Differentiation of *Prionchulus muscorum* (Dujardin, 1845) Wu & Hoeppli, 1929 and *P. punctatus* (Cobb, 1917) Andrássy, 1958 by egg-shell structure

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SUMMARY

The surface structure of eggs from cultures of *P. muscorum* and *P. punctatus* studied by light and scanning electron microscopy, showed an unexpected variation. The outer shell layer is a tertiary membrane produced by the *pars dilatata* cells of the gonoduct. In both species it is composed of a finely granulated base, surmounted by coarser granulated ridges. Only the elaboration of these ridges differs considerably between the two species. The structure of the ridges and their relation to the egg contour proved to be a sound additional criterion to separate the two related species. Smooth eggs can no longer be considered as typical for *P. muscorum* since they may occur in both species under unfavorable conditions in cultures, as well as, under natural conditions.

RÉSUMÉ

Distinction de Prionchulus muscorum *(Dujardin, 1845) Wu & Hoeppli, 1929 et de* P. punctatus *(Cobb, 1917) Andrássy, 1958 par la structure externe de la coque de leurs œufs*

L'aspect de la coque des œufs de deux espèces très proches, *Prionchulus muscorum* et *P. punctatus*, a été étudié au microscope optique ainsi qu'au microscope électronique à balayage à partir de matériel d'élevage. La couche externe de la coque est une membrane tertiaire secrétée par les cellules d'une zone bien déterminée du gonoducte, la *pars dilatata*. Chez les deux espèces, cette membrane se compose d'une couche de base finement granulée et divisée par un système irrégulier de fentes, le tout surmonté par des crêtes à plus forte granulation. Ce n'est que le parachèvement de ces crêtes qui diffère considérablement entre les deux espèces. Chez *P. muscorum* ces crêtes sont arrondies et gardent un aspect granuleux grossier, tandis que chez *P. punctatus* elles se transforment en projections filamenteuses et donnent cet aspect échinuleux si typique de l'espèce. L'élaboration de crêtes très typées chez *P. punctatus* semble être liée au type d'habitat. La structure de ces crêtes associée à la netteté du contour de l'œuf constitue un caractère optionnel complémentaire pour séparer les deux espèces. Les coques lisses ne peuvent par contre plus être retenues comme un critère spécifique de *P. muscorum*, étant donné leur présence chez les deux espèces, aussi bien chez les spécimens récoltés dans la nature que dans des conditions d'élevage, physiquement défavorables.

In the genus *Prionchulus* two species, *P. muscorum* and *P. punctatus*, are very common in temperate climates and mostly occur together. Characters to separate them are size of the buccal cavity, tail shape and structure of the egg-shell. They are nevertheless closely related and Andrássy (1958) even synonymized them. Several authors separated these species only on the basis of surface structure of the egg-shell, e.g. smooth, rugose or punctate, but a detailed description is lacking and the intraspecific variation is not known. The study of numerous *Prionchulus* specimens from France revealed a large variation of the commonly used characters, including the egg-shell (Arpin, Samsoen, Ponge & Khan, 1984). Therefore a detailed study of the egg-shell was necessary to clarify the status of the two species. To assess the intra-, as well as, the interspecific variability of the structure of the outer egg-shell layer, eggs of the two species obtained from cultures were studied with light and scanning electron microscopes.

Material and methods

Cultures were maintained on agar plates with a modified enchytraeid medium (Maertens, 1975) at 20°, with *Panagrellus redivivus* (Linnaeus, 1767) as prey organisms. *Prionchulus punctatus* was obtained from Bürgerspark, i.e. one of the localities from which the species was originally described (Brakenhoff, 1914). Other specimens came from France, from some localities already studied by Arpin (1979) : Sénart, Mont-Morin and Armainvilliers. Because the type locality of *P. muscorum* (Jardin des plantes,

Paris, France) is destroyed, we collected specimens at some of the localities in France that were already studied by Arpin (1979). These localities are Armainvilliers, Bois de la Tour and Mont-Cuvier. We produced several clones by culturing females separately in order to assess the possible variation of a character through the progeny. Cultures were renewed each month to reduce bacterial or yeast infections. At regular intervals, eggs were picked out from the cultures, washed twice in distilled water and fixed with hot 4% formaldehyde. For scanning electron microscopy they were post-fixed with 1% osmium tetroxide in cacodylate buffer (pH 7.4), dehydrated through a graded series of ethanol and a mixture of ethanol-amylacetate; critical-point dried with carbon dioxide as transition fluid and sputter coated with gold.

