no effect in the male. We have not been able to carry out similar experiments in summer months owing to the high mortality (over 90 %) following hypophysectomy, naturally on account of their already exhausted (postreproductive) physiological condition. Winter 1989-90 has been remarkably mild and temperatures reached 22° C. Consequently, the H.G. secretory activity was at its highest. Hypophysectomy performed in these animals was successful and provoked opposite results when compared to the previous ones : acinar cell height decreased and the protein fraction of 480 kd disappeared. Replacement therapy restored the control protein pattern.

3) *Sex hormones*. Before testing the possible sex hormone influence on the H.G. of the frog, *R. esculenta*, we first looked into the presence of sex hormone receptors. We found not only a large number of cytosolic and nuclear androgen receptors, but also reported a parallel annual profile between plasma androgen concentration and receptor number sites. Interesting was the finding of large numbers of androgen receptors in both sexes, which is not surprising if we consider that circulating androgens in the female are as high as in the male. No estrogen receptor was detected in the frog H.G. in either sex. The lack of a sex dimorphism in the frog H.G. might be related to the similar pattern of circulating androgens in both sexes, while in the golden hamster the clear sex dimorphism correlates well with the low levels of androgens in the female compared to those in the male.

Notwithstanding the presence of androgen receptors in the frog H.G., castration carried out in some, but not all, periods of the year, has no effect on the morphology of the gland. However, the influence of testosterone on the H.G. was observed at biochemical level. In fact testosterone treatment induced a marked increase of some protein fractions, which proved to be mostly glycoproteins and one of them has a MW of 480 kD, the same MW as the protein

fraction which is influenced by hypophysectomy (see above). CPA treatment, given alone or in combination with testosterone, prevent these changes.

The role of testosterone on the secretory activity of the frog H.G. was further studied by *in vitro* incorporation of ^{3}H-Uridine in presence or absence of the steroid. Since the hormonal treatment resulted in a preferential uptake in the poly (A) + fraction, it is supposed that testosterone can trigger the specific biosynthesis of some mRNAs.

FOOD INTAKE INHIBITION AND METAMORPHOSIS STIMULATION BY *SHEEP* CORTICOTROPHIN-RELEASING HORMONE (CRF) ADMINISTRATION IN *RANA PEREZI*, I. Corpas, B. Gancedo, A.L. Alonso Gomez, M.J. Delgado and M. Alonso-Bedate, Depto Fisiologia animal, Facultad de Biologia, Universidad Complutense, 28040 Madrid (Spain).

One of the most spectacular events in animal development is the process of metamorphosis. During premetamorphosis tadpoles grow Prometamorphosis comprises the developmental changes that occur between emergence of the hind limbs and emergence of the fore limbs. During the climax normal feeding is prevented and decreases fecal excretion. Biochemical, physiological, morphological and behavioral changes occur at climax, that prepare a long-tailed herbivorous, aquatic larva to become a tailess, carnivorous, terrestrial organism.

That the thyroid hormones are responsible for these events has been known for a long time. An interesting problem regarding the control of the thyroid system in amphibians development is the identity of the hypothalamic factor or factors which control TSH release by the pituitary gland. Recently, studies by Denver (1) in *Rana catesbeiana* adults suggest that ovine CRF produces a greater output of TSH than do hGnRH or TRH. On the other hand some studies in mammals indicate that CRF is an important and physiologically relevant feeding inhibitor. In the present investigation we pretend to study the effects of sheep CRF on metamorphosis events and food intake responses in *Rana perezi* tadpoles.

Tadpoles obtained by spontaneous breeding in June were kept in darkness at 10 ± 2° C temperature. They were fed with boiled spinach once a week in order to maintain the minimum metabolic requirements.

One week before the beginning of the experiments, tadpoles were acclimatized to the experimental conditions; 12L:12D photoperiod and 24 ± 2° C temperature. Spinach was weighed every day before and after tadpoles were fed and the excreta were evaluated. Three times during the course of the experiment tadpoles were anaesthetized with MS-222 (1:10000) and measured. The experiment was finished when some of the tadpoles reached metamorphic climax. Tadpoles were anaesthetized weighed and measured and then used for histological study of the thyroid gland.

Sheep CRF (Sigma) was dissolved in a minimum amount of Holtfreter solution and stored at − 30° C. For treatment, tadpoles were daily injected through the opercular opening into the dorsal part of the body near the eyes. Treatment was performed in October-November and tadpoles, eight per group, received 2µl of Holtfreter solution (controls) or 2µl of Holtfreter containing 1µg CRF per injection (total number of injections was 17).

The results obtained in the present study demonstrate that sheep CRF significantly stimulates hindlimb growth, cloacal tail piece disappearance and increases the number of thyroid follicles. Also we observed a significant decrease of food intake and fecal excretion and tadpoles were significantly smaller than controls. It is suggested that in amphibian

development a CRF-like factor, depending on the level reached at one particular stage, stimulates thyroid hormones, and consequently metamorphic events like hind limb growth, cloacal tailpiece disappearance and directly affects some hypothalamic nucleus which decreases appetite during the climax. Some experiments using hypophysectomized tadpoles are now under way in our laboratory.

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LOCALIZATION OF INSULIN IN THE PANCREAS OF *XENOPUS LAEVIS*, AND CHARACTERIZATION OF ITS LIVER PLASMA MEMBRANE INSULINE RECEPTOR, B. J. COWAN, R. A. FOTY and R. A. LIVERSAGE, Department of Zoology, University of Toronto, Ontario (Canada), M5S 1A1.

Insulin is known to play an essential role in sustaining growth and differentiation in adult urodele appendage regenerates. Amputation of a *Xenopus* forelimb results in the regeneration of a spikeshaped appendage unlike the perfect epimorphic regenerate in urodeles. As tissue regeneration in *Xenopus* is a cell proliferation event, our aim is to examine the role of insulin as a growth-promoting hormone in *Xenopus* forelimb regeneration. We are attempting to determine whether a regenerate ; (i) is a target organ for insulin, and if so (ii) does the insulin receptor capacity and affinity change during the regeneration process. Our initial research involving liver cells was performed in order that we might devise methods for *Xenopus laevis* forelimb regeneration studies. The methods include : aldehyde-fuchsin staining of the pancreas as well as PAP immunochemistry of adjacent sections . competitive inhibition and Scatchard analysis of partially purified liver cell membranes ; cross-competition assays to determine receptor specificity ; as well as affinity photolabeling of receptors to determine structure (and function). Results demonstrate the presence of aldehyde-fuchsin positive pacreatic islets, scattered throughout highly vascularized acinar tissue. PAP immunostaining utilizing a mammalian antibody shows corresponding clusters of insulin-positive cells, indicative of the site of insulin production. Homologous antigen immunoabsorbance and pre-immune serum controls were PAP negative, whereas pre-absorption of insulin antibody with heterologous antigens, including guinea pig insulin, insulin-like growth factor 1 (IGF-1), and glucagon results in PAP-positive staining, thus confirming the specificity of the antibody. Competitive inhibition and Scatchard analysis describes a two binding site receptor model ; a low affinity ($0.16 nM^{-1}$), high capacity (3.2 ± 0.9 picomoles/mg) binding site and a high affinity ($2.7 nM^{-1}$), low capacity (0.5 ± 0.3 picomoles/mg) binding site. Cross competition experiments show that radioactive insulin is more efficiently displaced by excess cold insulin than by guinea pig insulin and IGF-1. Glucagon does not cross react. This suggests that the receptor is specific. Photoaffinity labeling and immunoprecipitation demonstrate the presence of a receptor subunit with an apparent molecular weight of approximately 130 Kd. Presumably, this is the alpha (insulin binding) subunit of the receptor, under reduced conditions. ^{32}P-ATP labeling demonstrates the presence of a receptor subunit approximately 95 Kd, which undergoes insulin-stimulated phosphorylation. Currently, we are attempting to determine whether changes in receptor numbers occur at various stages of limb regeneration, and to examine the effects of blocking insulin action at the regeneration site using antibody blocking techniques. (Supported by NSERC of Canada Grant A-1208 to R.A.L.).

INFLUENCE OF THIOURACIL AND THYROID HORMONES ON TESTICULAR FUNCTION IN *RANA PEREZI*, J.L. DOMINGUEZ, I. CORPAS, M.J. DELGADO and M. ALONSO-BEDATE, Depto Fisiología Animal, Facultad de Biología, Universidad Complutense, 28040 Madrid (Spain).

A possible correlation between the gonadal and thyroidal axis has been proposed by SARKAR and RAO (1), who showed that chemical and surgical thyroidectomy prevent ovulation in *Rana cyanophlyctis*. It has been suggested that estradiol-17β silastic implants in female *Rana ridibunda* depress thyroid hormone concentrations in plasma and the in vitro 5'-monodeiodination activity of kidney homogenates (2). Thyroid hormones (THs) appear to be necessary for estrogen-induced synthesis of vitellogenin by the liver of *Xenopus laevis* (3).

The data about THs being required for male reproduction are very scarce. The objective of the present study was to determine how manipulation of the thyroidal status (thyroid hormones or thiouracil administration) modify testicular function as assessed by gonadosomatic index (GSI), plasma testosterone levels or testes responsiveness to gonadotrophins (hCG) in *Rana perezi*.

Adult male frogs (*Rana perezi* SEOANE, 1885) were captured in Galicia (NW of Spain) and sent to Madrid in March and May. They were maintained in plastic tanks with tap dechlorinated water in a room with natural photoperiod and temperature for one week after their arrival. The experiments were performed in a room with controlled photoperiod (12L:12D in March ; 14L:10D in May, lights on at 8:00 a.m.) and temperature (20 ± 2° C in March and 23 ± 2° C in May). Calliphora larvae were available three days a week and both — March and May frogs — were eating regularly.

Experiment 1 : Effects of thiouracil, human chorionic gonadotropin (hCG) or both on GSI and plasma testosterone levels. This experiment was performed in March. Frogs were divided into four groups consisting of eight frogs. Each group received two injections in the ventral lymphatic sac every second day. The first injection was given at 9:00 a.m. and the second one at about 30 minutes later. The first group (Control) was injected with 0.1 ml frog Ringer (FR) followed by 0.1 ml FR. The second was injected with 0.1 ml FR followed by 100 IU hCG/0.1 ml. The third was injected with 5μg 2-thiouracil/0.1 ml followed by 0.1 ml FR. Finally, the fourth group was injected with 5μg 2-thiouracil/0.1 ml followed by 100 IU hCG/0.1 ml. The treatment consisted of a total of six injections. Then, animals were anaesthetized with tricaine methanesulfonate (MS-222, Sandoz) and blood was collected directly from the heart, it was centrifuged and stored at −20° C until assayed for testosterone. Testes were taken to evaluate GSI (testes weight/body weight × 100). Testosterone was evaluated as described previously.

Experiment 2 : Effects of T_3 and T_4 on plasma testosterone levels and GSI. This experiment was performed in May. THs from Sigma were dissolved in a minimum amount of NaOH and then diluted in distilled water. THs were finally added to two liters of dechlorinated water and frogs were so treated by immersion. Concentration of NaOH was also taken into account in controls. The final concentration of NaOH in the tanks resulted to be 10^{-8} g/ml. Animals were divided into 3 groups of eight animals each. The first group was immersed in tap water containing 10^{-8} NaOH g/ml. The second group was immersed in 50 ng T_3/ml. The last group was immersed in 50 ng T_4 ng/ml. The water was renewed daily. After one month of treatment, animals were anaesthetized with MS-222 and blood was collected, stored and assayed for testosterone by RIA. Testes were weighed and GSI was calculated. The results obtained in the present work suggest that a long-term treatment with T_3, T_4 or thiouracil does not influence testosterone plasma levels and GSI and does not modify the

testicular response to gonadotropin administration at least in these experimental conditions in *Rana perezi.*

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ADHESION AND RECOGNITION MOLECULES DURING OOGENESIS IN THE AMPHIBIAN *TRITURUS CRISTATUS* CARNIFEX, C. Falugi [1], A. Contini [2], G. Faraldi [1], S. Fasulo [2], G. Tagliafierro [1], G. Zaccone [2], 1. Ist. Anatomia Comparata, Viale Benedetto XV 5, 16132 Genova ; 2. Dip. Biol. Animale ed Ecol. Marina, S. Agata, 98166 Messina (Italy).

Cell-cell and cell-environment communications play an important role in regulating differentiation. Communication is mediated by hormonal or, generally, chemico-physical signals, that must undergo changes at the passage through membranes to the intracellular medium, where they display their role. In this process membrane receptors are involved, such as glycocalix, for signal recognition and cell adhesion (1). During oogenesis, surface changes take place, which are needed for the reception of different signals ; they are also involved in the regulation of cell movement and shape, by interaction with the extracellular matrix (2). In this work, a study of cell surface changes was carried out on gonads of adult female specimens of the amphibian *Triturus cristatus carnifex*, by use of either FITC or HRP-labelled lectins : concanavalin A (ConA, from Sigma, USA), with affinity to carbohydrate residues of mannose and glucose ; wheat germ agglutinin (WGA, from Kem-en-tec, DK), with affinity to N-acetyl-D-glucosamine and sialic acid ; peanut agglutinin (PNA), with affinity to galactose-1-3-galactose-N-acetylgalactosamine ; soy bean agglutinin (SBA), which binds N-acetylgalactosamine and D-galactose ; *Griffonia somplicifolia* I (GS I), which binds α-D-galactose. Furthermore, we investigated the presence of fibronectin in the extracellular matrix, by use of an anti-human fibronectin antiboby (Behring, D). We found that WGA affinity sites are present in different locations in oocytes at different maturation stages. Small oocytes (20-40 μm) are weakly labelled in the cortical region, while 50-80 μm oocytes present binding sites in the cortical region and in the cytoplasm, in the form of rings, concentric to the surface ; maturing oocytes were mainly labelled in the zona pellucida. ConA binding was present in all the oocytes in the follicular envelopes, and in the cytoplasm, but not in the zona pellucida, as it was shown for mouse ovarian oocytes (3) ; SBA-binding sites are localized in the egg central region, mainly around yolk granules ; no binding was found either with PNA or with GS I. FN-like immunoreactivity was present on the outer face of the zona pellucida and in intercellular matrix of ovular envelopes close to the big oocytes. Probably, the presence of FN around big oocytes is linked to the behaviour of such oocytes, that must move towards the ovarian surface, to be released, as it was shown for migrating germ cells in embryos of amphibians (2). These data provide a first bulk of information for distinct components of the amphibian ovary and their developmental behaviour.

