SEPIA ARMS AND TENTACLES: MODEL SYSTEMS FOR STUDYING THE REGENERATION OF BRACHIAL APPENDAGES

B Rohrbach, H Schmidtberg

VIE ET MILIEU – LIFE & ENVIRONMENT, 2006, 56 (2) : 175-190

The cuttlefish *Sepia officinalis*
(N. Koueta, J.P. Andrade, S. v. Boletzky, eds)

SEPIA ARMS AND TENTACLES: MODEL SYSTEMS FOR STUDYING THE REGENERATION OF BRACHIAL APPENDAGES

*B. ROHRBACH, H. SCHMIDTBERG**

Institut für Allgemeine und Spezielle Zoologie, Bereich Entwicklungsbiologie, Justus-Liebig Universität Giessen, Stephanstrasse 24, 35390 Giessen, Germany
**Corresponding author: Henrike.Schmidtberg@allzool.bio.uni-gierssen.de*

REGENERATION
WOUND HEALING
TENTACLES
SUCKERS
SEPIA OFFICINALIS
CEPHALOPODA

ABSTRACT. – Macroscopic investigations on the capability of *Sepia officinalis* to regenerate lost tentacles reveal a process of renewal that can be divided into six stages of differentiation, corresponding to the studies on arm regeneration in the cuttlefish by Féral (1977, 1978): the protrusion of the central main nerve cord, the scarring of the wounded tissues, the formation of a hemispherical bud which is developing into a cone-like shape, the sucker-formation, and finally the recovery of the functional ability. Following Féral (1979) we applied a three-phase model based on histological-cytological aspects to evaluate degeneration, dedifferentiation, and re-differentiation processes of the wounded tissues in the regenerates. After amputation an intensive migration of amoebocytes into the wounded tissues begins, which is followed by the disintegration of cells at the wound surface. Inside the wound an autolysis of amoebocytes into a plasmoidal mass occurs and forms the primary wound occlusion which is replaced by a monolayered epithelium. The total differentiation of all tissues occurs in the latest phase starting in the central main nerve cord and proceeding to the peripheral regions of the tentacle. The sucker primordia become innervated only towards the end of the third month. Their regeneration is compared with sucker differentiation during embryonic development.

INTRODUCTION

Considering the enormous variety of tasks the arms and tentacles of cephalopods have to achieve (Naef 1928, Sanders & Young 1940, Messenger 1968, 1977, Kier 1982, 1996, Boletzky 1994), it seems likely that an animal that looses one or several arms while fighting with a prey, a conspecific or another predator, suffers a decrease of fitness. But this appears to be only a temporary handicap, because in all investigated cephalopod species a regeneration of the lost arms is possible.

The ability for regeneration was observed for the first time in *Octopus vulgaris* (Steenstrup 1856) and was initially denied for the Decabrachia (Brock 1886). Further investigations of *Sepia officinalis*, however, showed that the Decabrachia can regenerate the arms as well (Lange 1920, Aldrich & Aldrich 1968). Féral (1977, 1979, 1988) gives excellent observations on the regeneration of arms, tentacles and suckers in cephalopods. This author analysed in detail the wound healing processes and the morphology, histology, and cytology of arm regenerates in *Sepia*. Furthermore, the regeneration of amputated arms was proven in studies on *Ommastrephes bartrami* (Murata *et al.* 1981) and field investigations on *Architeuhtis dux* also showed that regeneration of tentacles occurs (Aldrich & Aldrich 1968).

Sepiidae as nekto-benthic animals stay close to the substrate or are buried in the sandy ground waiting for the prey to be caught by a surprise attack. Older *Sepia* can also seize prey only with its arms by jumping on it, a so called pouncing attack (Messenger 1968, Duval *et al.* 1984). This pattern of behaviour can be used when a part of the tentacle or the whole tentacle is lost. The growth rate of the animals is not affected by this changed behaviour (Murata *et al.* 1981). Thus, apparently the tentacles are not absolutely necessary for catching prey (Messenger 1968). Therefore, the question arises if sepiids totally regenerate their lost tentacles as in pelagic cephalopods, or do the regenerates remain imperfect because *Sepia* is able to catch prawn without tentacles? Another question is whether the regeneration process is age dependent. And finally, is the regeneration process much slower or faster than in arms?

The muscular configuration of tentacles of adult squids (Kier 1982, 1985, 1988, Budelmann *et al.* 1997) and the differentiation of the tentacle muscle fibres in squids (Kier 1996) and cuttlefish (Grimaldi *et al.* 2004) have already been extensively studied. The present investigations addition-

ally provide a morphogenetic analysis of tentacle regeneration processes in juvenile *S. officinalis* L. to compare those results with previous studies on arm regeneration and on developmental processes. The removal of one tentacle allows one to show the extreme adhesive efficiency of the suckers in the remaining tentacle club (Messenger 1968). Therefore, we discuss the functional ability of the regenerating tentacles according to the differentiation status and functional morphology of their regenerating suckers.

MATERIAL AND METHODS

Fertilized eggs of *Sepia officinalis* (Linné, 1758) were obtained from Arcachon (Atlantic Ocean, France) and Banyuls-sur-Mer (Mediterranean Sea, France) and reared in closed aquarium systems (filtered and recycled seawater, at 18°C) at the Institute of Zoology in Giessen. The animals were chosen according to their age without considering their dorsal mantle length (dml). Prejuvenile (hatching to 30 days) and juvenile (1 to 6 months) animals were used. The studies on the sucker development were carried out on embryonic stages, newly hatched, and juvenile *S. officinalis* from Banyuls-sur-Mer.

Once the animals were anaesthetized in a 2% ethanol-seawater solution, the club with a part of the shaft of one tentacle in each animal was dissected. The following dissections were defined (Fig. 1A):

amputation site 1: in that tentacle area where shaft length is equal to club length (prejuveniles ca. 2 mm shaft length)

amputation site 2: in the tentacle area where the shaft length is equal to one half of the club length

amputation site 3: at the base (3) and the centre (3*) of the club. At this position suckers exist in prejuvenile animals. This group serves as a positive control for the ability to regenerate.

amputation site 4: position of the tentacle shaft from where chromatophores are developed distally.

amputation site 5: shaft "base" (as far as the tentacle could be pulled out of the tentacle pocket)

For the observation of a progressive renewal of tissues a second amputation was performed on several animals at various time intervals after the first cut: 2 mm tissue samples were cut from the shaft tip where the regeneration of the tentacle takes place (Fig. 1B).

The time intervals chosen for sample preparation were 2 h, 5.5 h, 20 h, 26 h, 43 h, 62 h, 84 h, 100 h, 120 h, 140 h, 10 days, 15 days, 16 days, 21 days, 25 days, 30 days, 60 days, 90 days after dissection.

Tissue samples were fixed immediately after amputation, with preparation for scanning (SEM), transmission electron (TEM) and light microscopical (LM) embedding. For this purpose different fixation solutions were used. TEM investigations: prefixation in 2.2% or 3.8% glutardialdehyde in cacodylate buffer (pH 7.4, 1000 mosmol) and postfixation in 1% or 2% OsO_4 dissolved in cacodylate buffer. The material was embedded in Araldite (Durcupan®) or in Spurr's medium (Spurr 1969). Semithin sections (1 µm) were stained with toluidine blue (Böck 1984) and ultrathin sections were contrasted according to Reynolds (1963). LM investigations: fixation in Bouin's solution (Romeis 1968) or in 4% paraformaldehyde in seawater, and embedding in paraffin. Sections (7 µm) were stained with Masson's trichrome (modified by Goldner), Azan according to Heidenhain, and Bodian (Romeis 1968). For immune histochemistry samples were fixed in a 4% formaldehyde/seawater solution and were later embedded in paraffin. The sections were stained according to van Leeuwen (1986). The antibodies against serotonin (Serva) were used at a dilution of 1:5000 (overnight, 4°C). Control sections were incubated without primary antibodies. After incubation with the bridging antibody (dilution 1:20, 30 min at room temperature), sections were incubated with the PAP-complex (1:80, 2h) and made visible using substrate 3.3'-diaminobenzidine tetrahydrochloride (DAB).

For the fluorescence histochemical investigations unfixed cryostat sections were stained according to Barber (1982). The specific acetylcholinesterase (EC no. 3.1.1.7) was localized according to the direct thiocholine method of Karnowsky & Roots (1964).

Material for SEM investigations was dehydrated in ethanol or acetone, critical point-dried, and gold-coated.

RESULTS AND DISCUSSION

Morphology of untreated Sepia tentacles

The tentacle organization in Decabrachia has been described in numerous studies (Kier 1982, 1988, Budelmann *et al.* 1997). In particular the characteristic arrangement and the interaction of the different muscular systems, which differ from the configuration observed in the arms, play an important role for the rapid elongation of the tentacular shaft in squids (Kier 1982, 1985, 1991, 1996, Smith & Kier 1989, Kier & Schachrat 1992). The tentacles of squids and cuttlefish show similar organization. Therefore we present only a short overview of the gross morphology of an intact *Sepia* tentacle (Fig. 1C).