Results

Formation of the outermost layer of the egg-shell

Females of *Prionchulus* species are didelphic with gonoducts comprising three parts : a narrow oviduct, an enlarged part (= *pars dilatata*) and a chamber-like uterus ; the latter two parts separated by a sphincter. Normally one to four eggs are present in the genital track of mature females. The two species under consideration are parthenogenetic and egg production alternates between the two genital branches. Different numbers of eggs can be found in the gonoducts. In females where egg production has just started, there is usually one egg in one of the uteri while another egg may occur in the *pars dilatata* of the opposite genital branch. In well-fed and fully mature females the rate of ripening of the oocytes is somewhat increased and two to four eggs may be present in both uteri. This feature is apparently not restricted to cultured specimens since a similar situation has been observed in field specimens.

The outer layer of the egg-shell is formed in the *pars dilatata*. Evidence for this was found in a few specimens where a granular material was observed around the chitinous layer of the egg-shell. In other specimens where the ovary contains an almost fully-grown oocyte, the cells of the *pars dilatata* of the same genital branch are swollen and granular indicating an increased activity. Around the chitinous layer, which is secreted by the egg itself, an ornamented outer layer is deposited. The formation of this outer layer can be followed in the *pars dilatata* where almost smooth to ornamented eggs with few to numerous protuberances are observed. Hence these structures represent different stages in development rather than intraspecific variations. Variation in structure of the egg-shell is therefore only studied on eggs in the uterus or already deposited in the culture medium.

The structure of the outer shell layer based on eggs laid by cultured females

A large variation in the structure of the outer shell layer was observed on intra-uterine eggs of a large collection of *Prionchulus* specimens (Arpin, Samsoen, Ponge & Khan, 1984). Two groups were distinguished (see Fig. 3 in Arpin, Samsoen, Ponge & Khan, 1984) : one with spinelike protuberances *(P. punctatus)* and the other with scaled ones *(P. muscorum)*. However, the protuberances vary considerably in size and confusion is easy if not carefully studied. Two kinds of protuberances of the outer shell layer can be more clearly recognised on the laid eggs : echinulate for *P. punctatus* against scaled for *P. muscorum*. Even after two generations no overlap in the structure of these protuberances occurs. *P. punctatus* from Germany was reared for more than two years without producing a single egg resembling those of *P. muscorum*.

Smooth or seemingly smooth eggs, however, occur in both species. Some are without any surface structure, but most are ornamented either only at their extremities, or with very low protuberances scattered over the egg surface giving the impression that they are smooth (Fig. 1 B, 2 F, 4 A). Their number represents only 10-20% in *P. muscorum* and 6-13% in *P. punctatus*. This corresponds with the numbers of smooth eggs found in natural conditions (Arpin, Samsoen, Ponge & Khan, 1984). When egg producing females are transferred to distilled water, they produce two well ornamented eggs during the first 24 h. Later they start producing a higher number of progressively less ornamented eggs till, after two days, twice the normal amount of eggs is laid but all of them are smooth. Hatching capacity of these eggs remains intact. A higher number of smooth or almost smooth eggs is also found in old cultures (Tab. 1) where the culture medium has become more liquid. Even on well-ornamented eggs the protuberances diminish in size when the culture grows older.

The outer shell layer as observed under the light microscope

P. punctatus has punctate or echinulate eggs due to the highly variable shape of the protuberances which are sharply pointed, hooked, thorne-shaped or composed of a more or less cylindrical ridge. In the German population of this species the egg-shells are strongly ornamented with numerous flad cylindrical to paddle-shaped, crestlike protuberances. In