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HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY ON THE SPERMATOGENESIS OF *TRITURUS CRISTATUS CARNIFEX*, C. Falugi, G. Faraldi, G. Tagliafierro, Istituto di Anatomia Comparata dell'Universita di Genova, Viale Benedetto XV, 5, I-16132, Genova (Italy).

A developmental role for neurotransmitter systems localized in non-neuromuscular cells has been generally hypothesized (1, 2) ; in particular, these systems can be involved in regulating morphogenetic movements and cell differentiation (3). Since spermatogenesis is characterized by evident changes in cell shape, we have investigated if neurotransmitters are present and active during such a morphogenetic process. Our study was carried out on specimens of the amphibian *Triturus cristatus carnifex*, obtained from De Rosa (Napels). Cholinesterase and acetycholine esterase (ChE) activities have been detected on testicular cells by a direct thiocholine method, and catecholamines by the FIF method ; indirect immunofluorescence reactions have been carried out by the use of antibodies anti-ChE (Dako, DK), anti- ChAT (Seralab, UK), anti-ACh (Biosys, F), and anti-5 HT (Inr, USA).Furthermore, the presence of acetylcholine receptors or their precursors has been investigated by the use of bungarotoxin (BuTx), labelled with FITC (control by curare pretreatment), and its glycoprotein nature by use of lectins (fluorescent ConA, WGA and PNA) ; lectins were also employed to test cellular adhesiveness.

In the sperm lineage cells, the cholinergic system, including BuTx-binding sites, was found to be present mainly during sperm maturation. This system is implicated in regulation of intracellular cation changes (1), and consequently, in the rearrangements of the cytoskeleton. In mature sperms, the cholinergic system is still active in the sperm head, around the nucleus, and in the flagellum, probably with other functions, as it was shown for sea urchin sperms (4). Catecholamines, that are known to stimulate second messengers production in sea urchin eggs (5) have been found in cells undergoing early differentiation, while 5HT, implicated in the regulation of microfilament contraction during egg and blastomere cleavage (6), is present at every stage of spermatogenesis, but has not been found in maturing and mature sperms ; ConA and WGA binding sites are also present around the nuclei of maturing and mature sperms, showing the nature of oligosaccharidic residues (mannose/glucose and sialic acid/N-acetyl-D-glucosamine), which together with the BuTx binding, suggests the presence of proteins, similar to the α-subunit of the ACh receptor. Such report is in favour of a role for neurotransmitter systems in the control of sperm differentiation, through modulation of intracellular dynamics.

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IMMUNOCYTOCHEMICAL LOCALIZATION OF NEUROPEPTIDES AND SEROTONIN IN THE GUT AND PANCREAS OF THE ITALIAN PLETHODONTID SALAMANDER, G. FARALDI, G. TAGLIAFIERRO, Istituto di Anatomia comparata, viale Benedetto XV, 5, Universita di Genova, Genova (Italy).

The distribution and localization of gut endocrine cells and nerve fibres have been studied rather extensively in the anuran amphibians, but there is relatively little information on urodeles which represent the most ancient group of this taxon (1-5). For this reason we investigated by immonocytochemical methods, the presence of some neuropeptides and serotonin in the gut and pancreas of the italian cave salamander *Hydromantes (Speleomantes) ambrosii* LANZA (1954).

Immonoreactivity was detedcted by indirect immunofluorescence and peroxidase-antiperoxidase (PAP) methods. Dewaxed sections were incubated overnight at room temperature using different rabbit antisera against several mammalian gut and pancreatic peptides. Controls were performed by absorption of the primary antiserum with its corresponding antigen. A positive reaction followed the use of antisera such as : bombesin (1:400, CRB, UK); vasoactive intestinal polypeptide (VIP) (1/200, CRB, UK); peptide histidineisoleucine (PHI) (1/200, CRB, UK); substance P (1:250, CRB, UK); glucagon (1:200, Dako, DK); somatostatin (1:200, Dako, DK); insulin (1:200, Dako, DK); pancreatic polypeptide (PP) (1:200, Dako, DK); serotonin (5HT) (1:500, INC, USA).

Immunoreactive endocrine cells and nerve fibres can be detected in the gastrointestinal tract. Bombesin-, substance P-, and PHI-like immunoreactivities (IR) are localized in both endocrine cells and nerve elements, while somatostatin-, glucagon-, 5HT-like IR are seen only in endocrine cells, and VIP-like immunoreactivity only in nerve elements. Immunoreactive endocrine cells are located along the whole gastrointestinal tract; generally they are flask-shaped and exhibit a long, narrow process reaching the lumen, and a few basal processes running beneath neighbouring cells. However bombesin-like containing cells appear to be of a closed type. All endocrine cells show an enlarged nucleus but a restricted cytoplasmic area. Immunoreactive nerve fibres seem to be particularly abundant in the lamina propria beneath the epithelium, and around the smooth muscle fibres (muscularis mucosae, muscular layers and blood vessel wall). VIP, PHI, and bombesin immunopositive neurons can be detected in the submucous and muscular layers. Pancreatic endocrine cells are immunostained by insulin, glucagon, somatostatin, and PP antisera. Apparently they do not organize endocrine islets. Glucagon- and somatostatin-like immunoreactive cells are individually scattered while the fewer insulin- and PP-Like immunoreactive cells constitute cord-like formations near blood vessels.

Thus the morphological and distributive patterns of immunoreactive endocrine cells and nerve elements appear to be rather primitive even if some more advanced characters, such as concentration of bombesin-containing cells in the stomach, and the presence of VIP immunoreactivity in nerve elements only, can be found in the italian cave salamander.

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COEXISTENCE OF DIFFERENT NEUROACTIVE SUBSTANCES IN THE HYPOTHALAMUS OF AMPHIBIA, A. FASOLO [1], F. FRANZONI [1], B. MULATERO [1], C. ANDREONE [1], H. VAUDRY [2] and F. VANDESANDE [3], 1. Dip. Biologia Animale, Via Accademia Albertina, 17, Universita di Torino, 10123 Torino (I.); 2. Lab. Endocrinologie Moleculaire, Université Rouen (F.); 3. Zoological Institute, Universiteit Leuven (Belgium).

The coexistence of different neuroactive substances in the same neuron represent probably a significant way to hamper heterogeneity and signalling complexity of the nervous tissues.

Nevertheless extensive comparative studies on the coexistence of neuroactive substances are scanty, even if they might throw some light on the evolutionary mechanisms and functional significance of such coexistence.

In the present paper the distribution of many different neuroactive substances, including neuropeptides, biogenic amines, aminoacidic transmitters, is reviewed, focusing on the coexistence in the hypothalamo-hypophysial systels of Amphibia. As experimental models the green frog, *Rana esculenta* (and *Rana ridibunda*) and the crested newt (*Triturus carnifex*) were used.

In general, the large majority of the neuroactive substances appeared localized in separate neurons, but relevant coexistence phenomena were described, e.g. for different tachykinins, for SP-like and enkephalin-like immunoreactivities.

The innervation of the hypothalamo-hypophysial complex, and in particular of the pars intermedia, is a good example of coexistence, since in the frog dopamine, GABA and NPY seemingly are co-occurring in a small subset of nerve fibers.

On the whole such a study can help to redraw the mapping for neuroactive substances. It prompts as well some intriguing comparative problems, concerning stability versus plasticity of the basic neurochemical pattern in vertebrates.

THE DISTRIBUTION OF THE ATRIAL NATRIURETIC FACTOR IN THE CENTRAL NERVOUS SYSTEM OF THE CRESTED NEWT, *TRITURUS CARNIFEX* LAUR., M. F. FRANZONI, R. TAVOLARO*, A. FROVA and M. CANONACO*, Dipartimento di Biologia Animale dell'Universita di Torino, Via Accademia Albertina, 17, I-10123 Torino; *Dipartimento di Biologia Cellulare and *Dipartimento di Ecologia dell'Universita della Calabria, I-87036, Arcavacata di Rende, Cosenza (Italy).

The first evidence of the presence of an atrial natriuretic factor (ANF)-like immunoreactivity in the central nervous system of a non-mammalian vertebrate has been provided by NETCHITAILO *et al.* (1) in the frog *Rana ridibunda*.

Because of the interest in the Urodele brain as a simple model for increasingly complex vertebrate neural organization we have studied immunohistochemically the neuronal systems containing ANF and related peptides in the brain of the crested newt, *Triturus carnifex* Laur.

Using antibodies a-alpha ANF (1-28), a-porcine BNP (brain natriuretic polipeptide), a-alpha rat ANP (8-33), a rich innervation by ANF-containing nerve processes has been shown throughout all the brain stem of the newt (and also in the hypothalamo-hypophysial complex). The ANF-immunopositive neurons appeared clustered in two main paired groups extending from the preoptic recess of the preoptic area toward the basal telencephalic regions where they fused on the median line

In respect to the organization of the ANF-containing neurons described in the frog, the pattern of distribution of neurons in the newt seemed by large simplified and in some way primitive.

As we previously reported (2), the preoptic area and preoptic recess of the newt contain abundant dopaminergic neurons (labelled by the immunopositivity for the tyrosine hydroxylase, the rate limiting enzyme in the catecholamine metabolic pathway) together with a thick dopaminergic innervation.

As biochemical assessments (3) have indicated that in the mammalians the ANF may interact importantly with dopaminergic mechanisms, we have analysed, applying immunohistochemical double sequential or simultaneous methods, the interrelationship between ANF-containing neurons and dopaminergic ones in the preoptic area of the newt.

Our results have shown the absence of co-localization of ANF and tyrosine hydroxylase in the same neuron. However close relationships between dopaminergic fibres/terminals and ANF-like containing neurons have been observed.

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SUBCELLULAR RESPONSE OF AMPHIBIAN THYROTROPHIN- AND GH-PRODUCING CELLS TO SEVERAL HYPOTHALAMIC FACTORS, F. GRACIA-NAVARRO, M.M. MALAGÓN, A. RUIZ-NAVARRO, J. CASTAÑO, S. GARCÍA-NAVARRO, R. TORRONTERAS, Department of Cell Biology, Fac. Sciences, Univ. of Córdoba, Avda. San Alberto Magno s/n. E-14004 Córdoba (Spain).

The amphibian pituitary has been the subject of numerous endocrinological studies. Nowadays, the availability of purified antisera against some amphibian hypophysial hormones for RIA and the development of indirect methods (*i.e.* measure of T3 or T4) have considerably enlarged the knowledge about the dynamics of the amphibian pituitary under different experimental conditions. One of the most interesting features of the amphibian pituitary recently reported is the fact that one cell type may specifically respond to more than one hypothalamic releasing peptide, which is not so common in higher vertebrates, especially in mammals.

We have investigated the multiple response of some of these amphibian cell types to the *in vivo* administration of several hypothalamic factors from a morphological point of view. This includes the evaluation of the changes suffered by the cytoplasmic organelles that indicate the hormonal content of the cells (volume and numerical densities of the secretory granules) and the activity of the biosynthetic machinery (volume density of the rough endoplasmic reticulum and Golgi complex). The evaluation was carried out on micrographs corresponding to cells previously identified immunocytochemically in order to assess the specificity of the measure. Two methodological methods were applied, the point-counting method and area analysis (1), using an image analyser (IBAS Kontron). Statistical differences between morphometric data were established by nested analysis of variance and a U-test.

With this methodological approach, we have studied the time course response of *Rana perezi* TSH cells to daily administration of thyrotrophin-releasing hormone (TRH) and corticotrophin-releasing factor (CRF), as well as that of GH cells to TRH and growth hormone-releasing hormone (GHRH).

Thyrotropes. The administration of 7.5 μg/20 g bw synthetic TRH induced a short-term response in TSH cells. The tripeptide evoked significant decreases of the Vv and Nv of the SG of TSH cells indicating stimulated release of hormone, which has been also suggested to occur in other amphibian species (2, 3). The dose used also activated TSH cells to stimulate synthesis since the relative cytoplasmic volume (Vv) occupied by the RER and GC was significantly increased compared to control animals.

These ultrastructural modifications indicating the activation of TSH cells were less intense when ovine CRF was administered (1μg/20 g bw), which only induced loss of secretory granules. The ability of CRF to stimulate the hypophysis-thyroidal axis has been recently reported in other amphibian species (3, 4, 5) as well as in a reptile (6). In such species, TRH is also capable of enhancing the release of thyrotrophin which supports the dual control of the secretion of this hormone in these groups of vertebrates.

After a long-lasting treatment, TRH-stimulated TSH cells tended to recover the control values of the stereological parameters while in the case of CRF-injected animals the response of TSH cells was significantly increased.

These results indicate that each peptide induces a different response on TSH cells. TRH causes a short-term response while the effects of CRF are more noticeable after a long-term treatment.

In both experimental treatments, the degranulation of TSH cells always affected small and medium secretory granules, which probably represent new synthesized and stored thyrotrophin respectively (7).

Somatotropes. With respect to GH cells, they showed significant signs of activation after TRH (7.5 μg/20 g bw) or GHRH (1 μg/20 g bw) injection, featuring a diminution of SG and increased development of the organella related to synthesis. This response was much more marked after several days of GHRH treatment while it was considerably reduced in the case of long-term TRH administration. In both experimental treatments, the loss of hormonal content seemed to affect the newly synthesized and stored pools of hormone.

The stimulation of GH release by TRH and GHRH has been also demonstrated in birds (8), in which both peptides are considered to participate in the physiological control of GH. In mammals, TRH stimulates GH secretion in some circumstances such as hypothyroidism (9), acromegaly (10), during the neonatal period (11), and in the case of the bovine pituitary *in vivo* and *in vitro* (12, 13).

Similarly to what occurs in TSH cells, GH cells seem to be differentially affected by the two peptides, GHRH being more effective than TRH in stimulating this cell type.

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DEVELOPMENT SEQUENCES IN THE MORPHOGENESIS OF THE ADRENAL GLAND IN ANURAN AMPHIBIANS, A. Grassi Milano and M. Antonietta Bracci, Dip. Biologia Animale e dell'Uomo, Università di Roma « La Sapienza », Viale dell'Università 32, 00185 Roma (Italy).

Among anurans, morphology of the adrenal gland is subject to evolutionary variation according to the systematic position of the species, so that different types of adrenal gland may be distinguished in different anuran suborders. The position of the adrenal gland on the ventral renal surface of the adult anuran is the most evident differential characteristic between archaeobatrachians and neobatrachians as regards the morphology of this gland : it is medial in the former, lateral in the latter (1, 2). Analysis of the morphogenesis of these evolutionary variations may indicate how they originate.