The tentacles are surrounded by a monolayered prismatic epithelium, separated from the adjacent dermis by a lamina basalis (Fig. 2C). The dermis consists of three layers: a subepidermal connective tissue layer, a chromatophore containing layer, and a thinner internal connective tissue layer. In both connective tissue layers collagenous fibres, blood vessels and capillaries, nervous and muscular fibres, and loosely distributed fibrocytes are located (Fig. 2D). In contrast to the arms (Féral 1977) the muscular tissue in the dermis is more strongly developed. At least in the investigated animals an iridophore layer could not be located, neither in the tentacle shaft nor in the club. The

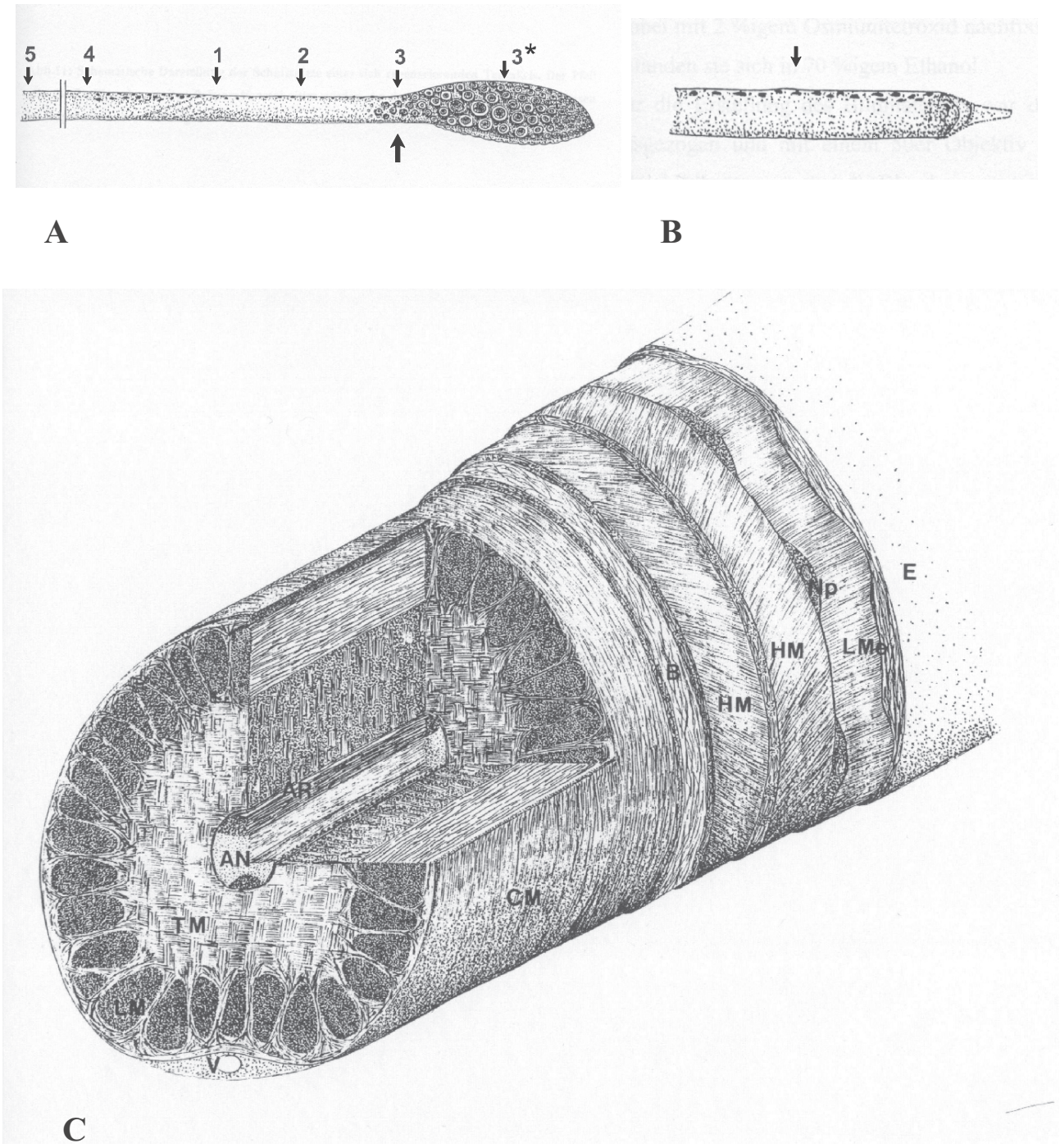

Fig. 1. – A, Schematic representation of tentacle club and shaft with various amputation sites, lower arrow: proximal sucker primordia on tentacle shaft. B, Stump of a regenerating tentacle, arrow: second amputation site. C, Cutaway diagram of a tentacle shaft of *S. officinalis*. AN axial main nerve cord, AR tentacle artery, B connective tissue, CM circular musculature, E epidermis, HM helical muscular system, LM longitudinal musculature, LMe longitudinal extrinsic musculature, Np peripheral nerve cord, TM transversal musculature, V vein.

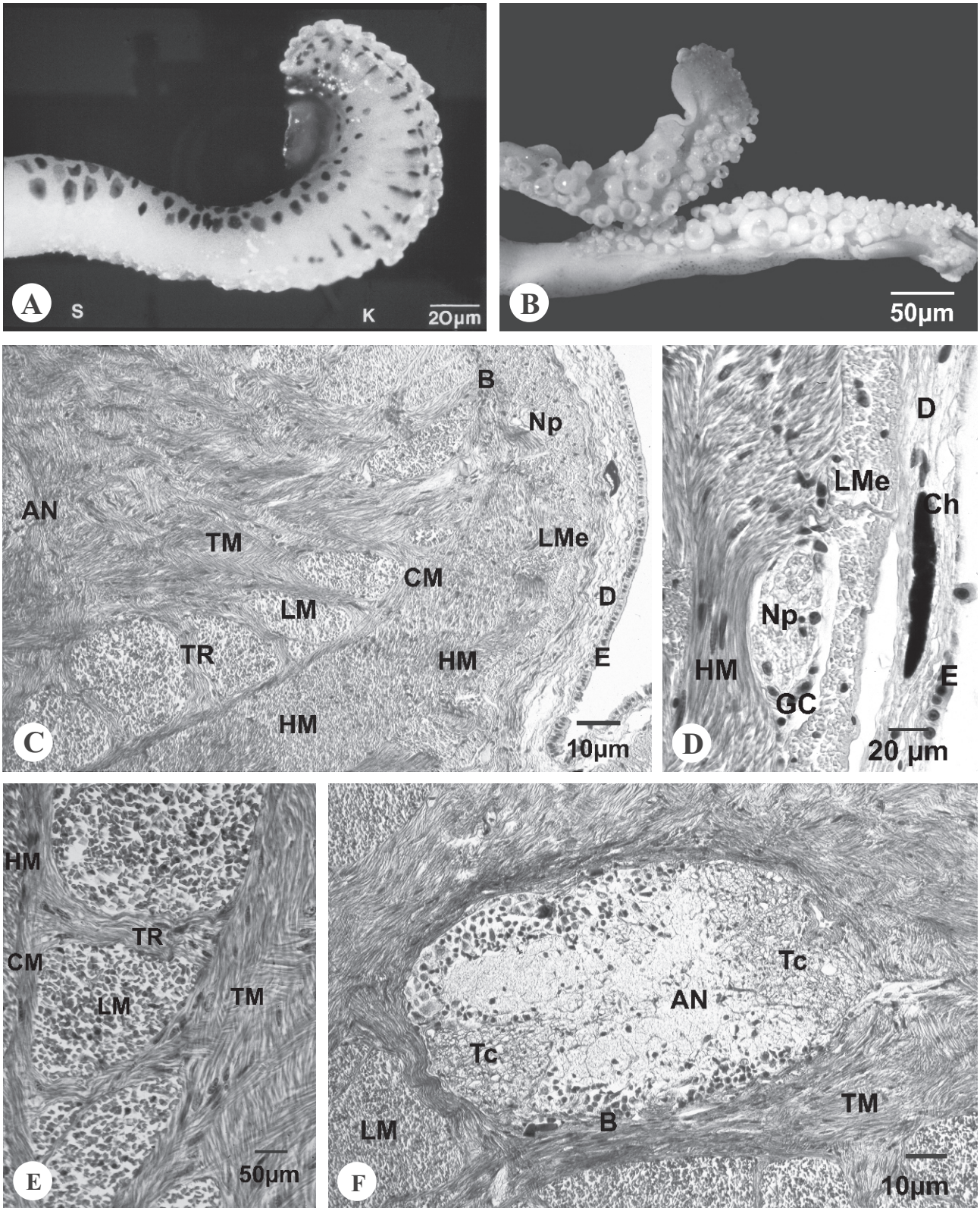

Fig. 2. – A, Aboral side of juvenile *Sepia* tentacle, B, Upper tentacle six months after amputation, stage VI, untreated tentacle below. C, Cross section of tentacle club with the different muscular systems. D, Extrinsic musculature. E, Intrinsic muscle system. F, Tractus cerebrotentacularis consisting of two fibers of different thickness on the oral and aboral side. AN axial main nerve cord, B connective tissue (adventitia), Ch chromatophore, CM circular musculature, D dermis, E epidermis, GC cortex, HM helical muscular system, LM longitudinal musculature, LMe longitudinal extrinsic musculature, Np peripheral nerve cord, Tc tractus cerebrotentacularis, TM transversal musculature, TR trabeculae.

chromatophores are loosely distributed in the aboral side of the distal tentacle shaft and are more densely packed in the tentacle club. They are well developed already in late embryonic stages (Fioroni 1963, 1990).