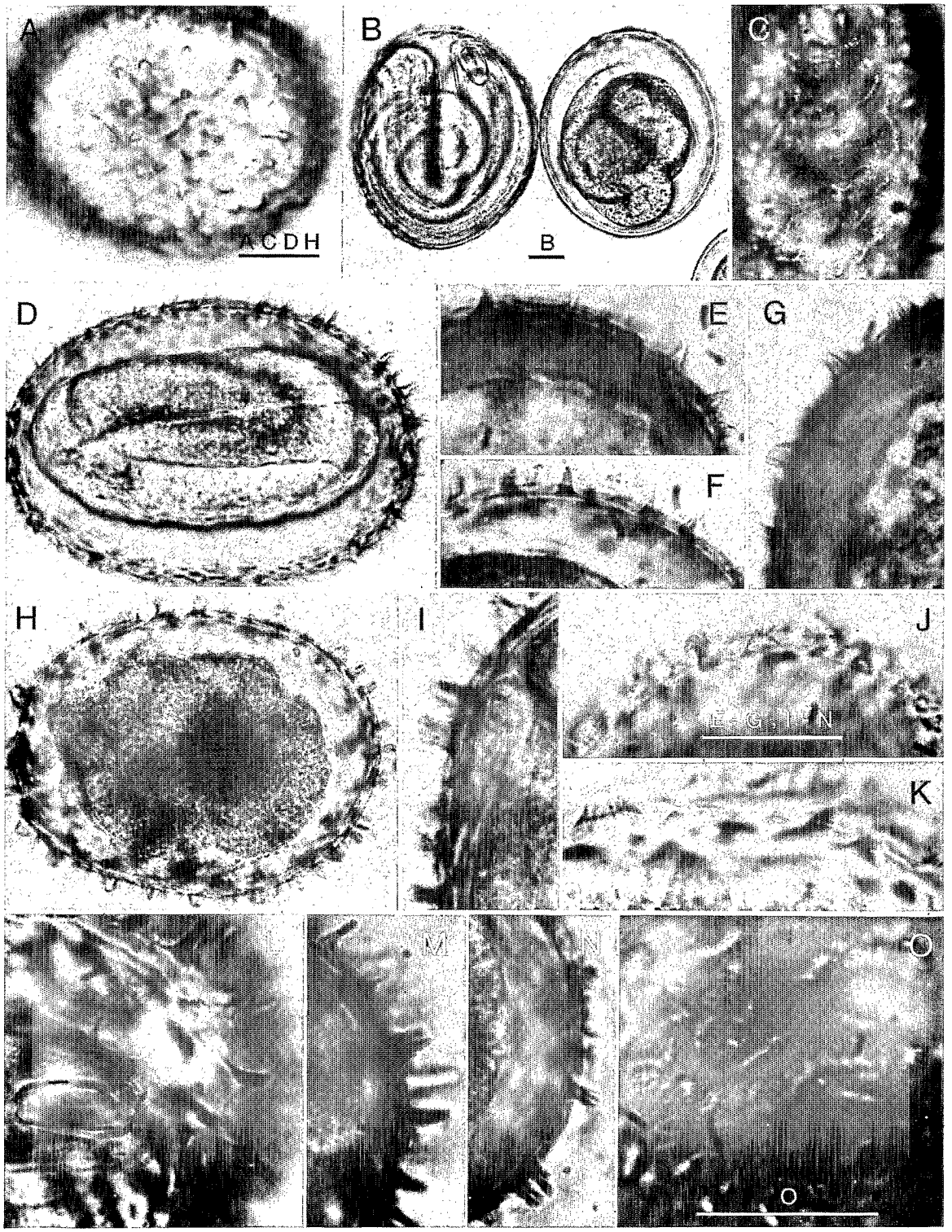

Fig. 1. Variation of egg-shell structure in *Prionchulus punctatus* (light microscope). C, H-K : from German specimens (topotypes). Remainder from French ones. A, C, O : surface view. L, K : view just below surface. Remainder : optical median section. Bars equal 20 µm.

surface view (Fig. 1 C) they appear as elongate processes ; in optical section (Fig. 1 H, I) they are columnar to triangular and appear to be composed of fused "fibres" giving them a striated appearance (Fig. 1 K). On some eggs the protuberances are sharper giving them a truly echinulate aspect (Fig. 1 E). The latter type is the most frequent in the *P. punctatus* population from France, especially from Mont-Morin and Sénart (Fig. 1, D-G). The study of eggs from the different cultures and localities in France revealed large variation in the aspect of the outer shell layer. Even prominently ornamented egg-shells with columnar protuberances as the German ones were observed among the French material (especially Armainvilliers. Fig. 1 H, M, N). Both, the German and Armainvilliers populations, come from heavy clay in a small ditch. Sharp and paddle-like protuberances can occur together in the same clone but always with a predominance of one of the two forms. Differences in the development of the protuberances found among the different cultures and localities are gradual. The most frequent types are the sharply pointed ones (Fig. 1 D, F, L), the broadly pointed ones (Fig. 1 D, G), the more or less paddle-shaped ones (Fig. 1 A, H) or crestlike ones furnished with a fan of fibres (Fig. 1 K, O). It is typical for *P. punctatus* that the contour of the egg remains sharp and easily observable in equatorial view (Fig. 1 H, D) even at higher magnification (Fig. 1 F, N) independent of the kind of protuberance. When the protuberances are thick or broad and depending on the observation angle, they may resemble the scaled protuberances of the egg of *P. muscorum*. However at some place of the egg-shell surface there are always some that appear as curved thick spines or thorns typical for *P. punctatus*, even on intra-uterine eggs.