During ontogenesis the position of the adrenal blastema varies. At the beginning of its differentiation it occupies a dorsal position between the dorsal aorta, the caval vein and the dorsal renal surfaces. Later the blastema gradually rotates from the dorsal to the ventral surface of the kidney. In neobatrachians the shift continues towards the lateral part of this surface.

The research on the stages of this process in archaeobatrachian and neobatrachian anurans, may allow us to determine whether adrenal gland development displays chronological differences in anuran species correlated to their systematic position, and whether the position of the adrenal gland in adults is attained by means of chronological changes of the development. For these purposes, we compare the variations in the position of adrenal gland during metamorphosis of four anuran species, the archaeobatrachians *Xenopus laevis* and *Discoglossus pictus* and the neobatrachians *Rana esculenta* and *Bufo bufo*.

Morphometric analysis of the rotation process demonstrates that it is characterized by numerous statistically significant chronological differences. Some of them are intragroup differences, *i.e.* within archaeobatrachians or neobatrachians, but most of them are observed among species belonging to both suborders and are related to their sytematic position. In particular, progression towards the ventral position is slower in archaeobatrachians than in neobatrachians. On the other hand, as the adult position on the ventral surface of the kidney is nearer to the medial renal margin in archaeobatrachians than in neobatrachians, at the end of the metamorphosis the gland of the former is closer to the definitive position. Morphogenesis proceeds after the end of the metamorphosis until this arrangement is reached. It is achieved in four months in *Discoglossus pictus*, whereas it proceeds for more than one year in *Rana esculenta*. Evolutionary modifications determined by chronological variations of morphogenesis are including among the cases of heterochronic development (3). Modification of the adrenal structure towards a more advanced arrangement is therefore achieved by means of a heterochronic modification of the morphogenesis.

Most of the dorso-ventral displacement occurs during the metamorphic climax. According to numerous authors (4-7) in many anuran species synthesis and plasmatic concentration

of aldosterone and corticosterone surge at the climax. It is therefore possible that the high levels of adrenal hormones enhance the accelerated rotation during this period.

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CONTIGUITY BETWEEN CHROMAFFIN AND STEROIDOGENIC CELLS IN ADRENAL GLAND OF AMPHIBIA, E. Grassi Milano, S. Contristano and H. Manelli, Dip. Biologia Animale e dell'Uomo, Università di Roma « La Sapienza », Viale dell'Università 32, 00185 Roma (Italy).

Analysis of the evolution of the adrenal gland of Vertebrates demonstrates that a plesiomorphic adrenal gland is a diffused organ, dispersed inside the kidney, with aminergic (chromaffin) and steroidogenic cells scarcely or not at all in contact. In the following evolution the adrenal gland tends to form a discrete and compact organ, external to the kidney, in which aminergic and steroidogenic cells strictly associate, frequently with precise localization. This is the apomorphic type of gland (1). The adrenal gland of Amphibia constitutes an intermediate stage in the evolution of this organ. In the « amphibian type » arrangement, both tissues, steroidogenic and aminergic, emerge from the kidney and associate, although in different degrees. Inside this general structural type, we can recognise a primitive subtype, found in apoda, many urodeles and archaeobatrachian Anura, and a more advanced one, in some neourodeles and in all neobatrachian Anura (2-4). It was proposed (5) that the contiguity between the two types of cell is lower in the primitive subtype. This hypothesis is now verified by morphometric analysis, comparing neourodele *Hydromantes supramontis* with neobatrachian *Rana esculenta*. Preliminary data are also collected on the paedogenetic urodeles *Amphiuma tridactylum* (neourodele) and *Siren lacertina* (primitive Urodele) and on the archaeobatrachian *Discoglossus pictus*.

A quantitative approach, integrated by statistical analysis, demonstrates that in *Hydromantes supramontis* only 50.7 % of aminergic chromaffin cells (CC) are in contact with the steroidogenic cells (SC), whereas this value is 98.5 % in *Rana esculenta*. The difference is highly significant. The cellular volume in anurans is less than in Urodeles, so that in the surface unity the SC + CC are 1.92 in *H. supramontis* and 5.95 in *R. esculenta*, thus improving the contact between the cells. Furthermore the ration CC/CS in the surface unity is 1/4 in *H. supramontis* and 1/1.78 in *R. esculenta*. In *Amphiuma tridactylum* the percentage of mingling is lower than in *H. supramontis*. In *Siren lacertina* the CC are in general isolated from the SC. In *Discoglossus pictus* the degree of mingling is intermediate between the values found in *R. esculenta* and in *H. supramontis*.

The adrenal gland of urodeles then displays a lesser degree of anatomical integration between aminergic and steroidogenic cells with respect to the anurans.

Since reciprocal control mechanisms between the two tissues have been demonstrated (6-8), a high degree of contiguity between CC and SC may improve the functionality of the gland. It is possible that the more complex organization of the anuran adrenal gland, promot-

ing more integrated secretion activities, may explain the wider distribution of the anurans with respect to the urodeles.

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IMMUNOCYTOCHEMICAL AND ULTRASTRUCTURAL STUDY OF *RANA DALMATINA* PARS DISTALIS DURING LARVAL DEVELOPMENT, A. GUASTALLA, E. CAMPANTICO, K. YAMAMOTO, T. KOBAYASHI and S. KIKUYAMA, Università di Torino, Dipartimento di Biologia animale, Via Accademia Albertina 17, 10123 Torino (Italy).

Cytodifferentiation and immunocharacteristics of prolactin (PRL) and growth hormone (GH) secreting cells in the adenohypophysial primordium of *Rana dalmatina* tadpoles at developmental stages 28 to 45 (1) were studied at the electron microscopic level.

Single and double simultaneous indirect immunogold techniques were applied to ultrathin sagittal sections of Epon-Araldite embedded tadpole heads. Rabbit anti-*Rana catesbeiana* PRL and rabbit anti-*Rana catesbeiana* GH were employed in the single method ; rabbit anti-*Rana catesbeiana* PRL and monkey anti-rat GH were used in the double simultaneous technique as primary antisera.

The intensity of the immunoreaction on the pituitary cells was very strong with the antisera anti-*Rana catesbeiana* PRL and GH and virtually all of the immunogold was confined to secretory granules ; it was much weaker and exhibited considerable non-specific binding with anti-rat GH.

At earlier developmental stages, a few hours after hatching, wide intercellular spaces are present in the hypophysial primordium ; the cells contain in their sparse cytoplasm numerous vitelline platelets, lipid droplets and melanine granules. At stage 28 are already identifiable some cells with few membrane-bound secretory granules, 70-90 nm in diameter, immunoreactive with anti-*Rana catesbeiana* PRL, and some others with few secretory granules, 80-110 nm in diameter, immunoreactive with anti-*Rana catesbeiana* GH.

With the progress of the development intercellular spaces become narrower, cytoplasmic inclusions undergo a progressive reduction and immunoreactive cells increase in number. The differentiation in both cell types is shown by an increase in the cytoplasmic volume and in number, size and immunoreactivity of secretory granules. Neither PRL nor GH cells, however, exhibit ultrastructural features specific enough to allow their identification on pure morphological bases, at least up to the end of premetamorphosis.

Neither comparison of adjacent ultrathin sections stained with single labeling technique, nor results of double simultaneous labeling showed coexistence of PRL and GH within the same cell.

Double simultaneous immunogold technique was only applied at development stages 38 and 45, when pituitary cells had reached a higher degree of differentiation. The affinity for

the amphibian hormone of the monkey anti-rat GH employed by us together with the rabbit anti-*Rana catesbeiana* PRL was indeed noticeably lower, probably because raised in different species.

The present findings reject the hypothesis of the existence of « somato-mammotrophic » cells since the earliest developmental stages of hypophysial primordium in *Rana dalmatina* larvae suggested by us in a previous study performed at the light microscope employing indirect immunofluorescence and unlabeled antibody enzyme methods (2).

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REGULATION OF PITUITARY FUNCTION IN AMPHIBIA : EFFECTS OF HYPOTHALAMIC PEPTIDES AND NEUROTRANSMITTERS, T.R. HALL, Biovet Unit, Ciba-Geigy S.A., Centre de Recherches Agricoles, 1566 St. Aubin FR (Switserland).

In common with other vertebrate species, the amphibian hypothalamus contains a set of factors that modulate the secretion of hormones from the pituitary gland, these factors including various peptides and amines. The interactions between neurotransmitters, releasing hormones and pituitary function has been studied for more than three decades, but only during the past few years, with the isolation of the pituitary hormones and subsequent development of specific radioimmunoassays, has a more profound understanding of amphibian neuroendocrinology been made possible. The purpose of the present review is to summarize these findings and to highlight neglected areas of research.

Early studies revealed that acid extracts of hypothalami from several anurans (*Rana, Xenopus* and *Bufo* spp.) have been shown to possess prolactin (PRL), GH and LH-stimulating activity *in vitro*, in common with most other vertebrate species (except that mammalian and some teleost hypothalamic extracts inhibit PRL release). The hypophysiotrophic peptides first isolated from mammalian hypothalami, namely thyrotrophin releasing hormone (TRH), luteinizing hormone releasing hormone (LHRH) and somatostatin (SRIH), are biologically active in Amphibia, though sometimes with surprising effects. Using immunological techniques, HPLC, etc., peptides identical or very similar to these have been found in amphibian brain. Other peptides isolated from mammalian brian or intestinal tract have also been found in Amphibia. In addition, amphibian species are an extremely rich source of neuroactive peptides that have subsequently been demonstrated in the mammalian CNS. Unfortunately, although the activities of these peptides have been extensively studied in mammals, little is known of their functions in frogs.

The prolactin-stimulating actions of TRH were first demonstrated, using relatively crude techniques, in 1975, and confirmed by RIA a number of times. The prolactin-releasing activity of hypothalamic extract is not due solely to its content of TRH, but also to the presence of other factors, separated by chromatography and HPLC, but otherwise not identified. Vasoactive intestinal peptide and the related peptide histidine isoleucine also stimulate prolactin release in bullfrogs, as they do in mammals and birds. Whether other prolactin-releasing agents are present has not yet been determined. Prolactin release-inhibiting agents also exist. Certainly dopamine, as in other vertebrate groups, inhibits secretion of prolactin. An intriguing possibility that GAP, a peptide formed on processing pro-LHRH and that is prolactin-inhibiting in mammals, is a vertebrate prolactin release-inhibiting hormone, has not yet been tested.

GH release is under the control of both releasing and inhibiting peptides. TRH has been shown to release GH *in vitro*, though no physiological role can yet be ascribed. The GH-releasing hormone has not been isolated, though it is likely to be similar to the releasing peptide found in mammalian hypothalami. The inhibitory peptide is SRIH or another similar peptide. In mammals, SRIH exists in two forms, including a larger (28 amino acids) that is not merely a precursor of SRIH-14, but a peptide with its own physiological actions and control. In the vertebrates, a number of SRIH peptides exist, but their roles are not fully understood, and it is not clear whether they are separate entities or artifacts of preparation.

In addition to its effects on prolactin, TRH can stimulate TSH release, as measured by increased thyroid activity, in ranid and at least one urodele (*Ambystoma*) species. TRH also stimulates MSH release from frog neurointermediate lobe, a response antagonized by meuropeptide Y (NPY). Clearly, TRH has an important multifunctional role in the amphibian hypothalamus in conjunction with other influences. LHRH also activates the thyroid axis in ranids and *Ambystoma*, indicating a functional correlation between the gonadal and thyroidal axes.

Serotonin, a neurotransmitter implicated in the stimulatory control of prolactin secretion in mammals and birds, apparently has the same role in the bullfrog. Co-incubations of pituitaries and hypothalami in the presence of serotonin produces a stimulation of prolactin secretion. Moreover, during metamorphosis, when prolactin levels rise, there is a parallel increase in brain serotonin turnover. In addition, monoamine oxidase type A (MAO-A), the enzyme responsible for serotonin inactivation, also increases during this period. Larval Amphibia only have MAO-A, the B from appearing during the transition to a terrestrial lifestyle, when other monoamine transmitters may become more important in brain function. Serotonin may also stimulate GH secretion. The mechanism through which serotonin acts on the pituitary is not known, though it may involve stimulation of release of TRH, and possibly other releasing peptides.

One unsolved problem is the neuroendocrine regulation of metamorphosis and the adaptations of this system on shifting to a terrestrial habitat. Clearly this adaptation involves changes in expression of key enzymes, thereby altering the levels of neurotransmitters which regulate the secretion of the regulatory peptides. This model may hold the key to understanding the physiological adaptations during hatch in birds and during parturition in mammals, both of which involve a relatively rapid shift from an aquatic environment.

THE PHYLOGENY OF THE INFLUENCE OF NONAPEPTIDES OF THE NEUROPHYPOPHYSIS ON THE INTERRENAL GLAND, W. Hanke and W. Kloas, Zoologisches Institut, Universität, D7500 Karlsruhe (F.R.G.).

Nonapeptides of the neurohypophysis are known as hormones with vasoconstrictor and antidiuretic activity in mammals. It has also been shown that vasopressin (AVP) stimulates the turnover of phosphatidylinositol. In connection with this AVP increases aldosterone synthesis in mammals. The extent of this response was found by some authors to be lower than the response on angiotensin II, the most potent stimulator of aldosterone production in mammals. Other authors reported a similar response on AVP than on angiotensin II. The most of the recent papers suggest that the physiological effect of nonapeptides on interrenal secretion is due to a paracrine action of these hormones because they are distributed in cells of the adrenal gland.

Experiments with interrenal *in vitro* preparations of Amphibia (different species) showed that the tissue responds quite strongly to arginine vasotocin (AVT). This was already reported

much earlier than the effect was found in mammalian preparations (1). The response was stronger and obtained with lower doses than that of angiotensin II. This observation is now further characterized (2) and confirmed by others (3).

This effect was not seen in teleost fish. Investigations with interrenal preparations of tilapia did not respond to different and quite high doses of AVT, isotocin or angiotensin II and its analogues. Interrenals of carps did not react on AVT but showed some changes of the secretion rate after high doses of angiotensin II (0.5 µM given fot 60 min). This is quite high compared with Amphibia where 10 nM (given for 5 min) are already effective. Mammalian glomerulosa cells respond already to 0.1 till 1 nM. The difference between tilapia and carp might be due to the adaptational type. The tissue of the stenohaline carp seems to be more sensitive than that of the euryhaline tilapia.

Among the Amphibia, there are different types of adaptation for the environment. Tissue from neotene axolotl responds quite clearly to AVT. 10 nM given for 5 min are already effective. The response to angiotensin II was not clear. 50 or 100 nM were necessary to induce a moderate answer.