Extrinsic and instrinsic muscle tissues that are separated by a connective tissue layer run across the *Sepia* tentacle. Transversal, longitudinal and circular muscles form the intrinsic muscular system (Fig. 1C, 2C, 2E). According to the situation found in squid tentacles (Kier 1982, 1985) the extrinsic muscular system consists of two layers of helical muscles overlain by outer longitudinal muscles (Fig. 1C).

Our ultrastructural findings show that in the cuttlefish tentacles the transversal and circular muscle tissues possess cross-striated muscles, as described by Kier (1985) for squid tentacles. The other muscle systems mostly contain obliquely striated muscles (for review see Budelmann *et al.* 1997).

The large axial nerve cord, which is separated from the surrounding muscular system by an adventitia, consists of an outer ganglion cortex and an inner neuropil (Fig. 2D). In the cortex lies the tractus cerebrotentacularis consisting of two fibres of different thickness, respectively on the oral and aboral side of the nerve cord. In accord with the description of the arms (Kier 1982), six smaller nerve cords – so called intramuscular nerve cords – lie peripherally and axially and are embedded in the fibres of the extrinsic muscular system that extends longitudinally and helically (Fig. 1C). This extrinsic nervous system is connected by nerve fibres to the main nerve cord.

The suckers of the tentacle club of adult *S. officinalis* are described in numerous studies (Girod 1884, Tompsett 1939, Graziadei 1959, 1964, 1965, Nixon & Dilly 1977, Schmidtberg 1997). They appear in various stages of individual development, and the largest ones show two different regions (Fig 7E): the infundibulum consisting of a soft horny ring underlaid by a muscular wall, and the proximal suction chamber, the acetabulum surrounded by a thick muscular wall. The outer surface of the infundibulum of the tentacle suckers shows rings of polygonal processes provided with a projecting peg. The inner part of the horny ring reveals projections or teeth in the form of cones. Radial, meridional and circular (sphincter) muscles form the intrinsic muscular system which activates the suctorial chamber. The sensory apparatus is represented by primary ciliated receptor cells which lie in the surface epithelium of the sucker. The subacetabular sucker ganglion is situated below the acetabular cup between the loose connective tissue and the extrinsic musculature of the sucker. Numerous nerve fibres exist beneath the infundibulum and the rim of the muscular wall, and a thick nerve bundle in the peduncle can be traced to the main nerve cord of the tentacle.

General observations on tentacle regeneration

Animals of different ages do not in general differ in their regeneration abilities. However, a second amputation of regenerating tentacles causes a delay of 2 to 3 days in tissue regeneration. Additionally, temperature has an influence: lower temperatures decelerate the healing process, as already demonstrated in earlier studies (Féral 1977, 1978). Both the regeneration and the development of a new tentacle club are independent of the existence of suckers on the remaining tentacle shaft. Tissue regeneration seems to proceed according to a given pattern: the tentacle club is starting to be renewed, and this is followed by the extension of the tentacle shaft. This pattern is realized regardless of which site is amputated. The closer the cut is located to the body the sooner the elongation of the shaft begins after the formation of the club primordium. A cut leads to a regeneration of a club at any rate and the developing cones look the same.

At all amputation sites the tentacle club was completely repaired after three months, and already before the whole tentacle length was achieved the functional efficiency of the tentacle club was re-established; this became obvious in the usability of the largest suckers and the general functioning of the regenerated tentacles.

The time of the complete tentacle renewal (i.e. club and shaft have the same size and form as the second untreated tentacle) depends on the site of amputation and results exclusively from the different growth rates of the tentacle shaft. The regeneration process of complete tentacles cut at amputation site 3 was completed about 3 months after amputation; tentacles cut at amputation site 2 after 5 to 6 months; tentacles cut at amputation site 1 between 6 and 8 months later. Tentacles without chromatophores on its shaft cut at amputation site 4 and 5 showed regeneration processes as well, but the total regeneration time could not be defined because the animals died (between 9 to 23 months) before regeneration was complete.

Macroscopic findings on regeneration

Based on the studies of Féral (1977, 1978) on the arms of *S. officinalis*, we propose a similar classification of the tentacle regeneration process in six stages, because of the highly visible morphological similarity (Table I).

In stage I (0 to 7 days after amputation) the main nerve cord emerges from the surrounding transversal muscle tissue while the rim of the wound contracts (Fig. 3A). 4 h to 8 h later the

Table I. – Comparison of time scales for morphological stages of regeneration processes in arms and tentacles of *S. officinalis*.

stages	*Sepia* arms (Féral 1977) rearing temperature 16 °C	*Sepia* tentacles rearing temperature 18 °C
Stage I	1 to 7 days wound healing, cicatrisation of wound	0 to 7 days wound healing, cicatrisation of wound
Stage II	5 to 14 days hemispherical shape of arm tip, bud formation	5 to 7 days hemispherical shape of tentacle tip
Stage III	10 to 21 days arm growth, cone formation	8 to 19 days cone formation
Stage IV	17 to 25 days sucker formation	15 to 25 days tentacle growth
Stage V	25 to 35 days first chromatophores	25 to 37 days first chromatophores, sucker primordia
Stage VI	> 30 days thickening of regenerate, recovery of functional ability	35 days to 3 months differentiation of suckers, thickening of regenerate, recovery of functional ability

wound constriction is dorsolaterally extended. An amputation in the immediate vicinity of suckers leads to a rejection of these suckers 2 h to 7 h later. Suckers that are not located in the wound area remain intact. In stage II (Fig. 3B), 5 to 7 days after amputation, the external and internal epidermis merge over the wound while the axial nerve cord is withdrawn. This process, which lasts from 5 to 16 days after amputation, is completing the phase of wound healing and starting the phase of regeneration. In stage III (Fig. 3C, 3D), 8 to 19 days after amputation, a thin cone is growing aborally. In stage IV (Fig. 3E), 15 to 25 days after amputation, a concave bulge appears on the oral side of the tentacle where the primordia of tentacle suckers emerge. At stage V (Fig. 3F, 3G), 25 to 37 days after amputation, the cone is considerably elongated, and chromatophores appear on the aboral tentacle side, dispersing from the base to the tip of the regenerate. The surfaces of the more basal suckers start to invaginate their epidermis where their infundibulum later appears. In the terminal stage VI (> 30 days) the regenerate extends after an intensive growth phase (Fig. 2B). At that time the tentacle has regained its functional ability, as demonstrated by the completed differentiation of the largest suckers.

A comparison of the observations by Féral (1977, 1978) on arm regeneration clearly shows a delay of regeneration processes to occur only in the formation of the tentacle suckers (Table I), even though the water temperature was higher than in the earlier study and thus some acceleration of growth was expected. It remains questionable if this phenomenon is caused by different rearing conditions (laboratory raised animals, addition of artificial seawater). Moreover, the dwarfism of animals observed in our aquarium system (Versen & Boletzky 1992) as a result of the rearing conditions (relatively small aquaria holding numerous animals) is unlikely to cause a decelerated sucker development. Several authors point out that diminutive animals show normal behaviour and reach sexual maturity (Boletzky & Hanlon 1983, Versen & Boletzky 1992). A partial answer to this question may be the observation that juvenile *Sepia* reared in the aquarium often do not use their tentacles after unsuccessful attempts at catching prey and therefore move forward by using their arms instead (Boletzky 1972, 1974a,b, 1993, Versen & Boletzky 1992, Chen *et al.* 1996). This means that the total regeneration of the tentacle suckers during this particular time of postembryonic development may not be absolutely necessary for the young animals, because another equally profitable behaviour exists, which is supported by fully differentiated and powerful suckers on the arms.

Structural reorganization after amputation

The regeneration of the tentacle may also be divided into three separate phases according to the histological and ultrastructual characteristics of amputated arms as described by Féral (1979):

Phase I starts with the amputation of the tentacle and lasts about five days. It consists of three main processes: closure of the wound associated with migration of blood cells; massive synthesis of collagen; degeneration, and dedifferentiation of the surrounding tissues. According to Féral (1979) the degeneration process is characterized by lysis of cells in the wound tissue. The dedifferentiation process is determined by cells that lose their morphological and physiological characteristics and acquire an embryonic nature. In phase II, between the 5th and 20th day after amputation, a blastema