In *P. muscorum* rounded ridges cover the egg margin resulting in a diffuse egg contour (Fig. 2 A, C). In this species the egg-shell does not really possess isolated protuberances but rather few to many short scaled ridges. These ridges are short (Fig. 2 A, B, D) or longer (Fig. 2 C, H, I). Their outer border is convex (Fig. 2 D, H) and they have a spongy appearance at high magnification (Fig. 2 E, H, J, K). However, the appearance of the egg-shell in *P. muscorum* varies also a lot from almost smooth (Fig. 2 F, right egg) to fully covered with ridges.

The outer shell layer as observed under the scanning electron microscope

Under SEM, the echinulate protuberances of *P. punctatus* appear as hairy projections of the egg-shell (Fig. 3). The shape, orientation and number of these projections account for the variation in shape of the protuberances observed under the light microscope. The paddle-like processes of the egg of the German specimens are either columnar projections (Fig. 3 E) or filamentous aggregates (Fig. 3 F). The pointed protuberances (Fig. 3 C, D, G) are similarly composed of a cluster of fibres, but with conical overall shape. The outermost shell layer consists of fine granular material covered with coarser granules at the site of the ridges. The fine

Table 1

Percentage of strongly, weakly or non-ornamented eggs from the original female of two clones and her F_1 and F_2 progeny

		P. muscorum *(Bois)*			P. punctatus *(Armainvilliers)*		
	eggs collected after *	*strong*	*weak*	*smooth*	*strong*	*weak*	*smooth*
P	few days	100			100		
	15 days	80	16.5	3.5	90.5	9.5	0
F_1	15 days	51	29	20	73.5	19.5	7
	30 days	41.5	41	17.5	50	33.5	16.5
F_2	15 days				76.5	17.5	6.0
	30 days	39	45	16			

* x days after starting the culture with adult females (n = 1 ♀ for P, n = 15 ♀♀ for F_1 and F_2).