The evolution of the regulatory capacity of nonapeptides compared to angiotensin II can be summarized. Teleost fish do mostly not respond to both types of stimulators, except the response of the interrenals of the stenohaline carp to high doses of angiotensin II. Urodelean Amphibia, like axolotl, show a good response to AVT, but angiotensin II has only a moderate action. Frogs and clawed toads react very well to AVT or angiotensin II but the latter is needed in higher doses. Mammalian glomerulosa cells are generally stimulated by lower doses of angiotensin II than AVT.

An extended analysis of the response of an *in vitro* preparation of the interrenals of the clawed toad to AVT has shown that 0.1 nM AVT given for 5 min caused already a clear stimulation of corticosterone and aldosterone release. This is the same range of concentration of two other powerful stimulators, ACTH and urotensin II. The increase of corticosterone and aldosterone secretion after AVT was also seen *in vivo* when the normal concentration in the serum was increased more than 4 fold. It needed 12 hrs to adjust the elevated levels to normal values. The relative increase of the serum values of aldosterone was much higher than that of corticosterone. The C/A ratio dropped from 10-12 to 5. This suggests that the terminal pathway of aldosterone biosynthesis was stronger activated than the primary steps. There is a clear dose dependent response *in vitro* from 0.1 to 50 nM added for 5 min to the incubation medium. In the case of 50 nM, aldosterone release is about 12 fold and corticosterone release about 2.5 fold of the normal secretion rate.

The judgement of the effectiveness of different nonapeptides resulted in the following rank of power : AVT > mesotocin = oxytocin. A similar stimulation of the terminal pathway was induced by phospholipase C which causes the formation of inositolphosphates and diacylglycerol. This suggests that AVT stronger affects the conversion rate of corticosterone to aldosterone.

A special focus of the study was to find the type of receptors being involved in this AVT response. Two types of AVP receptors are discussed in mammals : The V_1 type which is the vascular (vasopressoric) type and the V_2 type which is the renal (antidiuretic) type. It was clearly shown that a V_1-antagonist did not prevent the AVT reponse and a V_2-agonist was ineffective. This indicates that a different type of receptor must exist in this tissue. This is in accordance with some results in mammals where also other types of receptors are suggested.

Further experiments demonstrated clearly the dependency of the stimulation by AVT on extracellular Ca^{++}. A mediation of the AVT effect by the inositol triphosphate system is most probable.

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AMPLEXUS INDUCES LH SURGE IN MALE TOADS *BUFO JAPONICUS*, S. ISHII and M. ITOH, Department of Biology, School of Education, Nishi-Waseda 1-6-1, Tokuo 169 (Japan).

In our recent work, we found in a wild population of *Bufo japonicus* that a rapid increase in the plasma LH level, which is similar to the LH surge in females of higher vertebrates, takes place in males as well as in females at the height of the breeding activity. Furthermore, we showed that the mean plasma LH level in males in amplexus is higher than that in males not in amplexus. These results in combination with our classic knowledge of the Galimani test strongly suggest that the amplexus induces the LH surge followed by the spermiation in male toads.

In order to test this hypothesis, we conducted the following three series of experiments. In the first series, adult male and female toads that have just started the breeding migration but not yet in amplexus were captured from a wild population, and males were kept in plastic boxes with the same number of females or without females. The plasma LH level was monitored for each male toad at about 6 hour intervals over 48 hours by collecting blood samples from the heart without dissection and by determining LH in plasma by means of the radioimmunoassay developed by ourselves. As soon as males were put together with females in the box, they formed the amplexus that lasted for 48 hours. About ten-fold elevation in the plasma LH level was observed in males kept with females. The elevation lasted for 27 hours between 6 and 33 hours after they were put together. In contrast, no significant change in the plasma LH level was detectable in males kept without females throughout the period of 48 hours.

In the second series, solitary males and females were collected near or in the pond one to two weeks later, and similar experiments were conducted. Again, the LH surge was observed in males kept with females but not in males kept alone. In this case, however, the elevation of the LH level started as soon as males were put together with females and lasted for 12 hours. Thereafter, the level declined gradually over 36 hours to the initial level.

In the last series experiments, we attempted to know whether the amplexus itself or just the presence of females is the stimulus to induce the LH surge. We gave a dummy of the female to each male of the experimental group kept in a box. Males of two control groups were kept in boxes with and without females, respectively. The dummy was a block (10 × 6 × 1.5 cm) of « konyaku », which is Japanese food made from the root of a plant (*Amorphophallus conjak*). It has a milky colour and is as elastic as jelly. Experimental males clasped dummies immediately, and the amplexus with the dummy lasted for 12 hours. During this period, the LH surge was observed in the experimental group (as high as that in the control group with females). No significant increase in the plasma LH was detectable in the control group without females.

We concluded from results of these experiments that in males of *Bufo japonicus* the amplexus, or clasping the female for a certain period, forms the stimulus to induce LH surge which in turn results in the spermiation. In the natural condition, the proportion of male individuals is extremely high during the breeding season in this species. Accordingly, spermiation should take place only in successful males in synchronization with ovulation of the female in amplexus. In order to adapt to this condition, the above mentioned neuroendocrine reflex in association with behavior may be constructed in *Bufo* during the course of its evolution.

PERIPHERAL CONVERSION OF T_4 TO T_3 IN THE FROG. INFLUENCE OF THE PITUITARY ? G. JACOBS and E.R. KÜHN, Laboratory of Comparative Endocrinology, Catholic University of Leuven, Naamsestraat 61, B-3000 Leuven (Belgium).

The occurrence of thyoxine (T_4) 5' deiodinase (5'D) activity in amphibians was first demonstrated in anuran tadpoles by Leloup and coworkers. The T_4 to triiodothyronine (T_3) converting system which operates to a small extent at late premetamorphic and early prometamorphic stages in *Xenopus*, reaches maximal activity at midclimax (1). This peripheral conversion of T_4 to T_3 seems to play an essential role in thyroid hormone stimulation of metamorphosis, thus indicating that T_3 is the major metamorphic hormone in anurans (1). The existence of T_4 5'D activity has also been demonstrated *in vivo* in some adult anurans and urodeles (1-3).

Information about the location of 5'D activity in developing and adult frogs has been provided by *in vitro* studies. The enzyme system was detected in gut and skin from metamorphosing and adult *Rana catesbeiana* frogs as well as in the regressing tadpole tail, but could not be localized in other organs, such as liver, kidney, and heart from both larval and adult animals (4). Experiments performed in our laboratory also revealed the presence of T_4 to T_3 conversion in skin homogenates prepared from *Rana ridibunda* frogs (5).

In the present study we examined the T_3-generating capacity of *R. ridibunda* kidneys under different incubation conditions. Conversion of T_4 to T_3, which was demonstrated in the 6000-g supernatant fraction of kidney homogenates, was influenced by substrate and homogenate concentrations and by temperature and duration if incubation. Heating destroyed the T_4 5'D activity. Addition of dithiothreitol (DTT), as exogenous thiolcofactor, to the reaction mixture appeared to be essential to quantitate T_3 production, which even increased markedly in the copresence of EDTA. The DTT + EDTA-stimulated enzyme could partly be inhibited by propylthiouracil (PTU) (25-45 %). When comparing the PTU- and DTT-concentrations, used in this study, with data from the literature, we should have obtained almost complete inhibition if only a type I-enzyme had been responsible for the T_4 5'D activity. Therefore it is suggested that in the frog kidney at least a less PTU-sensitive deiodinase is present. T_4 5'D activity in frog liver was mostly low or undetectable.

Growth hormone (GH) stimulates the peripheral convension of T_4 to T_3 on several vertebrates, such as the eel (6), the chick embryo, adult chicken, rat and dwarf goat (7).

In the present study possible effects of ovine GH (oGH) and bullfrog GH (bGH) (gift from Prof. Dr. S. Kikuyama (Japan)) were investigated in the frog, *R. ridibunda*. An intravenous injection of respectively 1 and 10 μg oGH given on two consecutive days (in October) did not alter plasma T_3 or T_4 concentrations significantly, nor could a change be noted in the renal T_4 5'D activity 5 hours after the second injection. In a second experiment (October) frogs were injected subcutaneously with saline, 1, or 10 μg oGH during 6 days. In the animals which had received the high GH dose, the plasma T_3 and T_4 levels were slightly

increased ($P<0.05$) 5 hours after the last injection. However the T_4 5'D activity in the kidneys remained unchanged. Subsequently, 1.5 µg bGH was administered intravenously on two consecutive days (June), but neither the plasma T_3 and T_4 concentrations, nor T_4 5'D activity were influenced.

It is too soon to conclude that GH would not be involved in the regulation of peripheral deiodination activity in the frog. On the other hand, it seems reasonable to assume that the T_4 5' deiodination system already functions maximally in the adult frog and cannot be stimulated supplementary by exogenous GH. In that case, hypophysectomy experiments combined with GH and/or T_4 injections could give an indication. In order to support this hypothesis, pars distalis- and sham-ectomized frogs were injected intravenously with 10 µg oGH (on 2 consecutive days) starting 4 days after the operation (June). At that time, basal T_4 levels were 2.5 fold decreased, while basal T_3 levels were not different from those in the sham frogs. Unexpectedly, a sharp elevation of plasma T_4 was observed in both groups of animals 2 and 4 hours after oGH-injection ($P<0.0001$). The plasma T_3 level was increased only in the sham frogs. Since the oGH we used did not contain HPLC-detectable TSH-impurities, oGH seems to exert a thyrotropic action in the frog. Therefore, the use of homologous GH appears to be more appropriate for these studies.

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MULTIDISCIPLINARY ANALYSIS OF NEUROENDOCRINE INTEGRATION IN THE PARS INTERMEDIA OF THE AMPHIBIAN, *XENOPUS LAEVIS*, B.G. Jenks, H.P. de Koning, E.P.T.C. de Rijk, T.A.Y. Ayoubi, P.M.T. Deen, I.D. van Zoest, G.J.M. Martens, H.J. Leenders and E.W. Roubos, Department Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen (The Netherlands).

Melanotroph cells of the amphibian pars intermedia secrete α-melanophore stimulating hormone (αMSH), a peptide that regulates pigment dispersion in dermal melanophores during environmental (background) adaptations. Secretion from these cells is regulated by multiple factors, including neurotransmitters (e.g. dopamine, GABA) and neuropeptides (e.g. TRH, CRH, NPY). In our laboratory the mechanisms of neuroendocrine integration in the pars intermedia of the toad, *Xenopus laevis* are being investigated, using morphological, biochemical and molecular biological techniques. Given below is a summary of recent findings :

1) *Melanotroph Cell Recruitment* : Analysis of the activity of intermediate lobe melanotrophs from animals adapted to different backgrounds (white, black and shades of grey) reveals that the melanotrophs function as a heterogeneous cell population in meeting

the increasing demand for αMSH ; there is recruitment of progressively more melanotrophs from the inactive to the active state with increasing blackness of background. The proportion of active cells correlates positively with both the level of circulating αMSH and the degree of pigment dispersion in the melanophores of the animals. We are currently examining the underlying mechanisms for progressive recruitment ; initial studies indicate that there are differences among the melanotrophs in their threshold to respond to secretagogues.

2) *Intracellular Mechanisms of Integration* : Most secretagogues act directly on the melanotroph cell to regulate MSH release. The mechanisms of transmembrane signaling by the secretagogues are now under investigation. Two regulatory mechanisms are negatively coupled to the adenylate cyclase system, namely those activated by dopamine D_2 receptors and $GABA_b$ receptors. Such activation leads to an inhibition of αMSH secretion. CRH is positively coupled to adenylate cyclase and it stimulates secretion. TRH also stimulates secretion, an action which is associated with production of inositol phosphates in the melanotrophs. At least one ionotropic mechanism is present, namely that functioning through the $GABA_a$ receptor. Activation of this receptor leads to an influx of chloride and, consequently, an inhibition of αMSH secretion.

3) *Cellular Mechanisms of Integration* : Recent findings indicate the existence of an indirect mechanism in the regulation of MSH secretion, namely that exerted by neuropeptide Y (NPY). The evidence for this comes from *in vitro* superfusion experiments ; NPY inhibits αMSH secretion from the intact pars intermedia but has no effect on isolated melanotroph cells. There are two possible mechanisms for an indirect action of a secretagogue in the pars intermedia : it could function either presynaptically (*i.e.* stimulate release of inhibitory neurotransmitters from nerve terminals within the tissue) or act via a non-endocrine cell-type such as folliculostellate cells, which make intimate contact with melanotroph cells. In fact, the latter appears to be the case for NPY because : i) NPY inhibits αMSH secretion in tissue in which functional nerve terminals have been eliminated, ii) NPY containing neurons have been found to make synaptic contact with stellate cells and, iii) specific binding sites for NPY have been localized on stellate cells. Possibily, NPY induces release of an αMSH-release-inhibiting factor from the stellate cell.

4) *Morphological Basis for Regulation* : Within the neurointermediate lobe fibers are present that are immunopositive for each of the established MSH-secretagogues. In some cases the fibers are restricted to the neural lobe (*e.g.* those containing CRH and TRH), indicating a diffuse neurohormonal action on the melanotrophs. In other cases, the immunopositive fibers are found throughout the pars intermedia (*e.g.* GABA and NPY). At the ultrastructural level, the pars intermedia possesses a rich network of fiber varicosities, some of which make synaptic contacts with melanotrophs and folliculo-stellate cells. NPY and GABA have been found to be colocalized within these varicosities, NPY occuring primarily in dense core vesicles and GABA in electron-lucent vesicles.

5) *Different Secretory Pathways* : Evidence has been obtained for the functioning of different secretory pathways within melanotroph cells. One pathway involves newly synthesized peptides (secreted within 6h of their biosynthesis) and the other involves secretion of mature secretory material. The newly synthesized pathway secretes primarily the acetylated form of αMSH while desacetyl-αMSH is the main form secreted from the mature secretory pathway. The peptide content of these secretory pathways with respect to other proopiomelanocortin (POMC) derived peptides is now being studied, as is the possibility that the pathways could be independently regulated ; the latter situation would greatly enhance the potential of melanotrophs to generate diverse biological signals.