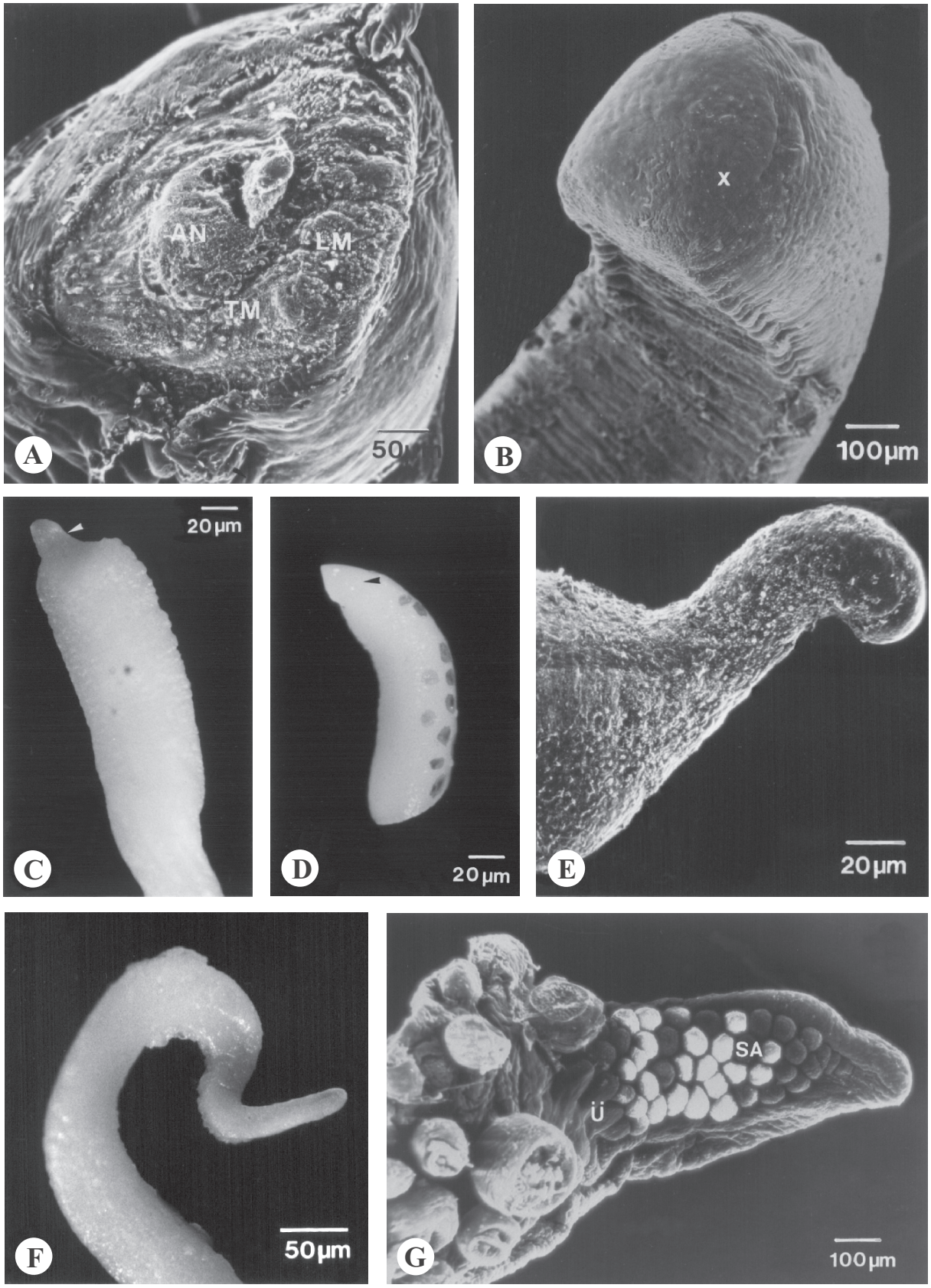

Fig. 3. – Stages of regeneration, macroscopic findings: A, Stage I, immediately after cut. B, Stage II: hemispherical shape of tentacle tip (x). C and D, Stage III, cone formation at aboral side. E, Stage IV, regenerate turns to the aboral side. F, Stage V, cone growth. G, differentiation of sucker primordia (SA) in a regenerating club. Ü amputation site in the middle of club.

develops. Numerous mitotic cells are indicative of an intensive growth phase. In phase III (20 days to 3 months) the cells start to differentiate, and tissues and organs develop. Finally, the tentacle regains its mobility and functional abilities. Let us now consider the different phases in greater detail.

Phase I: from the time of amputation to the fifth day, the central nerve cord protrudes from the amputated stump. This protrusion is caused by contractions of the surrounding longitudinal muscular tissue (Fig. 4A). At the edge of the wound bulges arise from the surrounding epithelium and cells disintegrate in the wound region (Fig. 4B). An intensive migration of amoebocytes takes place between and among all tissues of the tentacle (Fig. 4C). In parallel with an increased protocollagen synthesis inside the wound the autolysis and agglutination of amoebocytes into a "plasmoidal mass" begins. It forms a primary wound occlusion (Fig. 4D). At the transition from phase I to phase II, between 5 to 7 days, the wound tissue is covered by an epidermal layer consisting of flattened epithelial cells. Initially no lamina basalis is developed, and in contrast to other tissues no degeneration or dedifferentiation process is detectable in the epithelium (Fig. 4E).

Degeneration of tissue, revealed by a necrosis in the periphery of the wound, can be noticed immediately after the amputation, starting with the neuron cells of the cortex of the main nerve cord, and continuing with the musculature and up to the dermis. In comparison with other tissues, the degeneration of muscle cells, especially of the longitudinal muscle bundles, is weak. The extrinsic muscle system detaches the connection to the muscular system of the suckers of the wound area before they are discarded, which happens at a later date. During the dedifferentiation process the muscle cells disintegrate their myofilaments and degenerate their capillaries.

Phase II: between 5 - 20 days the epithelium cells show numerous mitoses and therefore appear to form multiple rows. Dedifferentiated cells of all tissues, except the unmodified cells of the epidermis, form a blastema that is continuously increasing in thickness by immigration of cells from deeper wound layers. After the re-establishment of blood capillaries, numerous mitoses occur in the regenerating nervous and dermis tissue at the end of the second week. With the beginning of cleavages in the dermis blastema mitoses occur in the epidermis and the regenerate starts growing.

Phase III: at the beginning of the fourth week the mitosis rate of the epithelium decreases. The cells differentiate and the lamina basalis is regenerated. On the oral tentacle side, the primordia of the suckers develop. The re-differentiation of tissues occurs between 20 days and 3 months and begins with the re-innervation of the blastema with invasion of nervous tissue originating from the remaining nervous stumps where increased cleavage activity of the neuroblasts starts. Furthermore, the tentacle vessels, the muscles and finally the cells of the dermis differentiate (Fig. 4F). Around the days 20/21 the chromatophores appear but they are not innervated until the end of the third week, when nerve fibres finally extend to the outer regions of the wound (Fig. 4G).

Following the nervous system, myofilaments in the intrinsic longitudinal muscle system differentiate about day 22 starting from the base of the regenerate and extending along the extrinsic muscular system. Afterwards the transversal muscles are reorganized. The sucker "anlagen" become innervated only towards the end of the third month. Before reaching its full length, the club's functional ability has already been restored. At this point the club is still slender and reaches its final diameter only when the tentacle has completed its extension and has stopped growing (Fig. 2B).

The histological and cytological differentiation of tissues in regenerating tentacles proceeds almost exactly, morphologically and timewise, as the renewing process in arms as described by Féral (1979).

Numerous invertebrates are able to regenerate central and peripheral neural connections (Moffet 1995, 1996, Bale *et al.* 2001). Our investigations on the regeneration of both nervous tissue and the main nerve cord proceed in agreement with those studies.

One of the first responses to neural injury in molluscs is the activation of blood cells such as macrophages, NK-like cells, and granulocytes which migrate to the body wall and then to wound surfaces: such processes have been characterized by morphological, histochemical, and immunohistochemical methods in numerous studies (*e.g.* Ruddell 1971, Sminia 1974, Glinski & Jarosz 1997, de Eguileor *et al.* 1999). In cephalopods earlier studies revealed that wounds are closed by local vasoconstriction and haemocyte aggregation, forming a blastema while the injured muscle and nerve cells are phagocytized (Stuart 1968, Browning 1979, Malham *et al.* 1997, Beuerlein *et al.* 2002a, b).

Our investigations yielded no chronological differences in the regeneration processes involving the three different muscular systems. In the de-differentiating process the myofibrils disintegrate, subsequently the sarcomeres of the muscular cell disintegrate. For re-differentiation a reverse process is presumed to exist, corresponding to the situation of developing myoblasts as described in the central heart of *S. officinalis* (Versen 1991): first of all the z-patches differentiate, followed by the formation of myofilaments. But more data are needed to accurately characterize the differentiation of myoblasts in *Sepia*.