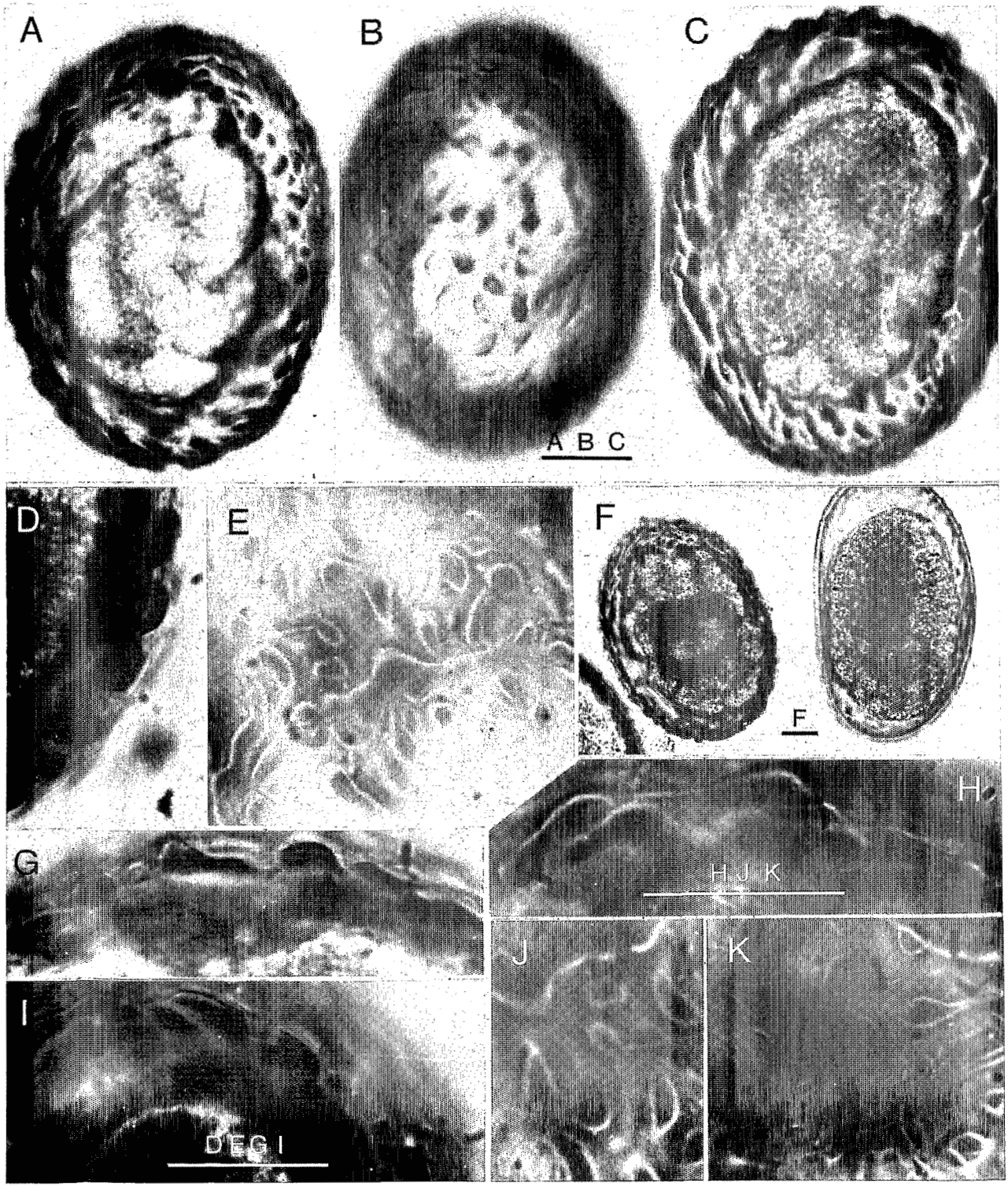

Fig. 2. Variation of egg-shell structure in *Prionchulus muscorum* (light microscope). B, E, J, K : surface view. A, C, F, G-I : view just below surface. D : optical median section. Bars equal 20 µm.