6) *Regulation of POMC Gene Expression* : The level of POMC mRNA is high in the pars intermedia of animals adapted to black background and low in white-adapted animals. These

differences are reflected in the levels of POMC biosynthesis found in the pars intermedia. In transferring black adapted animals to a white background the levels of both POMC mRNA and POMC biosynthesis fall only very gradually, taking several days to reach the level of white-adapted animals. These results could indicate either a slow inhibition of POMC gene transcription or a very stable POMC mRNA. That the latter might well be of importance is indicated by a kinetic analysis showing that the half-life of POMC mRNA is over 30 h. The effects of various secretagogues on POMC gene transcription are currently under investigation.

7) *Coordinate Gene Expression* : Differential hybridization techniques are being used to find genes that are co-expressed with the POMC gene during physiological adaptations. Such genes could code for proteins essential for the secretory function of the melanotroph cell, such as enzymes for the processing of POMC or proteins necessary for the proper functioning of the secretory machinery. One co-expressed product, namely the 7B2 protein, has been characterized. This 25 kDa protein is highly conserved during vertebrate evolution, is associated with secretory granules within the melanotroph and the 18 kDa processed product of the protein is co-secreted with POMC-derived peptides. At present, the possible role of 7B2 in the secretory process is being investigated.

EFFECTS OF CORTISOL ON CUTANEOUS WATER PERMEABILITY IN TOADS, C. B. JØRGENSEN, Zoophysiological Laboratory A, August Krogh Institute, 13 Universetetsparken, DK-2100 Copenhagen 0 (Denmark).

The increase in cutaneous water permeability in frogs and toads in response to dehydration is widely assumed to depend upon secretion of arginine vasotocin from the pars nervosa of the hypophysis. However, elimination of pars nervosa function only slightly affected the response to dehydration in the toad *Bufo bufo* (1). Moreover, effects may result from interference with pars distalis functions, particulary corticotropic function that arise as side effects of the operations (2). Therefore effects were studied of treatment with a corticosteroid, cortisol, on the cutaneous water permeability in toads, as well as its response to dehydration.

Cortisol pellets implanted under the skin of toads kept in water substantially enhanced the water influx, which approached a plateau 2-3 times higher than the initial within a week or more. Also, the cortisol implants affected the time constants of normalization of the cutaneous water permeability in rehydrating dehydrated toads. Thus, the mean halftime of decline in water influx increased from 100 ± 31 (S.D.) min (n = 15) to 190 ± 64 min (n = 9). The difference was highly significant, $P < 0.001$. By contrast, there was no demonstrable effect of the cortisol implants on the initial water influx response to dehydration.

The effects of implanting cortisol pellets in water-acclimated toads was similar to the effect of acclimation of the toads to a simulated terrestric environment, a terrarium with free access to water. The level of interrenal activity may therefore be one factor in the regulation of the cutaneous water permeability, by controling opening and/or number of epidermal water pores (3).

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CARBOHYDRATES IN AMPHIBIAN CUTANEOUS GLANDS : A LECTIN HISTOCHEMICAL STUDY , J.C. KALTENBACH, K.E. NYTCH and C.H. POTTER, Department of Biological Sciences, Mount Holyoke College, South Hadley, MA 01075 (U.S.A.).

In recent years considerable interest has focused on the chemical composition of exocrine glands in frog skin. Immunohistochemical staining has revealed the presence of a number of peptides in granular, serous glands, but not in mucous glands. For example, we have demonstrated such a distribution for the immunoreactive tripeptide, TRH. In addition, we have shown opposite glandular sites for polysaccharides (PAS technique), and acid mucopolysaccharides (alcain blue, toluidine blue), i.e., the absence of such components from serous glands, and their presence in mucous glands (1). The present study was undertaken to further characterize the carbohydrate components of frog cutaneous glands by means of peroxidase-conjugated lectins with affinities for specific sugar residues.

Pieces of dorsal skin from adult frogs (*Rana catesbeiana, Rana pipiens* and *Xenopus laevis*) and from *R. catesbeiana* tadpoles in representative developmental stages were fixed in Bouin's solution in preparation for light microscopy. Two peroxidase-labeled lectins were used : 1) soy bean agglutinin (SBA) from the soy bean *Glycine max*, with main specificity for N-acetyl-D-galactosamine and 2) wheat germ agglutinin (WGA) from the wheat species *Triticum vulgaris*, with specificity for N-acetyl-D-glucosamine and sialic acid. Sections were treated with hydrogen peroxide to block endogenous peroxidase, incubated with peroxidase-conjugated lectin, followed by H_2O_2-diaminobenzidine (H_2O_2-DAB) solution. Controls were performed 1) to assure specificity of the lectin binding (incubation with peroxidase-lectin solution containing N-acetyl-D-galactosamine, inhibitor for SBA, and N-acetyl-D-glucosamine and sialic acid, inhibitors for WGA) and 2) to check endogenous peroxidase activity (incubation of non-treated sections with H_2O_2-DAB).

SBA lectin conjugate stained all serous glands, as well as the outer epidermis. Stain was not visible in mucous glands, basal epidermis, or dermal connective tissue. Reactions were similar in the skin of the three species of frogs studied. Moreover, the same general pattern was apparent in the skin of developing tadpoles (*R. catesbeiana*) as soon as gland development became evident. Both types of controls inhibited the staining reactions. On the other hand, WGA lectin conjugate gave strong staining reactions in both serous and mucous glands, as well as in the epidermis and connective tissue. In the controls, staining was only inhibited by a peroxidase lectin solution containing N-acetyl-glucosamine, but was completely blocked by the addition of both the glucosamine and salic acid. H_2O_2-DAB controls were also negative. Our results suggest that serous, but not mucous, glands contain N-acetyl-galactosamine, while both types of glands contain sialic acid.

SBA and other lectins have been shown to bind to keratinocytes in the epidermis of *Rana perezi* and *Rana ridibunda* (2, 3). Our results confirm those findings. Moreover, to our knowledge the present study represents the first application of lectin staining to frog cutaneous glands. Our observations provide new information on the composition of stored secretions in these glands. Further studies to localize a variety of sugar moieties in the epidermis and cutaneous glands, which release their contents onto the epidermal surface, will serve as a basis for understanding the functions of the amphibian skin. This research was supported in part by a grant from the Research Corporation.

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COMPARISON OF BINDING SITES FOR ATRIAL NATRIURETIC FACTOR (ANF) AND VAL^5-ANGIOTENSIN II (A II) IN DIFFERENT AMPHIBIAN SPECIES,
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The osmomineral regulation of vertebrates is largely under endocrine control. Integration and coordination of the complex osmoregulatory processes are controlled by adrenal steroids and catecholamines, posterior pituitary peptides, the renin-angiotensin-system (RAS) and atrial natriuretic factor (ANF), by influencing the kidney and the central nervous system (CNS). Furthermore, the adrenal secretion is influenced by other hormones. In mammals, it is well known that the physiological effects of ANF and A II in kidney, adrenal and CNS directly oppose one another (1). The occurrence of these peptides in amphibians leads to the question whether a control of osmomineral regulation, similar to that found in mammals, does exist. Therefore we investigated the distribution and the properties of binding sites for ANF and A II in the kidneys, adrenal tissue and the CNS of different amphibian species by quantitative *in vitro* autoradiography to see if the binding sites are colocalized, which may imply an opposite function. The amphibian kidney is strongly connected to the adrenal tissue which is located on the ventromedian side. Earlier studies showed that in *Xenopus laevis* the binding sites of ANF and A II are colocalized only in the glomeruli of the kidney but not in the adrenal tissue (2). To see, if this finding could be used as a general model for Amphibia we studied the aquatic urodele *Ambystoma mexicanum*, the secondary aquatic anuran *Xenopus laevis* and the semiterrestrial anuran *Rana temporaria*.

Quantitative *in vitro* autoradiography : frozen sections were thaw-mounted and incubated either with ^{125}I-rANF (99-126) or ^{125}I-Val^5-A II as described elsewhere (2). Localization and quantification of binding sites were determined by computerized microdensitometry.

Specific ANF-binding sites were found in all three amphibian species but with a different distribution and quantity, respectively. In the CNS of *Xenopus laevis*, the highest density and quantity of ANF-binding sites occur in the *nuclei* (*n.*) *habenulares* and the *pars nervosa* of the pituitary, but also in the thalamic area and the *n. interpeduncularis*. A moderate number of binding sites is determined in the *bulbus olfactorius, pallium, n. accumbens septi, striatum*, lateral forebrain bundle, *tectum* and *n. infundibularis ventralis*. *Rana temporaria* shows a similar pattern, but the density of binding sites is lower than in *Xenopus*. In *Ambystoma mexicanum*, the highest density is found in the *pars nervosa*, but density and quantity of all binding sites are less than in *Rana remporaria*.

In the kidneys of all three species, ANF-binding sites are found in glomeruli and additionally in tubules of *Ambystoma mexicanum*. The diffusely distributed adrenal gland of *Xenopus laevis* shows certainly binding sites in the interrenal tissue and probably in chromaffin cells, too. In *Rana temporaria*, the tissue surrounding the adrenal gland has ANF-binding sites like some of the adrenal cells. In the adrenal tissue of *Ambystoma mexicanum* we could not detect ANF-binding sites.

The localization of specific A II-binding sites was successful only in *Xenopus laevis* and *Rana temporaria*, while in *Ambystoma mexicanum*, we observed a lack of A II-binding sites in all investigated organs.

In the CNS of *Rana temporaria* A II binding sites of high density are located in the *pars nervosa* and the area of the *amygdala*. A lower number and density of binding sites are found in the interventricular organ and the *striatum* as well as in the *tectum*. In *Xenopus laevis*, a similar pattern exists, but with much lower density and quantity. Only in the *pars nervosa* and the area of the *amygdala*, distinct binding sites could be observed and a diffuse area around the interventricular organ.

The glomeruli of both anurans contain A II binding sites, but *Rana temporaria* has a higher binding capacity than *Xenopus laevis*. The adrenal tissue of *Xenopus laevis* lacks A II binding sites, while the adrenal gland of *Rana temporaria* shows a similar pattern to ANF-binding sites.

CONCLUSIONS : Our results clearly show that a general model of osmoregulation in Amphibia does not exist. Three different species show three different patterns of binding sites for ANF and A II. Opposite physiological effects of ANF and A II could exist in the anuran glomeruli by affecting the glomerular filtration rate and in the anuran *pars nervosa* by influencing the secretion of AVT and/or mesotocin. Furthermore, in *Rana temporaria* a physiological antagonism of both peptides might act on the adrenal tissue. The lack of A II-binding sites in the organs of *Ambystoma mexicanum* and their occurence in *Xenopus laevis* and, more pronounced, in *Rana temporaria* implicates that the importance of the renin-angiotensin-system might have been developed during the metamorphosis from aquatic to terrestrial life style, while ANF seems to be a very conservative hormone playing an important role for osmoregulation in all amphibian species.

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VASOTOCIN ACTS AS A LOCAL REGULATOR OF CORTICOSTEROID SECRETION IN AMPHIBIANS, A. Larcher(1), C. Delarue(1), F. Vandesande(2), G. Pelletier(3) and H. Vaudry(1), (1) Laboratory of Molecular Endocrinology, CNRS URA 650, University of Rouen, Mont-Saint-Aignan (France) ; (2) Zoological Institute, Naamsestraat 59, Leuven (Belgique) ; (3) Laboratory of Molecular Endocrinology, Laval University, Québec (Canada).

The neurohypophysial neuropeptides vasopressin (AVP) and oxytocin (OXT) have been identified in various organs other than the brain, such as testis, placenta and ovary. The occurence of AVP- and OXT-like peptides has recently been shown in both adrenal cortex and medulla of various mammalian species. In addition, AVT has been shown to stimulate steroid secretion in the rat and bovine adrenal cortex. In amphibians, the peculiar structure of the adrenal (interrenal) gland which is composed of intermingled chromaffin and steroid-producing cells, favours interactions between the two types of cells. In fact, we have recently shown that in frogs, various neuroendocrine factors produced by chromaffin cells, such as vasoactive intestinal peptide, atrial natriurectic factor, dopamine and serotonin are involved in the regulation of adrenocortical cells.

The aim of the present study was to investigate the presence of arginine-vasotocin (AVT), the amphibian couterpart of mammalian AVP, in the frog adrenal gland and to examine the possible role of this neuropeptide in the control of corticosteroid production. We have applied the indirect immunofluorescence technique to examine the occurrence of AVT in the frog interrenal gland. Labeling of consecutive sections with antisera against AVT, tyrosine

hydroxylase (TH) and phenylethanolamine-N-methyl-transferase (PNMT), revealed that an AVT-like peptide was localized in both adrenaline- and noradrenaline- producing cells. In contrast no labeling of frog adrenal slices was observed using antibodies against mesotocin (MT). At the ultrastructural level, the immunogold technique revealed that the AVT-immunoreactive peptide is sequestered in chromaffin granules with various electron densities. Filtration of frog adrenal tissue extracts on Sep-Pak C-18 cartridges showed that the elution profile of AVT-like peptide was similar to that of synthetic AVT. The role of AVT in the regulation of frog adrenocortical cells was studied *in vitro* using a perifusion system technique. Graded doses of AVT (10^{-10} M to 10^{-7} M) induced a dose-dependent stimulation of both corticosterone and aldosterone production. Half-maximum stimulation was obtained with a concentration of 4×10^{-10} M. All other neurohypophysial peptides were able to elicit corticosteroid production but the potencies of OXT, AVP and MT were approximately 100, 400 and 1500 times lower than that of AVT. AVT was also capable of stimulating steroid secretion from acutely dispersed frog interrenal cells. These data show that AVT acts directly on adrenocortical cells to stimulate corticosteroid release. Iterative doses of AVT (10^{-9} M) given at 120 min intervals induced identical peaks of corticosterone and aldosterone. In contrast, prolonged administration (4 h) of AVT (10^{-9} M) induced a transient increase of corticosteroid secretion followed by gradual decline of steroid output suggesting the existence of a rapid desensitization phenomenon.

The mechanism of action of AVT has also been investigated in our model. The cyclooxygenase inhibitor indomethacin (5×10^{-6} M) which totally blocks angiotensin II (A II) — or acetylcholine (ACh) — induced corticosteroid secretion did not affect the response of the interrenal gland to AVT. Similarly the production of PGE_2, which was stimulated by A II or ACh, was not affected by AVT. These results indicate that the mechanism of action of AVT on corticosteroid secretion, in contrast to that of A II or ACh, is not mediated through prostaglandin synthesis. To investigate the type of receptor involved in the stimulatory action of AVT, interrenal slices were stimulated with synthetic AVT in absence or presence of various antagonists. The effect of AVT (5×10^{-10} M) was blocked by both the antidiuretic antagonist V2 ([d(CH_2)5,D-Phe^2,Ile^4,Ala^9-NH_2] AVP ; 10^{-6} M) and by the oxytocinergic antagonist ([d(Ch_2)5,Tyr(OMe)^2,Orn^8] vasotocin ; 10^{-6} M). In contrast vasopressor antagonists of type 1 such as [Asu^{1,6},Arg^8)-vasopressin] did not affect the response of the interrenal gland to AVT.