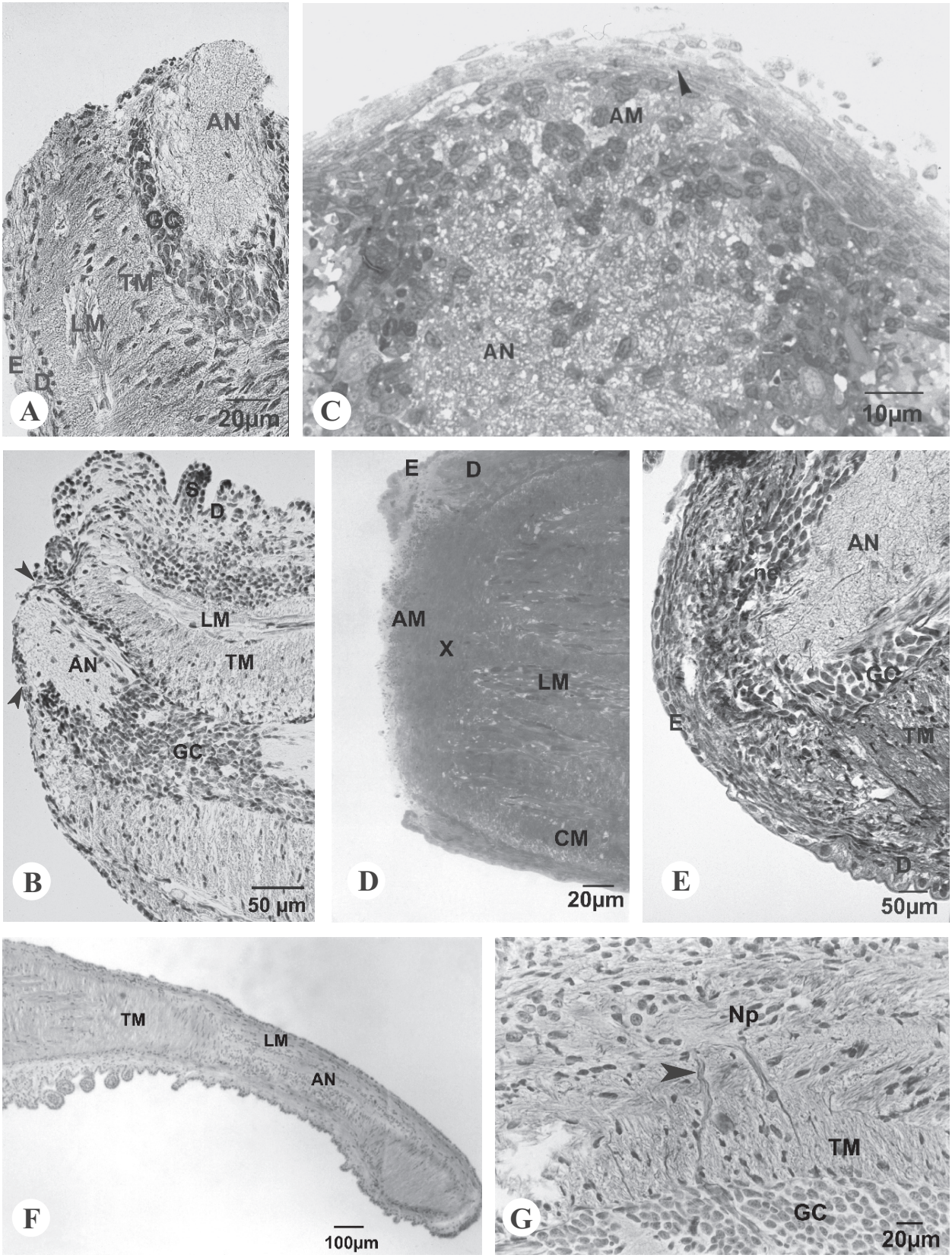

Fig. 4. – Structural reorganization. A, Phase I, protruding nerve cord immediately after cut. B, Phase I, beginning disintegration of cells in the wound region (arrowheads). C, Phase I, migrating amoebocytes inside the wounded tissue. D, Phase I, plasmoidal mass of amoebocytes (X). E, End of phase I, wound tissue covered by an epidermal layer. F, Phase II, cone growth. G, Phase III, nerve fibers (arrowhead) proceeding from the main to peripheral nerve cord. *Abbreviations*: see Fig. 2.

The role of neurotransmitters

Generally, the reaction of the regenerating neurones is influenced by neurotransmitters (Chiasson *et al.* 1994), and this appears to be true also in tentacle regenerates of *Sepia*.

Acetylcholine-esterase (AchE) as an indicator for the presence of acetylcholine was detected in neuromuscular end-plates (Fig. 5A) of the transversal (Fig. 5B) and circular muscular system as well as in the synapses of the tractus cerebrotentacularis (Fig. 5A) in untreated tentacles and regenerates of late phase III. This corresponds to the results obtained by Bone *et al.* (1982) who described the presence of acetylcholine in the inner longitudinal muscles of the arms and tentacles. The localization of the acetylcholine esterase in different areas of the central nervous system (Chichery & Chichery 1974) and in the central circulatory organs (Kling 1986, Schipp *et al.* 1986) suggests that acetylcholine is a common neurotransmitter not only in *S. officinalis* but in cephalopods in general as suggested by Messenger (1996) and has an excitatory effect on the muscles of the arms, tentacles, head retractors and siphon (Bone *et al.* 1982).

Studies on non-amputated parts of the tentacle, performed by means of glyoxilic acid induced fluorescence, demonstrate the presence of catecholamines in the neuropil of the main nerve cord (Fig. 5C) and in the tractus cerebrotentacularis (Fig. 5E). Investigations on 25-day old regenerates indicate the presence of catecholamines on maximum emission (480-490 nm) primarily in the neuropil of the main nerve cord (Fig. 5D). These

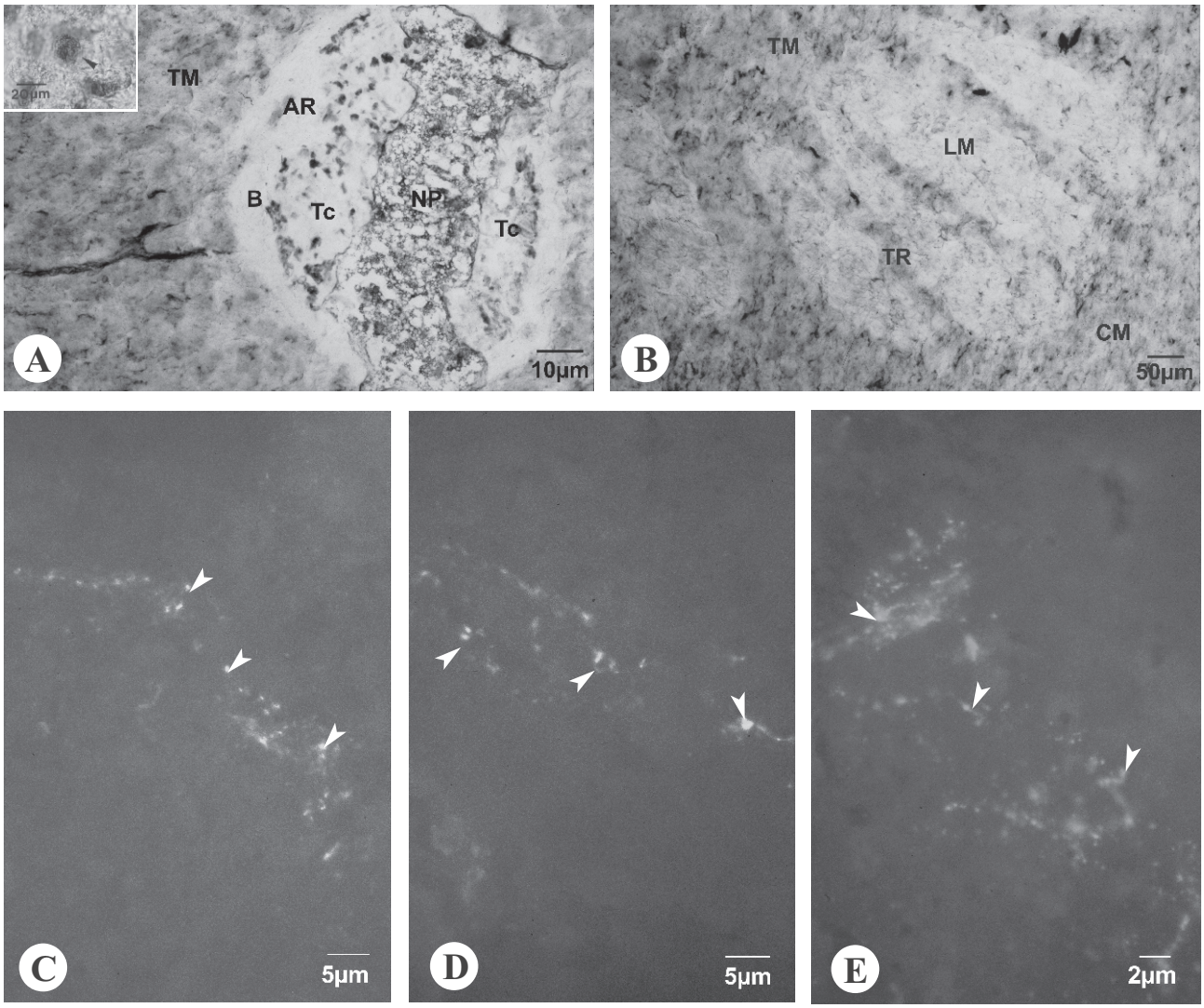

Fig. 5. – Neurotransmitters. A, AchE in the axial nerve cord, inset: AchE in neuromuscular end-plates B, AchE in the muscular system. C, Catecholamines in intact tentacles in the neuropil. D, Catecholamines in 25-day old regenerates primarily in the neuropil. E, Catecholamines in the tractus cerebrotentacularis of untreated tentacle. AR tentacle artery, B connective tissue, CM circular musculature, LM longitudinal musculature, NP neuropil, Tc tractus cerebrotentacularis, TM transversal musculature, TR trabeculae.

results correspond well with other findings demonstrating the presence of catecholamines in the nervous system of *Sepia* (Juorio 1971, Tansey 1980, Kling 1986). But the above results also indicate that the transmitter status with monoamines in the newly formed tissues is completed only at the end of the regeneration process.

No serotonin fluorescence (5-HT, 520–530 nm) could be traced in the stumps of any treated *Sepia* tentacle, in contrast to the situation observed in untreated tentacles where serotonin is detectable. According to our results the tentacles of *Sepia* possess a dual cholinergic/catecholaminergic innervation. Additionally, a peptidergic mechanism regulated by FMRF-amides, as described for cephalopods in earlier studies (Westermann *et al*. 1997, 2002, Schipp *et al.* 1991, Marschinke 1997), is supposed to also function as a neurotransmitter. But this and the possible role of further peptides in intact and regenerating tentacles has to be proved in further studies.