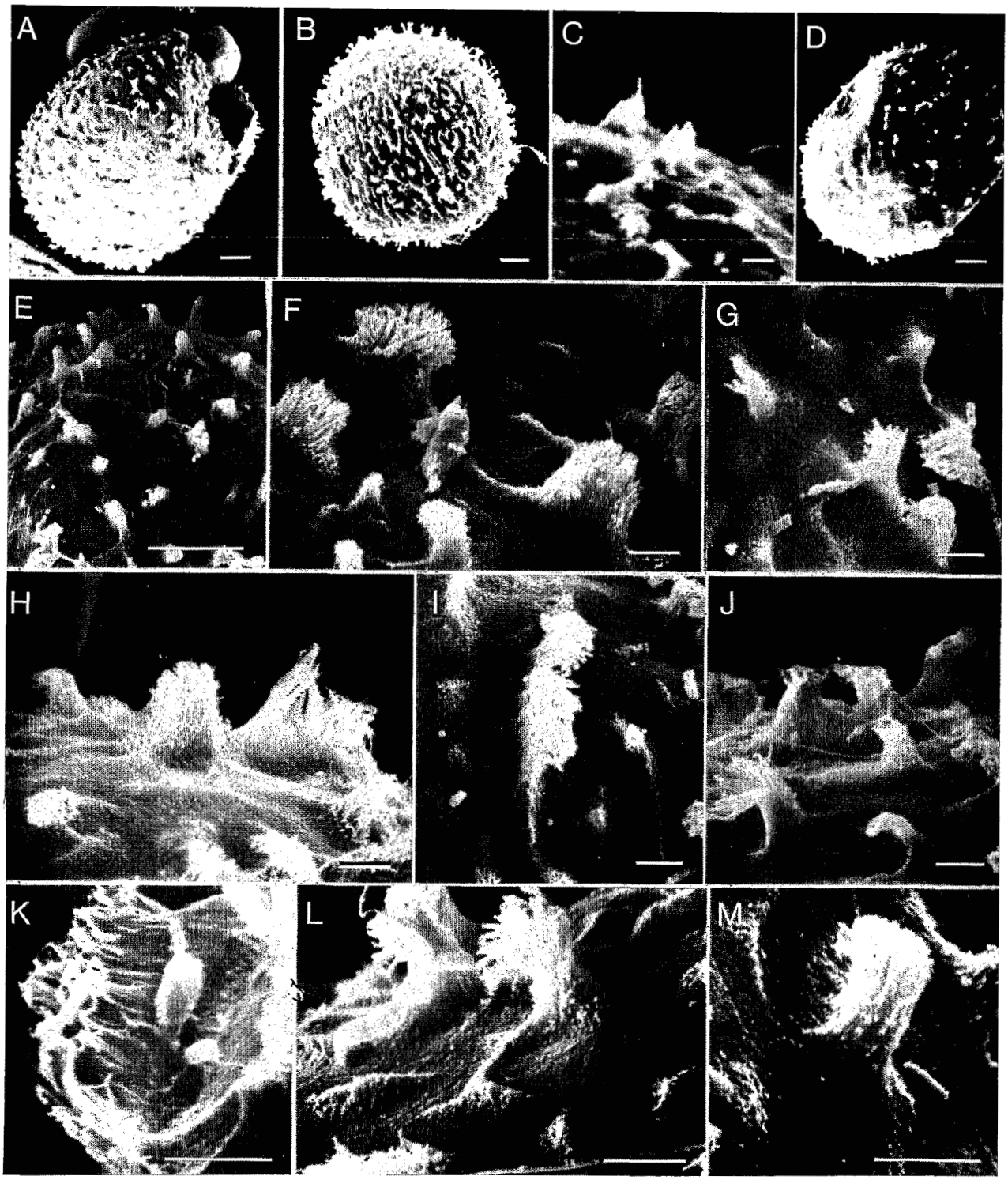

Fig. 3. Variation in structure of the egg-shell of *Prionchulus punctatus* (SEM). B, E-G : from German specimens (topotypes). Remainder from French ones. Bars equal 10 μm : A, B, D, E ; equal 2 μm : C, F-M. Rod-like structures on the egg (see : L and M) are bacteria from the culture medium.

granules are interrupted by an irregular pattern of fine grooves giving the impression that the layer is composed of interlocking plates (Fig. 3 L).

The outer shell layer of *P. muscorum* eggs consists of a similar fine granulated basic layer also interrupted by an irregular pattern of fine grooves (Fig. 4 E, G-I). Coarser granules form ridges on top of the basic layer (Fig. 4 H-J). The ridges vary considerably in shape, size and position ; occasionally they are branched or may run over the grooves (Fig. 4 E, G). Thus, it can be concluded that the outer egg-shell layer in both species is constructed in the same way : a fine granular base surmounted by irregular ridges and other protuberances on top of them. The finer structure of these ornamentations, however, differs between the two species : with coarse granules in *P. muscorum* and with filamentous projections in *P. punctatus*. It gives the impression that the ornamentations of the latter are a further elaboration of those in the former species.

On some eggs a few to several bacteria adhere to the surface of the egg-shell because of their presence in the rich culture medium. No alternations of the structure of the egg-shell were observed due to these bacteria.

Discussion

The egg-shell of nematodes is composed of three basic layers secreted by the egg itself, namely, an inner lipid layer, a middle chitinous layer and an outer vitelline layer (Bird, 1971). The latter, which is beyond the resolution of the light microscope, is covered in *Prionchulus* by a so-called protein coat (Christenson, Jacobs & Chitwood, 1950) or uterine layer (Bird, 1971). Such a layer has been reported and/or illustrated in a number of animal parasitic nematodes, where it may be highly ornamented and useful for taxonomic purposes (Ubelaker & Allison, 1975 ; Warthon, 1980 ; Kazacos & Turek, 1983), as well as in free-living (e.g. *Tobrilus* and *Anaplectus* cf. Christenson, Jacobs & Chitwood, 1950) and plant-parasitic nematodes (e.g. *Dolichodorus* cf. Smart, Schreiber & Perry, 1976).

The overall structure of the outermost layer is similar in both species of *Prionchulus* studied. It consists of a basic layer of aggregates of fine granules surmounted by ridges composed of coarser granules. The elaboration of the ridges, however, differs markedly between the two species. Since this outermost layer is secreted by the cells of the *pars dilatata*, one could think of a modelling influence of these cells (cf. also Crofton, 1966), but it may also be a self assembly process as suggested by Warthon (1979 *a*) for oxyurids. In ascarids the surface pattern of interlocking ridges on the egg-shell is the result of an outfolding and modification of the chitinous layer of the egg ; the surface layer thus reinforces the chitinous layer (Ubelaker & Allison, 1975 ; Warthon, 1979 *b*). No such outfoldings of the chitinous layer have been observed in *Prionchulus*. Furthermore the ridges on the eggs of *Prionchulus* do not form an interlocking pattern as in ascarids ; instead they are irregular in shape and position, so that a reinforcement function is less probable.