Since the anatomical organization of the frog interrenal gland favours cross-talks between corticosteroidogenic cells and chromaffin tissue, our data strongly support the concept that AVT produced by chromaffin cells may act as a local regulator (paracrine factor) of corticosteroid secretion.

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THE ROLE OF PARS DISTALIS/PARS INTERMEDIA AND THE ADRENAL IN MOULTING AND COLOUR OF THE SKIN, STRESS AND OSMOREGULATION IN TOADS, L. Olesen Larsen, Zoophysiological Laboratory A, August Krogh Institute, Universitetsparken 13, DK 2100 Copenhagen (Denmark).

Examples of interrelationships between the pituitary gland, the adrenal, skin moulting and osmoregulation will be given. I intend to provide a framework at the level of the integrated organism, in which details with regard to molecular and cellular aspects can and should be fitted. Moulting of the skin in *Bufo bufo* is especially suitable as a sensible and

easily observed criterion of function of the hypothalamic-pituitary-interrenal axis. Results obtained by experimental manipulation at all levels have been reviewed (1-5). The corticotrophin releasing hormone seems to be related to pars nervosa hormones, and corticosteroids are necessary for moulting ; all tested corticosteroids are able to elicit a moult in toads deprived of pars distalis.

Moulting of the skin, colour of the skin and stress factors. At the first symposium, I reported results obtained in crowded and non-crowded (single) *Bufo bufo* (6). Crowded toads kept on a black background showed a markedly lower melanophore index (MI) (2.0 versus 4.0) indicating increased sympathetic activity. Average interval between moults was the same in the 3 groups, indicating similar levels and patterns of circulating corticosteroids (3-5). However, the two crowded groups included more toads with irregular moulting intervals than the uncrowded group. Similar results were obtained recently with 3 groups of 5 toads each : 1. Nearly undisturbed, 2. Length, weight and MI recorded frequently, 3. As 2, but force-fed with amounts of mealworms equal to those eaten by 1 and 2. Average MI was 4.3, 3.9 and 3.6 respectively. Average interval between moults was the same in the 3 groups. Irregularity of moulting intervals increased with increasing level of disturbance. So crowding and manipulation had similar effects on the 3 parameters. Possible interpretations will be discussed.

Salinity and moulting interval. In 3 groups of *Bufo viridis* kept in tap water, 230 or 400 mOsm NaCl, moulting intervals were 4.3, 5.7 and 6.7 days (Uri KATZ, thesis, Hebrew University of Jerusalem, 1973). Possible interpretations will be discussed.

Pituitary-interrenal axis. In a recent review (7) I collected evidence for the role of interrenal steroids in sexual maturation in lampreys and other vertebrates. Here I also discussed the possibility that pars intermedia of the pituitary gland may stimulate interrenal secretion, by means of a POMC-derivative. In this connection data published by RODRIGUEZ and coworkers (8-10) become of interest. They demonstrated that when the pars distalis of *Bufo arenarum* was extirpated, interrenal cells underwent atrophy, but 60 and 90 days after the operation, they looked normal or even more stimulated, at a time when pars-distalis-like cells appeared in the pars intermedia. In spring similar cells appear spontaneously. In this light our first report on moulting in *B. bufo* becomes of interest. JØRGENSEN and NIELSEN (11), reported that toads with extirpated median eminence did not moult spontaneously, but when lysine-vasopressin was injected, moults were induced (in 12 out of 12 toads). When also the pars distalis was extirpated, lysine-vasopressin had no effect (in 0 out of 18 toads). However, injections of Insipidin (extract of neurointermadiate lobes) induced moults (in 11 out of 12 toads). We postulated that « pars distalis activity » was present as an impurity in Insipidin, but it may also be a POMC-derivative from the pars intermedia. Similar results, indicating corticotropic activity in neurointermediate lobes, were obtained with whale or toad preparations (11).

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SKELETAL GROWTH IN THE TOAD, *BUFO BUFO*, TREATED WITH BOVINE OR HUMAN GROWTH HORMONE, L. OLESEN LARSEN and ANDERSEN, Zoophysiological Laboratory A, August Krogh Institute, Universitetsparken 13, DK Copenhagen (Denmark) and Section for Biochemistry, Agricultural University of Norway, P.O. Box 36, N-1432 Aas-NLH (Norway).

Two unpublished studies showed that growth in length (reflecting skeletal growth) took place in small toads fed with carbohydrate (sucrose or starch) or lipid (pork fat). This indicates that skeletal growth and net protein synthesis can be separated. A separation of growth in body mass and in body length occured in long term studies of feeding and growth patterns (1, 2). It was therefore decided to test whether growth hormone (GH) in toads not fed at all could induce skeletal growth. It was further decided to compare bovine GH (b-GH) with human GH (h-GH), which has been shown to stimulate growth in crocodiles (ANDERSEN *et al.*, to be submitted).

Four groups of toads with body lengths ranging from 49 to 63 mm were subjected to (a) no food and injections of saline, (b) feeding ad libitum on mealworms, (c) no food and injections of 10 μg b-GH 5 days per week, (d) no food and injections of 10 μg h-GH 5 days per week. In group 1 one toad did not grow, the other 4 grew 1-2.5 mm during the first 2 weeks ; then growth stopped. In group 2 all 5 toads grew, and after 5 weeks growth had only stopped in one ; the range of increase in body length was at that time 7-16 mm. Group 3 grew for ca 3 weeks, range 2.5-6 mm ; 4 toads grew more than any starved control. Group 4 grew for ca 2 weeks, range 0.5-3.5 mm ; 3 toads grew more than any starved control.

It can be concluded that despite lack of food, control toads showed skeletal growth, further stimulated by GH, b-GH being slightly more effective than h-GH. Skeletal growth must be based on mobilization of small amounts of endogenous protein for matrix and probably on mobilization of calcium salts present in endolymphatic sacs in the brain and along the spinal cord (3).

In toads fed live food, GH only prolongs the period in which growth occurs, but does not cause supranormal growth rate (*Bufo boreas* (4) ; *Bufo bufo* (5). In these studies it was not possible to decide whether GH primarily acts at the level of anabolic processes and secondarily on the level of food intake (appetite) or vice versa. In the present study GH certainly stimulated anabolic processes in the skeleton.

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NEW INSIGHT INTO THE REGULATION OF FROG ANDRENOCORTICAL FUNCTION, F. LEBOULENGER, M. MORRA, F. HOMO-DELARCHE*, P. NETCHITAÏLO and H. VAUDRY, Lab. Endocrinologie Moléculaire, CNRS URA 650, Université de Rouen, 76134 Mont-St-Aignan (France) ; * Lab. Immunologie Clinique, INSERM U25-CNRM LA 122, Hôpital Necker, 75743 Paris (France).

In amphibians, the morphological organization of the interrenal gland, where paraneurons and steroidogenic cells are tightly intermingled, favours functional interactions between the two tissues. Steroidogenic cells are also under the influence of nerve afferences supplying the interrenal gland. In previous investigations, we have shown by immunohistochemical and biochemical methods that frog interrenal chromaffin cells contain proenkephalin A-derived peptides and vasoactive intestinal peptide (VIP). Using the perifusion technique, we have demonstrated that VIP stimulates corticosteroid production by frog interrenal slices whereas enkephalins exert no effect. More recently, we have shown that chromaffin granules also store serotonin (5-HT) and arginin-vasotocin (AVT) which both stimulate corticosteroid output in a dose-dependent manner. We have previously shown that acetylcholine (ACh) exerts a dose-dependent stimulatory action on corticosteroid production and that atrial natriuretic peptide (ANP), which is present in splanchnic nerve inputs, strongly reduces ACTH- and A II-evoked corticosteroid secretion. Several of these putative signals may interact with each other at the adrenocortical cell level. When infused simultaneously, VIP and 5-HT exert synergistic effects on corticosterone and aldosterone productions whereas concomitant administration of VIP and ACh induces stimulations of corticosterone and aldosterone release which are strictly additive. In contrast, simultaneous infusion of ACh and 5-HT causes a total blockage of the stimulatory effect of 5-HT. ACh exerts its inhibitory action upon 5-HT-induced steroidogenesis through muscarinic receptors since muscarine also blocks the steroidogenic action of 5-HT whereas nicotine does not. Recently, we have investigated a possible role for catecholamines in the regulation of corticosteroid secretion in the frog. Using the perifusion technique we found that exogenous dopamine (DA), for concentrations ranging from 5×10^{-8} to 10^{-3} M, caused a dose-dependent inhibition of corticosterone and aldosterone secretion by frog interrenal slices. Noradrenaline and adrenaline also inhibited corticosteroid release but were respectively 100 and 2000 times less potent than DA in our model. HPLC analysis combined with electrochemical detection revealed that DA is contained in substantial amounts in frog interrenal extracts (24.1 ± 3.7 ng/mg wet tissue) and that it is released by perifused interrenal slices (42 ± 5 pg/min per gland). The inhibitory effect of DA was reproducible and did not induce any desensitization phenomenon. DA also inhibited corticosteroid production by dispersed interrenal cells, indicating that DA exerts its effect directly on adrenocortical cells. Prolonged infusion of DA which induced a sustained inhibition of basal corticosteroid production did not alter the stimulatory effect exerted by ACTH or 5-HT but reduced by 50 % the angiotensin II-evoked stimulation of corticosteroid secretion. DA induced also an important reduction of prostaglandin (PGE_2 and PGI_2) release which preceded inhibition of corticosteroid output by 20 min, indicating that DA might affect arachidonic acid metabolism. Using [^{3}H] myoinositol or [^{3}H] arachidonic acid-prelabeled interrenal slices we verified that DA actually inhibits the formation of inositol phosphates (IP, IP^2 and IP^3) as well as that of diacylglycerol and arachidonic acid. These results indicate that in amphibians DA might directly depress corticosteroidogenesis via receptors coupled to phospholipid turnover by inducing inhibition of inositol phosphate formation. Immunohistochemical studies using antibodies directed against substance P (SP), a member of the tachykinin family, showed that frog interrenal gland is innervated by an abundant network of immunoreactive fibers. These fibers do not derive from splanchnic nerve since bilateral transection of this nerve or total lesion of celiac ganglion resulted within 15 days in

an increase in SP-like immunoreactivity in the fibers. Using the perifusion technique, we found that exogenous SP, for doses ranging from 10^{-8} to 5×10^{-5} M, induced a dose-dependent stimulation of corticosterone and aldosterone productions by frog interrenal slices. Corticosteroid secretion was also enhanced by a series of tachykinin-related peptides although SP was the most potent peptide of the tachykinin family; especially neurokinin A was 10 times less potent that SP in stimulating frog corticosteroid production. Repeated or prolonged infusion of SP induced a rapid and prolonged desensitization phenomenon, characterized by an attenuation of the steroidogenic response of the interrenal slices. Whether SP stimulates directly or not adrenocortical cells remains unknown since this effect was not found using dispersed interrenal cells. SP also enhanced prostaglandin (PGE_2 and PGI_2) release in the perifusate medium and this effect preceded by 10-15 min the increase in corticosteroid output, suggesting that SP may stimulate steroidogenesis by activating arachidonic acid metabolism. Characterization and identification of the tachykinin-related peptide present in the frog interrenal gland is in progress. Taken together our previous and recent results support the concept of a neuroendocrine control of corticosteroidogenesis in amphibians, involving locally produced paracrine and neuronal signals. This regulatory system might be activated during neurogenic stress. Supported by a grant from DRET (n° 87-135).

IDENTIFICATION OF AN UNKNOWN GASTRIN-34 (1-10)-LIKE PEPTIDE IN THE HYPOTHALAMO-HYPOPHYSEAL SYSTEM OF THE FROG, G. MAREELS and F. VANDESANDE, Laboratory for Neuroendocrinology, Zoological Institute, Naamsestraat 59, B-3000 Leuven (Belgium).

An immunocytochemical study with an antiserum raised against the NH_2-terminal sequence (Pyr-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu) of human gastrin-34 on brain slices of the frog *Rana temporaria* showed immunoreactive neurons in the nucleus preopticus pars magnocellularis. This neurons projected through the hypothalamo-hypophyseal tractus immunoreactive fibres to the median eminence and to the pars nervosa of the pituitary.

When such brain slices were stained with an anti-CCK-8 antiserum, recognizing the COOH-terminal of both CCK and gastrin, the immunoreactivity in the pars nervosa could not be found. So this reaction is due to the presence of an — up to now — unknown peptide related to gastrin-34 (1-10).

To identify this unknown peptide we developed large amounts of specific monoclonal antibodies in ascites tumor. After purification of the ascites fluid by Protein-A Sepharose affinity chromagraphy, the antibodies can be coupled on a CNBr-activated Sepharose-4B gel.

The peptide isolation starts by homogenization, extraction and centrifugation of a hundred frog pituitaries and this supernatant can be loaded on the immunoaffinity column. Reversed phase HPLC will be used for further purification of the eluent. The last and most exciting step will be the determination of the amino acid sequence using gasphase sequenator.

URODELE PROLACTIN : PURIFICATION AND RADIOIMMUNOASSAY, K. MATSUDA (2), K. YAMAMOTO (1), S. TANAKA (2) and S. KIKUYAMA (1), (1) Department of Biology, School of Education, Waseda University, Tokyo 169 (Japan); (2) Institute of Endocrinology, Gunma University, Maebashi 371 (Japan).

A urodele prolactin (PRL) was purified from the pituitary glands of the newt (*Cynops pyrrhogaster*) by subjecting the acid acetone extract to anion-exchange high-performance liquid chromatography (HPLC) (Mono-Q), gel filtration HPLC (Superose-12), and reverse-phase HPLC (TSK-gel ODS). The newt PRL had a molecular weight of 23 KD as determined by SDS-PAGE. The isoelectric point of the newt PRL was 4.7. Its amino acid composition is in good agreement with the results of amino acid analysis of PRLs of anurans and mammals. The amino acid analysis also revealed that newt PRL possesses six half-cystines as in anurans and mammals, whereas teleost PRLs have four.