Sucker differentiation

The expression of suckers in untreated embryonic arms and tentacles starts with a single row of ridgelike buds on the oral side of the tentacle tip at stage XI of Naef (1928) (see also Nolte & Fioroni 1983, Haas 1989). Histologically the sucker primordia can be detected by the bulging ectodermal epithelium. Below the epithelium a mesodermal tissue is developing. In stage XII the proximal suckers arrange themselves in two rows and furthermore in four rows on the newly developing tentacle club (Fig. 6A, 6B). In histological sections the suckers become spheroidal due to the narrowing sucker base (Fig. 6C). In stage XIII the proximal suckers are arranged in six to seven rows (Fig. 6C). All of them show the same form and size. In developmental stages XIV to XVI the epithelium of the apical sucker surface is differentiating (Fig. 6D), then invaginating the prospective region of the infundibulum and finally the acetabulum. The connective tissue and the muscular systems emerge, as well as the tentacle peduncle. In stage XVII to XVIII (Fig. 6E, 6F, 7C) the typical asymmetry of decabrachian suckers appears, and a high prismatic sucker epithelium is lining the sucker chamber. At stage XIX (Fig. 7D) adult-like suckers with a well-differentiated infundibulum with pegs are present. First muscle and nerve fibres differentiate but the intrinsic and extrinsic muscular and nervous systems are not totally finished before the hatching stage (Fig. 6G, 6H, 7E). All in all the differentiation of functional suckers depends also on the ambient temperature. At 20°C the embryonic development of the cuttlefish lasts about 40 to 50 days. The last embryonic phases (stage XV to XX) take 50% longer than the whole embryonic development and the last growing phase (stage XVIII to XX) actually requires 80% of the whole embryonic development (Fioroni 1964). Thus, generally the sucker development during embryogenesis up to hatching lasts about 30 days.

As mentioned above in tentacle regenerates the development of suckers begins for the first time in phase III, approximately four weeks after amputation, and becomes visible by the ridge-like buds of the epidermis. Thus, time of appearance and the structural organisation of sucker primordia in untreated and regenerating tentacles are similar. But in comparison with the situation in embryonic tentacles where the proximal suckers initially are the largest (Fig. 6B, 6C), knoblike tiny buds also appear in that region, where the tentacle shaft terminates and the prospective proximal part of the regenerating club develops (Fig. 1A, lower arrow). In tentacles of adult animals, no matter whether amputated or untreated tentacles are considered, these suckers are already incorporated into the tentacle club.

The differentiation of the various tissues in the suckers of regenerates proceeds in the same way as in embryonic development (Fig. 7F), it is delayed, however. In particular the innervation of the suckers, the connection between nerve cord of the sucker peduncle, and the main nerve cord in the tentacle is re-established only in the third month (see above: phase III), which means that the usability is delayed in regenerates.

Prey-capture behaviour

The transverse muscles of the intrinsic muscular system are responsible for the extremely rapid extension of squid tentacles, as described by Kier (1982). In comparison to the obliquely striated muscle cells of transverse muscles of the arms, they are cross-striated muscle cells that form a specialized ultrastructure with relatively short sarcomeres (Kier 1985, 1991, 1996). Kier (1996) states for the tentacles of *Sepioteuthis lessoriana* that the transverse muscles are obliquely striated for the first three weeks after hatching. Afterwards they change to cross striation like in adult animals. This muscle cell differentiation is correlated with behaviour. Immediately after hatching, animals perform the pouncing attack using the arms, and only after the transformation of the musculature the tentacles are used. In our investigations on the cuttlefish the transverse muscle cells of the tentacles are likely cross striated and possess short sarcomeres already at hatching. At this time hatchlings are already able to use their tentacles but they have to exercise this behaviour, as suggested by many failing attempts. Generally the animals show an individual behaviour independent of the length

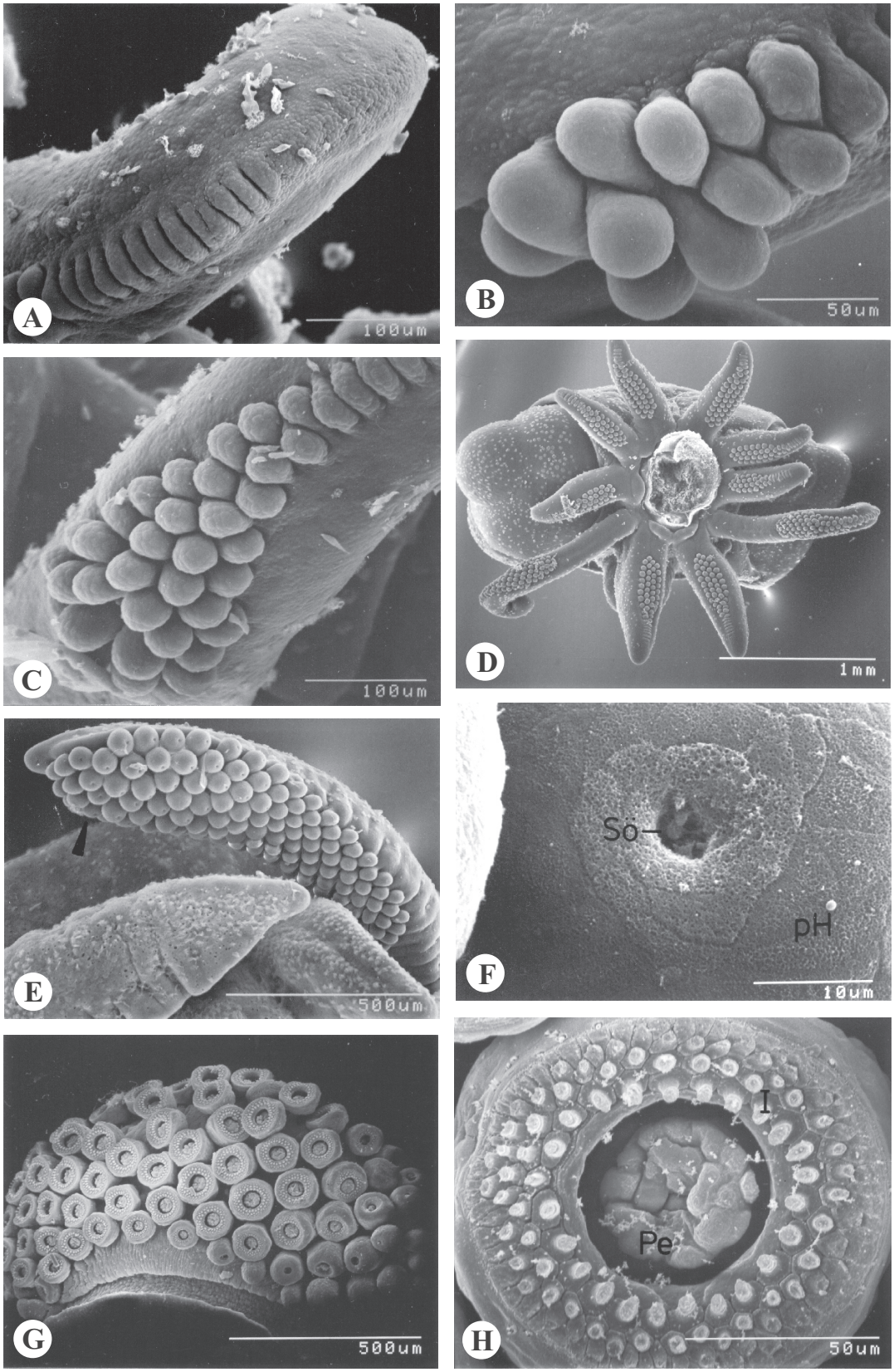

Fig. 6. – Sucker differentiation during embryonic development (stages according to Naef 1928). A, Stage XII, tentacle tip with lamellae-like sucker primordia in one longitudinal row. B, Stage XII, proximal tentacle region, bud-like sucker primordia in two rows. C, Stage XIII, proximal tentacle region with suckers beginning to organize in eight rows. D, Arm crown of *Sepia* at stage XIV-XV. E, Stage XVIII, distal suckers with first invagination of apical surface (arrowhead). F, Stage XVIII, largest tentacle sucker with opening of prospective sucker chamber, surrounded by polygonal infundibulum cells. G, Tentacle club at hatching stage XX. H, Differentiated tentacle sucker at stage XX, polygonal processes with pegs cover the infundibulum; the inner horny ring is still smooth and shows no teeth or cones, in contrast to suckers of adult animals. I infundibulum, Pe piston epithelium, pH polygonal infundibulum cells, Sö sucker opening to acetabular chamber.