In fixed specimens an intimate contact can be observed between the *pars dilatata*-cells and the protuberances on the egg-shell. It is not certain that this is the normal situation, but probable, as a result of the turgor pressure in the body. The fact that the same female may produce eggs with different ornamentation patterns may point to self assembly rather than to modelling by the wall. However, the wall of the *pars dilatata* is not a rigid one ; it collapses when the egg leaves that portion of the gonoduct and expands again when a new oocyte enters. This could account for different patterns of ornamentation moulded on the same cells. Furthermore, the differences found between the two closely related *Prionchulus* species can be better explained on the basis of modelling than of self assembly. Indeed, it seems easier to accept differences of the inner surface texture of the *pars dilatata* among species than differences in a self assembly mechanism. The production of less ornamented or smooth egg-shells under less favorable conditions can be explained in several ways. Less active cells may protrude less into the lumen and secrete less coating substance, but unfavorable conditions could also hinder the self assembly process. The fact that the number of such eggs increase in more liquid cultures or in water points, however, to another possibility. In such a medium, movement of a relatively large nematode as *Prionchulus* is quite ineffective. Despite the increased activity of the body musculature and sinusoidal bending they make little progress. The increased muscular activity results in a quicker passage of the eggs through the sphincter, that separates the *pars dilatata* from the uterus and normally holds the egg till the egg-shell is completely formed. This results in higher number of eggs with a less developed outermost layer.

Eggs with a smooth or poorly ornamented outermost layer have no diagnostic value since they can occur in both species. The same is true for the number of eggs in the uterus. In both species more than two eggs can occur in a uterus and this was observed in specimens from the field as well as in cultures. Accumulation of eggs seems to be related with the age of the females rather than with the species.