Antiserum against newt PRL was raised in a rabbit. Employing the antiserum and newt PRL, a specific and sensitive homologous radioimmunoassay for newt PRL was developed, several dilutions of plasma and pituitary homogenate of newts yielded dose-response curves that paralleled the standard curve. Plasma from hypophysectomized newts showed the least amount of crossreaction. Pituitary homogenates of other species of urodeles gave inhibition curves that parallel the standard curve, whereas purified PRLs of anurans gave inhibition curves that did not parallel the standard. Mammalian PRLs showed no inhibition of binding. The radioimmunoassay was applied to the determination of plasma PRL levels in newt captured in every month of the year. In the adult newts of both sexes, plasma PRL levels were relatively low after the breeding season (early spring) and during summer and early autumn. In the male, the levels rose markedly in March, while in the female, the levels became high in February and November.

PRL secreted prior to and during the breeding season may contribute to the development of the cloacal glands, tail fin, and Mauthner's neuron in the male and of the oviduct in the female, as suggested by the results of earlier experiments in which mammalian PRLs were exclusively used.

SEXUAL DIFFERENCES AS ADAPTATION TO THE DIFFERENT GENDER ROLES IN *XENOPUS LAEVIS*, S. MERKLE, GSF-Institut für Säugetiergenetik, Ingolstädter Landstr. 1, 8042 Neuherberg (F.R.G.).

Sexual dimorphism is a well-described phenomenon in amphibians. One of the most striking, obvious differences is the body size of males and females. An extensive study showed that in 90 % of all anuran species studied, females are larger than males (1). Moreover, sex-related differences were found in physiological parameters such as plasma concentration of calcium and lipids and were associated with differences in gametogenesis (2). However, the higher body weight loss and higher nitrogen excretion rates observed in females compared to males as seen in starving *Xenopus laevis* have not yet been explained (3). In order to test the hypothesis that this feature is a consequence of a higher turnover rate due to a higher motor activity and/or basal metabolic rate of females, fed adult animals of both sexes were investigated for oxygen consumption, motor activity, food consumption and several other physiological parameters in plasma and tissues. They were acclimated to 20° C with a light/dark cycle of 12/12.

As expected, female animals exhibited an approximately 35 % higher total oxygen consumption and motor activity increased by similar magnitude. Consequently, the difference

between the sexes in total metabolic rate observed in *Xenopus* seem to be mostly due to different motor activities. However, the difference in metabolic rate persisted even when the oxygen consumption at the same level of activity was compared. The calculated difference at an activity level of zero, for instance, was approximately 30 %, indicating that in addition, females have a higher basal metabolic rate. Furthermore, females ingested about 30 % more than males. The higher food intake and motor activity in females compared to males seem to be associated with a higher body growth and a higher substrate requirement for gametogenesis in this sex. As a consequence, in the natural environment *Xenopus* females have to be more active predators than males. This more extensive foraging behaviour would provide a good explanation for the higher motor activity of females which on one hand represents a caloric expenditure adding to the food requirement for growth and gamete production, and on the other hand increases the total metabolic rate compared with male animals.

Despite the considerably higher food and dietary protein intake of female *Xenopus*, the wasted nitrogen was not significantly increased in females compared to males. The time course of excretion of urea and ammonia during an observation period of 1 week was similar in both sexes except for the excretion of urea during the 7th day after feeding, which in males considerably exceeded urea excretion of females by about 100 %. Thus, the results suggest a relatively lower deamination rate and a higher storage rate of ingested proteins in females compared with males as well as sex-dependent differences in the course of digestion or/and the course of protein catabolism. Similarily, the sex-related differences observed in the activity of hepatic enzymes glucose-6-phosphate dehydrogenase, fructose-1,6-diphosphatase and phosphoenolpyruvate carboxykinase might be interpreted. The decreased activity levels of these enzymes in liver of females possibly reflect a relatively lower rate of lipogenesis and gluconeogenesis in this sex. The higher fat body-somatic index and glycogen concentration in liver of males, however, rather seem to reflect the absence of an additional storage organ analogous to the ovaries.

Finally, the differences found in the concentration of lipids and calcium in plasma confirm previous findings. In contrast, the higher aldosterone concentration in plasma of females is not well understood but may also be related to vitellogenesis since it is thought that corticoids act together with oestrogens in regulating the synthesis and secretion of vitellogenin in the liver of *Xenopus* (4).

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SEX DETERMINATION IN OVIPARE ANIMAL SPECIES, P. Querinjean, C. Blum, C. Gilles-Godfrin and Ph. Herman, a.s.b.l. BIOCLUB ®, avenue des Combattants, 24 B-1340 Ottignies-LLN (Belgique).

Vitellogenin (Vg) is known as a female characteristic protein, the synthesis of which is governed by œstrogen regulation. In oviparous animal species from helminth up to reptiles, this protein is detectable in some conditions in the plasma by electrophoretic and/or immunological techniques.

Massive production of *Xenopus* Vg was obtained by 4 to 6 injections of 17-β-œstradiol of male animals. After treatment, the dorsal lymph sac was filled with several ml of a greenish

liquid containing more than 5 g/100 ml of proteins, of which up to 95 % was Vg. The fluid could be easily recovered with a syringe.

Antigen (Vg) was prepared by conventional EDTA-$MgCl_2$ precipitation or by immunoaffinity chromatography. Specific rabbit polyclonal and LOU rat monoclonal antibodies were prepared. Detection of *Xenopus* Vg was performed by agarose electrophoresis, ELISA or immunofixation. Plasma samples from treated and untreated male animals were chosen as positive and negative controls.

The different techniques were used to sex some fish species, i.e. *Oncorhynchus mykiss* (rainbow trout) and *Sarotherodon nilauticus* (tilapia). Altough the presence of Vg could be identified by simple agarose electrophoresis, no cross reactivity of the anti-*Xenopus* Vg could be obtained. But Vg identification was obtained for most trout specimens as early as 12 months old, thus well before sexual maturation and possible anatomic sexing.

Fish sexing is thus feasible on young animals and appropriate antisera may help to detect Vg in several physiological fluids without killing the animal.

IMMUNOHISTOCHEMICAL LOCALIZATION OF NEUROPEPTIDES IN THE CHROMAFFIN TISSUE OF ANURANS, M. REINECKE[1], H. SEGNER[2] and W. KLOAS[2], [1] Department of Anatomy, University of Zürich, Zürich (CH); [2] Department of Zoology II, University of Karlsruhe, Kaiserstr. 12, 7500 Karlsruhe (FRG).

The chromaffin cells of the adrenal glands of all vertebrates do not only produce catecholamines. Additionally, they contain other neurotransmitters and regulatory peptides. A participation of these peptides in the regulation of steroid secretion by the adrenocortical cells in lower vertebrates, which possess intermingled chromaffin and interrenal tissue, is discussed (1).

The aim of the present study is to extend our knowledge of the occurrence of regulatory peptides in chromaffin cells of amphibia by a comparative investigation of three anuran species : *Rana esculenta, Caldula pulchra* and *Bufo marinus*. Adrenal tissue from individuals of these species has been fixed in Bouin's solution without acetic acid, and embedded in paraffin. Serial sections have been stained for peptides using the biotin avidin technique (2). Single sections have been stained by double immunofluorescence for coexistence of peptides. To identify chromaffin cells, antisera against the marker enzymes dopamine-beta-hydroxylase (DBH) and tyrosin-hydroxylase (TH) have been employed.

Pronounced interspecific differences with respect to the presence of neuropeptides in chromaffin cells were found. The atrial natriuretic factor (ANF) was present in cells of the adrenal organ of all three species. ANF-immunoreactivity (IR) was obtained only using antisera against the 28 AA carboxy-terminal sequence of mammalian ANF, which represents the circulating form of ANF. The finding that antisera against this part of the ANF molecule show specific cross-reactivity with adrenal cells as they did also with atriocytes of the anurans investigated suggests that this sequence has been highly conserved during evolution. For brain natriuretic peptide (BNP), positive immunoreactivity was obtained only in the adrenal organ of *Caldula pulchra*. BNP-IR was located in cells other than those showing ANF-IR.

Recently, LIHRMANN *et al.* (3), provided evidence of ANF-positive nerve fibers in the adrenal tissue of *Rana ridibunda*. Moreover, these authors showed that ANF decreased the ACTH-stimulated secretion of corticosterone and aldosterone by interrenals. KLOAS *et al.* (4) demonstrated the presence of specific binding sites for ANF in the adrenal organ of *Xenopus laevis*. In addition, *in vitro* ANF inhibited the basal secretion of aldosterone by the interrenal

cells of *Xenopus laevis* as well as the ACTH-stimulated secretion of aldosterone and corticosterone. In mammals, specific ANF receptors in adrenals and ANF effects on corticosterone secretion are reported, too (5).

Comparing the localization of ANF-IR and DBH-IR, three populations of cells could be distinguished :

a) cells being positive for DBH only
b) cells being positive for ANF only
c) cells being positive both for ANF and DBH.

Congruent results arised comparing the distribution of TH-IR and ANF-IR.

Using double immunofluorescence technique, the coexistence of the following peptides in the chromaffin tissue of the investigated anuran species could be demonstrated :

— ANF/neuropeptide Y (NPY)
— ANF/Leu-enkephalin (ENK)
— Leu-ENK/Met-ENK-Arg-Phe
— Calcitonin-gene-related peptide (CGRP)/substance P.

Coexistence of CGRP/substance P was also found in nerve fibers. In nerve fibers of *Caldula pulchra*, additionally, NPY and ANF showed coexistence.

The results complete earlier data of DELARUE *et al.* (1990) who showed that adrenochromaffin cells of *Rana ridibunda* contain, in addition to catecholamines, neurotransmitters such as serotonin and neuropeptides such as vasoactive intestinal peptide, met-ENK, and met-ENK-Arg-Phe. Further studies must now establish the importance of these peptides for the regulation of the adrenal organ.

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IMMUNE REGULATION DURING ANURAN METAMORPHOSIS, Laurens N. RUBEN, Richard H. CLOTHIER and Michael BALLS, Biology Department, Reed College, Portland, OR. 97202 (USA) and Department of Human Morphology, Queen's Medical Centre, University of Nottingham, NG 7 2UH (UK).

The period of anuran metamorphosis offers investigators a unique vertebrate paradigm. The adult cells that arise within the immunocompetent larval body are immunologically incompatible with those of the larva. Thus, the animal should undergo immunologic self-destruction during this transition period. Unresponsiveness to these modified-self cells is required in order to avoid this. At the same time, protection against environmental pathogens must be provided. We have been exploring the regulation of these two distinctly different demands being made on the immune system during this period with *Xenopus laevis*, the South African clawed toad.

The most obvious potential regulator of immunity during metamorphosis is the extremely high corticosteroid serum titer. It has long been known that the glucocorticoids can serve as powerful immune inhibitors by effecting the viability and function of thymus-derived lymphocytes (T cells) which individually serve as effector or regulatory immunocytes. We have shown that glucocorticoid receptors increase in immunocytes during metamorphosis and that all regulatory and effector functions of T cells become impaired. Because injection of the human cytokines, interleukin-1, interleukin-1 (IL-2) or IL-2, was able to restore T cell function, we have suggested that the corticosteroids inhibit IL-1 production by antigen-presenting cells, and consequently, IL-2 production by amplifying T cells. No immunological impact can be achieved by T cells in the absence of clonal expansion and IL−2 is required for T cell clonal expansion.

But, if T-lymphocyte functions are inhibited, how can the animal protect itself from environmental pathogens? Immune responses to all proteinaceous and cellular immunogens require regulatory T cell intervention for amplification and for suppression. We have tested a number of possible mechanisms with regard to how antobody producing (B) cells may provide protection from pathogens in an internal environment of compromised T cell function, *e.g.* 1. is the antibody producing cell population enhanced, either in number or responsivity during metamorphosis, 2. is the amplifying T cell population corticosteroid resistant, 3. is the high thyroxine titer of metamorphosis responsible for increasing antibody producing cell function, and 4. with impaired suppressor function, is T cell enhancement necessary? None of these were supported by our data.

However, during this transition period, the two immune systems, larval and adult, with the capability of reacting to each other, co-exist within the metamorphic animal. A reasonable outcome of this interaction could be the secretion of a cytokine capable of bypassing corticosteroid inhibition of the IL−2 sensitive T-amplifier cells, by affecting antibody producing cells directly. We have recently identified an enhancing factor that is released by metamorphic, but not by adult splenic cellular suspensions, after a period between 12 hrs. to 3 days *in vitro*, in a fetal calf serum-free medium. The factor will amplify *in vitro* immune responses in adult spleen cell suspensions and it appears to enhance B cell function directly.

We also have recent HPLC evidence that the metamorphic spleen is temporarily lacking in norepinephrine (NE), a neurotransmitter with the capacity to regulate immunity in adults of this species. Our current functional studies with adults suggest that NE will stimulate T cell activities, but inhibit B cell functions. Thus, the absence of this reagent represents an ideal solution to the immunological problems faced by the metamorphic anuran which must be unable to respond to modified-self antigens, a T cell function, but fully able to protect against xenogeneic environmental pathogens, largely a B cell function.

We propose that there are at least three ways that immunity is regulated during metamorphosis that will allow for survival during this transition period; a period that not only exposes the larve to new adult differentiation antigens, but will expose the immature adult to a new range of environmental pathogens, as a consequence of evolving from an aquatic to a terrestrial habitat. They are : a high corticosteroid level, a reduced norepinephrine level, and the *in vivo* production of a cytokine.

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PROLACTIN mRNA LEVELS IN THE BULLFROG PITUITARY GLAND, N. TAKAHASHI, D. UCHIDA, K. YOSHIHAMA, K. YAMAMOTO, K. WAKABAYASHI*, Y. KATO* and S. KIKUYAMA, Department of Biology, School of Education, Waseda University, Tokyo 169 (Japan), *Institute of Endocrinology, Gunma University, Maebashi 371 (Japan).