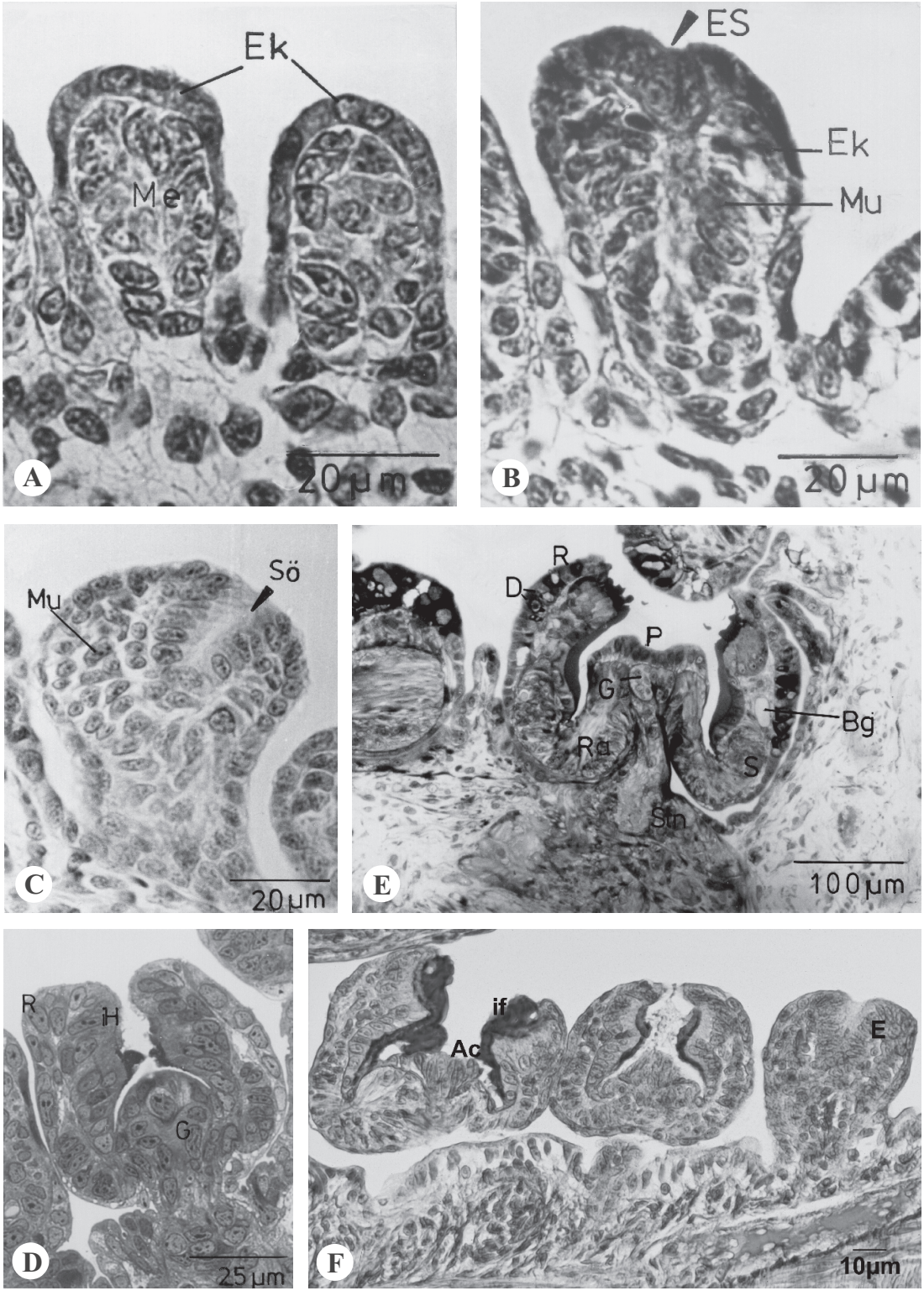

Fig. 7. – Sucker differentiation during embryonic development (stages according to Naef 1928) and during tentacle regeneration. A, Stage XV, tentacle sucker primordia with ectodermal epithelium and mesodermal tissue. B, Transverse section of a tentacle sucker in stage XVI, invagination of apical sucker epithelium, first differentiation of muscle tissue, and tapering of sucker stalk. C, Transverse section of tentacle sucker in stage XVIII, high prismatic cells are lining the prospective sucker chamber. D, Tentacle sucker in stage XIX with well-differentiated infundibulum and acetabulum. E, Tentacle sucker at hatching stage XX, highly differentiated, asymmetrical sucker with fully functional acetabulum and infundibulum, like in suckers of adult animals. F, Suckers of different developmental stages on a regenerating tentacle (stage VI); the morphological and histological organization is comparable with the sucker development during the different embryonic stages. Ac acetabulum, Bg blood vessel, D mucous cells, Ek ectodermal epithelium, Es invagination of apical sucker epithelium, G prospective sucker ganglion, if infundibulum, iH inner horny ring, Mu muscle tissue, P piston epithelium, Ra radial muscle tissue, R sucker rim with numerous chemoreceptor cells, Sö, sucker opening to actebular chamber.

of the tentacle stump or the stage of regeneration: some of them prefer the "pouncing" attack, others use the ejectible tentacles or the regenerates. And even a change of food from dead *Crangon crangon* to living, fast moving *Mysis* spec. does not cause a change in behaviour: some animals retain the slower "pouncing" attack with the arms even if failing attempts accumulate.

CONCLUSION

The structural organisation of the tentacle of *Sepia officinalis* is comparable to conditions described in the literature for other decabrachian cephalopods. Processes during regeneration (after amputation) such as degeneration, dedifferentiation and re-differentiation correspond to the observations on arm regeneration in the cuttlefish reported by Féral (1977, 1979). But in contrast to his results the full regeneration of the tentacle, which is defined by reaching full length and motility of the tentacle shaft and innervation of suckers, takes at least three months.

With the arms *Sepia* has the ability to achieve all vital functions; prey capture, warning, camouflage, defence etc. The tentacles, however, seem to be an auxiliary equipment that enable the animals to catch their prey from some distance. Thus, tentacles are of great importance for the animals to survive in their natural environment. In laboratory investigations the natural prey-capture behaviour can not be reproduced entirely but it is well known that in their natural habitat animals absolutely rely on tentacles to catch fast fish, shrimp or other cephalopods. The extreme velocity of the tentacle movement and the possibility to catch prey from some distance provides a great advantage in the interspecific competition. For this reason *Sepia* cannot abandon the possibility of tentacle regeneration which is an essential advantage of survival for these highly developed decabrachians.

DEDICATION. – We dedicate this work to Professor Pio Fioroni (1933-2003) who conveyed his enthusiasm for cephalopods to us.

REFERENCES

Aldrich FA, Aldrich MM 1968. On regeneration of the tentacular arm of the giant squid *Architeuthis dux* Steenstrup (Decapoda, Architeuthidae). *J Can Zool* 46(5): 845-847.

Bale SD, Howard TA, Moffet SB 2001. Neuronal and non-neuronal responses to nerve crush in a pulmonate snail, *Melampus bidentatus*. *Invert Neurosci* 4: 105-117.

Barber A 1982. Monoamine-containing varicosities by glyoxylic acid histofluorescence. *Cell Tissue Res* 226: 267-273.

Beuerlein K, Löhr S, Westermann B, Ruth P, Schimmelpfennig R, Schipp R 2002a. Components of the cellular defense and detoxification system of the common cuttlefish *Sepia officinalis* (Mollusca, Cephalopoda). *Tissue Cell* 34: 16-22.

Beuerlein K, Ruth P, Westermann B, Löhr S, Schipp R 2002b. Hemocyanin and the branchial heart complex of *Sepia officinalis*: are the hemocytes involved in hemocyanin metabolism of coleoid cephalopods? *Cell Tissue Res* 310(3): 373-381.

Böck P 1984. Der Semidünnschnitt. JF Bergmann Verlag, München.

Boletzky S von 1972. A note on aerial prey-capture by *Sepia officinalis* (Mollusca, Cephalopoda). *Vie Milieu* 23(1): 133-141.

Boletzky S von 1974a. Élevage de Céphalopodes en aquarium. *Vie Milieu* 24(A): 309-340.

Boletzky S von 1974b. Effets de la sous-nutrition prolongée sur le développement de la coquille de *Sepia officinalis* L. (Mollusca, Cephalopoda). *Bull Soc Zool Fr* 99(4): 667-673.

Boletzky S von 1993. The arm crown in cephalopod development and evolution: a discussion of morphological and behavioural homologies. *Am Malac Bull* 10(1): 61-69.

Boletzky S von 1994. Defensive "folding" response in the shortfin squid, *Illex conidetii* (Mollusca, Cephalopoda). *The Veliger* 37(3): 280-283.

Boletzky S von, Hanlon RT 1983. A review of the laboratory maintenance, rearing and culture of cephalopod molluscs. *Mem Nat Mus Victoria* 44: 147-187.

Bone Q, Packard A, Pulsford AL 1982. Cholinergic innervation of muscle fibres in squid. *J Mar Biol Assoc UK* 62: 193-199.

Brock J 1886. Indische Cephalopoden. *Zool Jhrb* 2: 591-593.

Browning J 1979. *Octopus* microvasculature: permeability to ferritin and carbon. *Tiss Cell* 11: 371-382.

Budelmann BU, Schipp R, Boletzky von S 1997. Cephalopoda. *In* Harrison FW, Kohn AJ eds, Mollusca II, Microscopic Anatomy of Invertebrates 6A, Wiley-Liss Inc: 119-414.

Chen DS, Van Dykhuizen G, Hodge J, Gilly WF 1996. Ontogeny of copepod predation in juvenile squid (*Loligo opalescens*). *Biol Bull* 190: 69-81.

Chiasson BJ, Baker MW, Croll RP 1994. Morphological changes and functional recovery following axotomy of a serotonergic cerebrobuccal neurone in the land snail *Achatina fulica*. *J Exp Biol* 192: 147-167.

Chichery M, Chichery R 1974. Histochemical studies of the localization of cholinesterases in the central nervous system of *Sepia officinalis*. *Cell Tiss Res* 148: 551-560.

Duval P, Chichery MP, Chichery R 1984. Prey capture by the cuttlefish (*Sepia offficnalis* L.): an experimental study of two strategies. *Behav Processes* 9: 13-21.