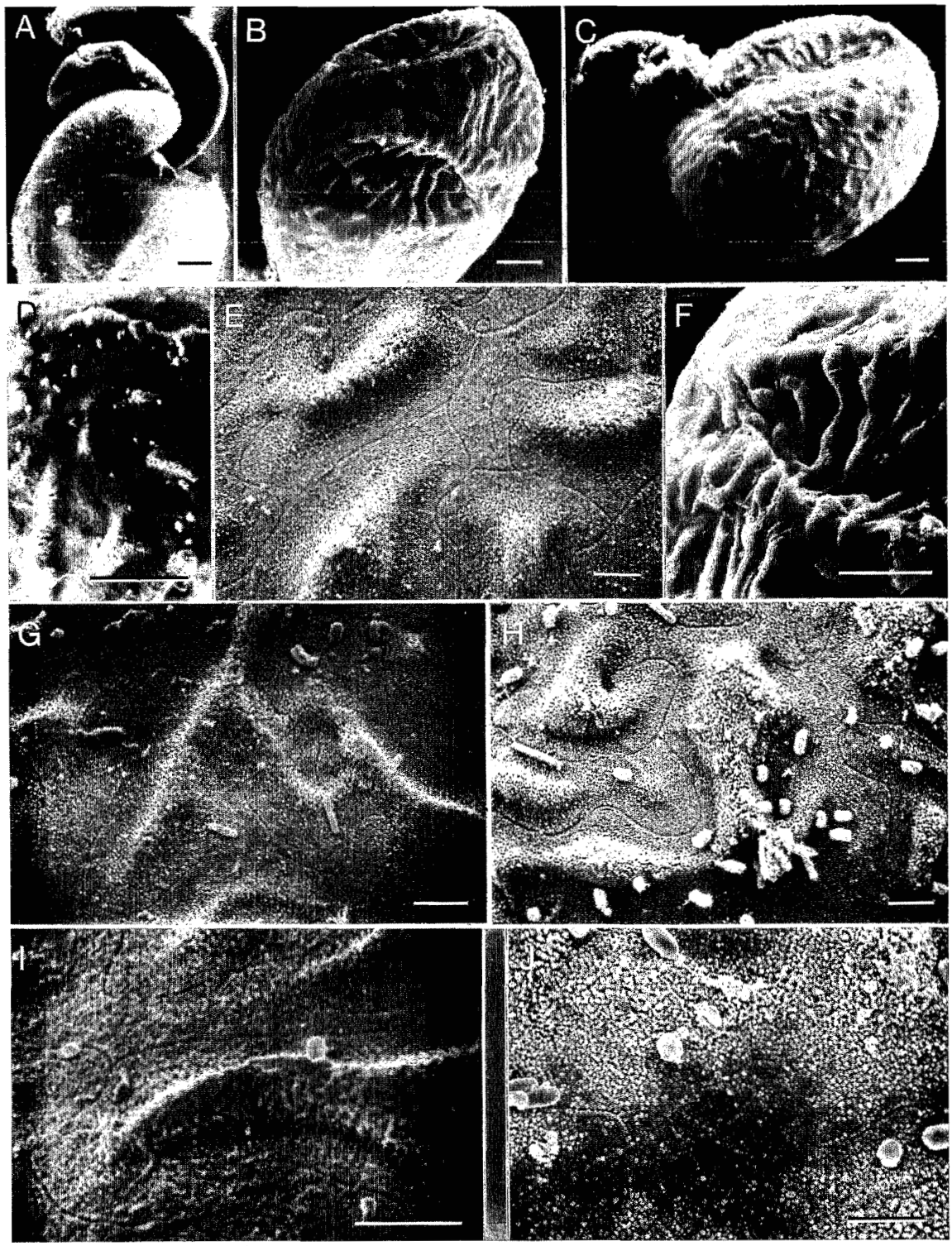

Fig. 4. Variation in structure of the egg-shell of *P. muscorum* (SEM). Bars equal 10 μm : A-D, F ; equal 2 μm : E, G-J. Rod-like structures on the egg (see : D, G-J) are bacteria from the culture medium.

On the basis of light and scanning electron microscopic observations it can be concluded that the normal structure of the outermost egg-shell layer is a good character to differentiate between *P. muscorum* and *P. punctatus*.

Ornamented eggs are a rarity among free-living nematodes and an explanation about the function of such ornamentations is therefore difficult. One possibility is that rough egg-shells do not sink as deeply in the sediment as smooth ones and this may be an advantage in the case of heavier eggs or surface dwelling nematodes. In this respect it is noteworthy that *P. punctatus* not only has well ornamented eggs when it is found in wet sediments, but generally this species is collected in larger numbers in the spring when the soil is more wet. At present this hypothesis is based on very little evidence but worth further investigation.

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REFERENCES

ANDRÁSSY, I. (1958). Über das System der Mononchiden (Mononchidae Chitwood, 1937 : Nematoda). *Annls. hist.-nat. Mus. natn. hung.*, 50 : 151-171.

ARPIN, P. (1979). Ecologie et systématique des nématodes Mononchides des zones forestières et herbacées sous climat tempéré humide. 1. Types de sol et groupements spécifiques. *Revue Nématol.*, 2 : 211-221.

ARPIN, P., SAMSOEN, L., PONGE, J. F. & KHAN, S. H. (1984). Ecology and Systematics of the Mononchid nematodes from wood- and grassland areas in wet temperate climate. II. The genus *Prionchulus* Cobb, 1916. *Revue Nématol.*, 7 : 417.

BIRD, A. F. (1971). *The Structure of Nematodes*. New York and London, Academic Press, 318 pp.

BRAKENHOFF, H. (1914). Beitrag zur Kenntnis der Nematodenfauna des nordwestdeutschen Flachlandes. *Abh. naturw. Ver. Bremen*, 22 : 267-311.

CHRISTENSON, R. O., JACOBS, L. & CHITWOOD, B. G. (1950). Nemic Ova. In : Chitwood, B.G., and Chitwood, M. B. (Eds), *Introduction to Nematology*, University Park Press, Baltimore : 175-187.

CROFTON, H. D. (1966). *Nematodes*. London, Hutchinson University Press, 160 p.

KAZACOS, K. R. & TUREK, J. J. (1983). Scanning Electron Microscopy of the Eggs of *Baylisascaris procyonis*, *B. transfuga* and *Parascaris equorum*, and their comparison with *Toxocara canis* and *Ascaris suum*. *Proc. helminth. Soc. Wash.*, 50 : 36-42.

MAERTENS, D. (1975). Observations on the life cycle of *Prionchulus punctatus* (Cobb, 1917) (Nematoda) and some culture conditions. *Biol. Jaarb. Dodonaea*, 43 : 197-218.

SMART, G. C., Jr., SCHREIBER, M. L. & PERRY, V. G. (1976). An outer layer on eggs of *Dolichodorus heterocephalus* (Tylenchida). *J. Nematol.*, 8 : 89-90.

UBELAKER, J. E. & ALLISON, V. F. (1975). Scanning electron microscopy of the eggs of *Ascaris lumbricoides*, *A. suum*, *Toxocara canis* and *T. mystax*. *J. Parasit.*, 61 : 802-807.

WHARTHON, D. A. (1979*a*). The structure and formation of the egg-shell of *Syphacia obvelata* Rudolphi (Nematoda : Oxyurida). *Parasitology*, 79 : 13-28.

WHARTHON, D. A. (1979*b*). The structure of the egg-shell of *Porrocaecum ensicaudatum* (Nematoda : Ascaridida). *Int. J. Parasit.*, 9 : 127-131.

WHARTHON, D. A. (1980). Studies on the function of the oxyurid egg-shell. *Parasitology*, 81 : 103-113.

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