In amphibians, prolactin (PRL) is known to act as an antimetamorphic hormone. Accordingly, it has been hypothesized that PRL levels are high during pre- and pro-metamorphosis and decline at early climax to facilitate metamorphic changes by thyroid hormone. However, PRL levels do not necessarily decline at metamorphic climax, but rise during late climax period when the animals are completing metamorphosis. Moreover it has also been known that the prolactin release is enhanced by a hypothalamic stimulation rather than a release from inhibition by the hypothalamus. Recently, we have obtained a cDNA clone coding the full length of bullfrog PRL. Using the PRL cDNA as a probe, PRL mRNA levels in the pituitary gland of bullfrog tadpoles of various developmental stages were measured. Effect of a hypothalamic factor (fPRF) having a potent PRL-releasing activity on the pituitary mRNA levels was also studied. Cytoplasmic RNA was isolated from the pituitary gland using the guanidium isothiocyanate/cesium chloride density gradient ultracentrifugation method. Dot-blot hybridization of cytoplasmic RNA was carried out according to the method of WHITE and BANCROFT (1982). Hybridization with chicken actin cDNA was used to correct for the variability in the amount of RNA blotted onto each dot. In the prometamorphic tadpoles (stage 18) the pituitary levels of PRL mRNA were relatively low. At advanced climax stages, the PRL mRNA levels were significantly elevated. At stage 23, the mRNA levels reached 300 % of those at stage 18. At stage 24, the levels remained considerably high. At the end of metamorphosis (stage 25), a decline of mRNA levels was observed. We have previously reported that both plasma and pituitary PRL levels are relatively low during pre- and prometamorphosis and rise as metamorphosis progesses, reaching maximum at stage 24. These results indicate that the pituitary PRL cell function is enhanced at advanced climax stages. When the bullfrog pituitary glands were incubated in the presence of fPRF (40 ng/ml), a significant elevation of both PRL levels in the medium and PRL mRNA levels in the pituitary was observed. It was concluded that fPRF stimulates both release and synthesis of PRL.

MECHANISMS OF ACTION OF DOPAMINE ON FROG MELANOTROPH CELLS, M.C. TONON, L. DESRUES, J. VALENTIJN, L. CAZIN and H. VAUDRY, Groupe de Recherche en Endocrinologie Moléculaire, CNRS URA 650, Unité Affiliée à l'INSERM, Université de Rouen, 76134 Mont-Saint-Aignan (France).

The intermediate lobe of the pituitary is a remarkable model of neuroendocrine communication. The pars intermedia is composed of a major population of endocrine cells, called melanotrophs, which synthesize the multifunctional precursor protein proopiomelanocortin (POMC). Specific proteolytic cleavage of POMC gives rise to a number of biologically active peptides such as alpha-melanocyte-stimulating hormone (αMSH) and β-endorphin. Pituitary melanotrophs receive direct innervation by hypothalamic fibers which release neurohormones in the immediate vicinity of the endocrine cells. In amphibians, the intermediate lobe of the pituitary is innervated by different populations of nerve fibers containing either classical neurotransmitters (mainly dopamine and GABA) or various neuropeptides including TRH, NPY, mesotocin and CRF. The spontaneous secretory activity of the pituitary melanotrophs is extremely high and inhibitory factors such as dopamine, GABA or NPY are thought to

play a pivotal role in the physiological control of hormonal secretion. The present study will focus on the mechanism of action of dopamine on frog pituitary melanotrophs. In perifused neurointermediate lobes (NIL), dopamine (10^{-10} to 10^{-6} M) was responsible for a dose-related inhibition of α-MSH secretion. The effect of dopamine was rapid and reversible. Both apomorphine and bromo-2-ergocryptine, two D_2 agonists, mimicked the inhibitory effect of dopamine on melanotropin release. In addition, dopamine antagonists such as haloperidol and pimozide blocked dopamine-induced inhibition of α-MSH release. In contrast, α-flupentixol, a specific D_1 antagonist, was devoid of effect on the response of NIL to dopamine, suggesting that dopamine modulates melanotroph activity through activation of a D_2-receptor subtype. In order to investigate the mechanism of action of dopamine on melanotropin secretion we have examined the effect of the neurotransmitter on the turnover of inositol phospholipids. Apomorphine (10^{-6} M) inhibited the incorporation of ^3H inositol into phospholipids. The inhibitory effect was significant at 3 hours and reached a maximum after 7 hours of incubation. In prelabelled NIL, dopamine decreased the rate of formation of all inositol phosphates, suggesting that dopamine might act through inhibition of the phospholipase C. Using the patch-clamp technique, on frog melanotrophs in primary culture, dopamine (10^{-6} M) was found to inhibit the generation of spontaneous action potentials by hyperpolarizing the cell. The hyperpolarisation was due to the activation of a voltage-dependent potassium outward current. In addition two distinct voltage-dependent calcium currents, namely a nifedipine-sensitive L-current and a nifedipine-insensitive N-Current, as well as a voltage-activated sodium current were reduced by dopamine. The effect of dopamine on spontaneous firing was blocked by the D_2-antagonist sulpiride, and unaffected by the D_1-antagonist SKF-83566.

Taken together, these data demonstrate that the frog pars intermedia, which is composed of a homogenous population of endocrine cells is a well suited model to investigate the mechanisms of action of dopamine. The data show that stimulation of dopamine D_2 receptors in this model causes marked electrophysiological effects and inhibition of polyphosphoinositid turnover. Studies are in progress to determine whether these cellular events are involved in the secretory response of the pituitary melanotrophs to dopamine.

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IMMUNE STATUS DURING NATURAL METAMORPHOSIS IN *RANA TEMPORARIA* DISTRIBUTION OF LEUCOCYTE TYPES IN CIRCULATION AND IN AREAS OF TAIL RESORPTION, A. Ph. Ussing (1 & 2) and P. Rosenkilde (1), 1) Zoophysiological Laboratory A, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen ; 2) Immunological Laboratory, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen.

Amphibian metamorphosis is a developmental period, during which immunity, and its counterpart, tolerance, towards new « self » and « non-self » antigens are established. « New », adult, and « old », larval, tissue antigens are present concomitantly in the individual during metamorphosis, thus composing an animal with « chimeric » composition. Since tolerance can be induced more readily during metamorphic development, the metamorphosis as an immunologically privileged period has been proposed. Does this exclude the possibility of autoimmune reactions, or are adult immunocytes actually involved in destruction of larval tissues ?

In the present work, we studied *Rana temporaria* tadpoles, metamorphosing individuals, and froglets. We studied blood cell pattern with a special emphasis on changes in immunocompetent cells during metamorphosis. Imprints of tail tissue from late metamorphic stages were analysed. Results : during metamorphic climax, blood lymphocyte count is low. With completion of tail resorption, lymphocytes become abundant in circulation. In tail resorption areas, an occasional eosinophilic granulocyte is seen, other granulocytes and lymphocytes are rare. Macrophages are abundant during late metamorphic stages, where they appear to be engaged in phagocytosis of striated muscle fibers. We suggest that the immune system is not directly involved in destruction of larval tissue. The role of macrophages is thought to be scavenging of cells already in the process of deterioration, and not a specific event in an immunological reaction.

STUDY OF THE THYROID FUNCTION IN RELATION TO THE OVARIAN OOCYTE DEVELOPMENT IN FEMALE *DICROGLOSSUS OCCIPITALIS*,
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A thyroidal-gonadal interrelationship has been suggested for most vertebrate classes. In the class of Amphibia, an effect could be demonstrated of a prolonged administration of estradiol-17β (E_2) or testosterone on the thyroidal axis of male and female adult frogs. However, little or no information was available regarding thyroid function in relation to the ovarian oocyte development and changes in sex hormone levels of female frogs under normal physiological conditions. The well known annual cyclicity of the thyroidal and the gonadal axis as well as the strong effect of external factors (e.g. photoperiod-temperature) on both axes in adult frogs, must be considered because their influence on both axes together could lead to a false interpretation of a thyroidal-gonadal interaction. In order to avoid these problems female giant swamp frogs (*Dicroglossus occipitalis*) were collected during a short time period in an equatorial region (Kinsangani, Zaïre) where the climatic factors are nearly constant throughout the year. Based on the developmental stage of the oocytes in the ovary, all the frogs could be subdivided in six different groups (stage I to V : going from the least developed to the full grown oocytes ; stage VI : postovulation). At a given time of the year, all the stages are represented in different frogs. Two experiments were set up in which thyroid and sex hormone concentrations, peripheral monodeiodination of T_4 (thyroxine) into T_3 (triiodothyronine) and some morphological gonadal parameters (GSI : gonadosomatic index = weight of the gonads/body weight in percent ; oviduct weight in percent of the body weight) were studied in giant swamp frogs, subdivided in the six different groups.

Going from stage I to V a progressive increase of the GSI and the oviduct weight was seen, together with a decline of these morphological parameters in stage VI. Considering the plasma E_2 concentration the same pattern is observed in the two experiments : an increase from stage I to V, followed by a drop in stage VI. However, the increase of plasma E_2 occurs faster and preceeds the weight gain of the ovary and the oviduct. This illustrates the stimulatory role of the estrogens on oviduct growth and on the process of vitellogenesis, resulting finally in an important weight gain of the oocytes. The plasma testosterone concentration is low in stage I, II, III and VI, but reaches a high level in stage IV and V. Looking at the E_2 levels in the ovaries, it was noticed that the total E_2 content increases progressively from stage I to V and declines in stage VI. However, when the E_2 concentration per gram ovary was considered, no differences could be detected between the distinct stages.

Quite different results were obtained at the level of the thyroidal axis. In neither of the two experiments was a significant difference noticed between the plasma T_4 concentrations obtained for the distinct stages. In the second experiment nearly equal plasma T_3 values were found in the six different groups. In the same experiment a slightly higher T_3 and T_4 concentration was found in the thyroids of the animals belonging to stage II and IV but this could not be related to the hormonal or morphological changes taking place at the level of the gonadal axis. In the kidney and skin homogenates, obtained from the frogs of the second experiment, the *in vitro* 5'-monodeiodination activity was estimated by measuring the produced T_3 which is derived from conversion of extra added T_4. Neither in kidney or skin homogenates the measured T_3 values were different in the six distinct groups.

Our results indicate that ovarian egg development, together with the hormonal changes at the gonadal level, are not connected with fundamental changes at the level of the thyroidal axis.

IDENTIFICATION AND CHARACTERIZATION OF AN ALDOSTERONE RECEPTOR IN THE TAIL EPIDERMIS OF BULLFROG TADPOLES, K. YAMAMOTO and S. KIKUYAMA, Department of Biology, School of Education, Waseda University, Nishiwaseda, Shinjuku-ku, Tokio 169 (Japan).

Corticoids such as aldosterone and corticosterone have been known to potentiate the action of thyroid hormone on the tadpole tail by augmenting the binding capacity of thyroid hormone (1, 2). Plasma corticoid levels rise during early climax (3-5) and administration of an inhibitor of corticoid synthesis retarded metamorphosis (6). These observations strongly suggest that endogenous corticoids are involved in metamorphosis. Accordingly, it is probable that corticoid receptors exist in the tadpole tail. There is one report concerning binding of glucocorticoid by tadpole tissue (7). Experiments were conducted to demonstrate the presence of aldosterone receptor in the tadpole tail. The temperature-dependent association experiments show that the specific binding of [^{3}H]aldosterone to the tail cytosol is thermolabile. The optimum assay conditions required for reaching equilibrium were defined as being 0° C and 3-hr incubation. Separation of bound and free hormone was performed by using a hydroxylapatite method. Specific binding of aldosterone was observed in the tail epidermis but not in the tail mesenchyme. Sucrose density gradient analysis of crude cytosol revealed a specific peak of radioactivity in 8S area. Saturation analysis revealed that specific binding of [^{3}H]aldosterone to the epidermal cytosol reached maximum between 20 and 40 nM. Scatchard plot analysis for the cytosol of the tail epidermis from tadpoles of stage XVIII yielded a straight line with a dissociation constant (Kd) of 8.1 ± 0.3 nM and the maximum number of binding sites (NBS_{max}) of 54.2 ± 2.5 fmol/mg protein. The dissociation of specifically bound [^{3}H]aldosterone from the tail epidermal cytosol obeyed first-order kinetics. The rate of dissociation of aldosterone from epidermal cytosol displayed a $t_{1/2}$ of 122 ± 2 min. The calculation of a molecular weight and a Stokes radius of aldosterone receptor gave values of 143 KDa and 4.54 nm, respectively. Steroid-binding specificity revealed a significant displacement of the [^{3}H]aldosterone by both radioinert aldosterone and corticosterone and, to a lesser extent, by cortisol, whereas 17β-estradiol and testosterone com-

peted very poorly. The number of binding sites was somewhat reduced as metamorphosis progressed with no appreciable changes in the Kd value.

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ABSTRACT

COMPARATIVE CYTOLOGY
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ULTRASTRUCTURE DES SENSILLES ANTENNAIRES DE *PHORACANTHA SEMIPUNCTATA* (COLEOPTERA CERAMBYCIDAE)

par

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Ce travail décrit l'ultrastructure des trois principaux types de sensilles antennaires de *Phoracantha semipunctata*. Il fait partie d'un programme de lutte intégrée contre ce ravageur de l'eucalyptus. Il vise à donner des indications sur la fonction probable de ces organes sensoriels et à servir de base pour leur étude électrophysiologique.

Les antennes sont très développées : leur surface varie de 20 à 100 mm^2 selon la taille et le sexe de l'insecte. La densité moyenne des sensilles y est d'environ 800/mm^2.

Les sensilles « chaetica fortes » sont les plus abondantes sur l'antenne (env. 600/mm^2). Elles sont couchées le long du tégument qu'elles recouvrent et articulées dans une cavité étroite et profonde de la cuticule. Leur hampe cannelée, d'environ 50 µm de long, est limitée par une paroi épaisse (> 2 µm) et dépourvue de pore. Les chaetica fortes sont innervées par au moins un neurone à la base mais dépourvues de dendrite dans la hampe. Elles sont toujours associées à une structure d'apparence glandulaire qui débouche dans la cavité d'articulation.

Leurs fonctions probables sont tectrice, tactile et peut-être exocrine.

Les sensilles trichodea sont nettement moins denses (10-15/mm^2). Elles sont dressées sur le tégument au-dessus des chaetica et articulées dans une cavité de la cuticule. Leur hampe sigmoïde (de 80-100 µm de long) et creusée de deux sillons longitudinaux est limitée par une paroi épaisse (> 2 µm) et percée d'un pore apical. Elles sont innervées par 5 ou 6 neurones qui se prolongent jusqu'à l'extrémité de la hampe.

Ces caractéristiques sont celles de sensilles gustatives.

Les sensilles basiconica sont abondantes (150-300/mm^2) et principalement concentrées dans de légères dépressions de la cuticule. Elles sont recouvertes par des chaetica fortes « tectrices » et insérées sans articulation sur un dôme de la cuticule. Leur hampe droite ou courbe à surface lisse (rarement cannelée) de 5-25 µm de long est limitée par une paroi mince (env.

0,5 µm) percée de nombreux pores. Un nombre variable de neurones (fréquemment deux) se prolongent dans la hampe.

Ces caractéristiques sont celles de sensilles olfactives.

Travail réalisé avec une bourse de l'OTAN N° 0369/87.

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