Eguileor M de, Tettamanti G, Grimaldi A, Boselli A, Scari G, Valvassori R, Cooper EL, Lanzavecchia G 1999. Histopathological changes after induced injury in leeches. *J Invertebr Pathol* 74(1): 14-28.

Féral JP 1977. La régénération des bras de *Sepia officinalis* L. (Mollusque, Céphalopode, Coléoide). *Inaug Diss Univ P.-et-M.-Curie, Paris.*

Féral JP 1978. La régénération des bras de la seiche *Sepia officinalis* L. (Cephalopoda: Sepioidea). I. Etude morphologique. *Cah Biol Mar* 19: 355-361.

Féral JP 1979. La régénération des bras de la seiche *Sepia officinalis* L. (Cephalopoda: Sepioidea). II. Etude histologique et cytologique. *Cah Biol Mar* 20: 29-42.

Féral JP 1988. Wound healing after arm amputation in *Sepia officinalis* (Cephalopoda: Sepioidea). *J Invert Path* 52: 380-211.

Fioroni P 1963. Zur embryonalen und postembryonalen Entwicklung der Epidermis bei zehnarmigen Tintenfischen. *Verh Naturforsch Ges Basel* 74: 149-160.

Fioroni P 1964. Zum embryonalen Größenwachstum bei Tintenfischen. *Rev Suisse Zool* 71(40): 777-804.

Fioroni P 1990. Our recent knowledge of the development of the cuttlefish (*Sepia officinalis*). *Zool Anz* 224: 1-25.

Girod P 1884. Recherches sur la peau des Céphalopodes. La ventouse. *Arch Zool Exp Gén* 2(2): 379-401.

Glinski Z, Jarosz J 1997. Molluscan immune defenses. *Arch Immunol Ther Exp (Warrz)* 45(2-3): 149-155.

Graziadei P 1959. Sulla presenza di elementi nervose negli epiteli di rivestimento della ventosa di *Sepia officinalis. Z Anat Entw Gesch* 121: 103-115.

Graziadei P 1964. Receptors in the suckers of the cuttlefish. *Nature* 203: 384-386.

Graziadei P 1965. Sensory receptor cells and related neurons in cephalopods. *Cold Spring Harb Symp quant Biol* 30: 45-57.

Grimaldi A, Tettamanti G, Rinaldi L, Brivio MF, Castellani D, Eguileor M de 2004. Muscle differentiation in tentacles of *Sepia officinalis* (Mollusca) is regulated by muscle regulatory factors (MRF) related proteins. *Develop Growth Differ* 46: 83-95.

Haas W 1989. Suckers and arm hooks in Coleoidea (Cephalopoda, Mollusca) and their bearing for phylogenetic systematics. *Abh Naturwiss Ver Hamburg* (NF) 28: 165-185.

Juorio AV 1971. Catecholamines and 5-hydroxytryptamine in nervous tissue of cephalopods. *J Physiol* 216: 213-226.

Karnovsky MJ, Roots L 1964. A 'direct-colouring' thiocholin method for cholinesterase. *J Histochem Cytochem* 12: 219-221.

Kier WM 1982. The functional morphology of the musculature of squid (Loliginidae) arms and tentacles. *J Morphol* 172: 179-192.

Kier WM 1985. The musculature of the squid arms and tentacles: ultrastructural evidence for functional differences. *J Morphol* 185: 223-239.

Kier WM 1988. The arrangement and function of molluscan muscle. *In* Wilbur KM ed, The Mollusca. Vol. 11. Academic Press, San Diego, 211 p.

Kier WM 1991. Squid cross-striated muscle: the evolution of a specialized muscle fiber type. *Bull Mar Sci* 49(1-2): 389-403.

Kier WM, Schachrat FH 1992. Biochemical comparison of fast- and slow-contracting squid muscle. *J Exp Biol* 168: 41-56.

Kier WM 1996. Muscle development in squid: Ultrastructural differentiation of a specialized muscle fiber type. *J Morphol* 229: 271-288.

Kling G 1986. Histochemical localization of cholinesterases and monoamines in the central heart of *Sepia officinalis* L. (Cephalopoda). *Histochemistry* 85: 241-250.

Lange MM 1920. On the regeneration and the finer structure of the arms of cephalopods. *J Exp Zool* 31(1): 1-40.

Leeuwen F van 1986. Pitfalls in immunohistochemistry with special reference to specific problems in the localization of neuropeptides. *Am J Anat* 175: 363-377.

Malham SK, Runham NW, Secombes CJ 1997. Phagocytosis by hemocytes from the lesser octopus *Eledone cirrhosa* (Lam.) (Cephalopoda). *Dev Comp Immunol* 22(1): 27-37.

Marschinke A 1997. Localization of an extracerebral NSV-ganglion in the cephalopod *Sepia officinalis* (L.). *Experientia* 43: 511-525.

Messenger JB 1968. The visual attack of the cuttlefish, *Sepia officinalis*. *Anim Behav* 16: 342-357.

Messenger JB 1977. Prey capture and learning in the cuttlefish, *Sepia*. *Symp Zool Soc Lond* 38: 347-376.

Messenger JB 1996. Neurotransmitters in cephalopods. *Neuroscience* 2: 95-114.

Moffet SB 1995. Neural regeneration in gastropod molluscs. *Progr Neurobiology* 46: 289-330.

Moffet SB 1996. Nervous system regeneration in the invertebrates. Zoophysiology. Springer Verlag, Berlin, Vol 34.

Murata M, Ishi M, Osako M 1981. On the regeneration of tentacle of the oceanic squid, *Ommastrephes bartrami* (Lesueur). *Bull Hokkaido Reg Fish Res Lab* 46: 1-14.

Naef A 1928. Die Cephalopoden (Embryologie). *Fauna e Flora del Golfo di Napoli. Roma, Bardi, Monogr* 35(1,2): 1-364.

Nixon M, Dilly PN 1977. Sucker surfaces and prey capture. *Symp zool Soc Lond* 38: 447-511.

Nolte K, Fioroni P 1983. Zur Entwicklung der Saugnäpfe bei coleoiden Tintenfischen. *Zool Anz Jena* 211(5/6): 329-340.

Reynolds ES 1963. The use of leadcitrate of high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* 17: 208-217.

Romeis B 1968. Mikroskopische Technik. R Oldenbourg Verlag, München.

Ruddell CL 1971. The fine structure of oyster agranular amebocytes from regenerating mantle wounds in the Pacific oyster, *Crassostrea gigas*. *Invertebr Pathol* 18(2): 260-268.

Sanders FK, Young JZ 1940. Learning and other functions in the higher nervous centers of *Sepia*. *J Neurophysiol* 3: 501-526.

Schipp R, Schmidt HR, Fiedler 1986. Comparative cytochemical and pharmacological studies on the cholinergic innervation of the branchial heart of the cephalopod *Sepia officinalis* (L). *Experientia* 42: 23-30.

Schipp R, Jakobs P, Fiedler A 1991. Monaminergic-peptidergig interactions in neuroregulatory control of the cephalic aorta in *Sepia officinalis* L. (Cephalopoda). *Comp Biochem Physiol* C 99: 421-429.

Schmidtberg H 1997. The structure of suckers of newly hatched *Sepia officinalis*, *Loligo vulgaris*, and *Octopus vulgaris*. *Vie Milieu* 47(2): 155-159.

Sminia T 1974. Haematopoiesis in the freshwater snail *Lymnaea stagnalis* studied by electron microscopy and autoradiography. *Cell Tissue Res* 150(4): 443-454.

Smith KK, Kier WM 1989. Trunks, tongues and tentacles: Moving with skeletons of muscle. *Am Sci* 77: 28-35.

Spurr AR 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26: 31-43.

Steenstrup JJ 1856. Hectocotyldannelsen hos Octopods laegterne *Argonauta* og *Tremoctopus* opylst ved lagttagelse af lignende Dannelser hos Blaeksprutterne I Almindelighed. Kgl. Danske Videns- kab. Selskab Skrifter 5 R *Naturw Mat Afdel* 4: 186-215.

Stuart AE 1968. The reticulo-endothelial apparatus of the lesser octopus *Eledone cirrosa*. *J Pathol Bacteriol* 96(2): 401-412.

Tansey EM 1980. Aminergic fluorescence in the cephalopod brain. *Philos Trans R Soc Lond Biol* 291: 127-145.

Tompsett DH 1939. L.M.B.C. Memoirs on typical British marine plants and animals. XXXII *Sepia*. The University Press of Liverpool.

Versen B 1991. Versuche zur Primärkultivierung embryonaler Zentral- und Kiemenherzen von *Sepia officinalis* L. (Cephalopoda; Coleoidea). Dipl thesis, JL-Univ Giessen.

Versen B, Boletzky S von 1992. *Sepia officinalis* L. as a laboratory animal: rearing and maintenance in small, closed aquarium systems. *Verh Deutsch Zoolges* 85(1): 243.

Westermann B, Schipp R, Hempelmann G 1997. Histochemical detection of different neurotransmitters in the digestive tract of *Nautilus pompilius* L. (Cephalopoda). *Vie Milieu* 47(2): 131-136.

Westermann B, Beuerlein K, Hempelmann G, Schipp R 2002. Localization of putative neurotransmitters in the mantle and siphuncle of the mollusc *Nautilus pompilius* L. (Cephalopoda). *J Histochem* 34: 435-440.

Received October 5, 2005
Accepted December 15, 2005