

## Behavioural and neurohistological changes in aging *Sepia*

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(Accepted 15 October 1991)

**Key words:** Aging; *Sepia officinalis*; Cephalopod; Learning; Neuroethology; Terminal degeneration; Predatory behavior

The life cycle of the cuttlefish in the English Channel is characterized by a succession of homogeneous population cohorts. These conditions provide an excellent opportunity for the study of aging in this cephalopod. In a first longitudinal study, we considered the oldest animals and compared their success rates at the first capture attempt. During the first weeks of the study, the results remained constant and then, during the weeks immediately preceding the natural death, a dramatic drop was observed. This deterioration may be due to defects of visuomotor coordination. In a second study, we used an associative learning protocol with negative reinforcement and the performances of young and old animals were compared. The most striking results showed that the performances of the oldest animals during the retention test were very mediocre. Such results suggest that the long-term memory process is affected. Finally, a modification of the Fink-Heimer silver stain enabled us to draw a map of spontaneous terminal degeneration in the central nervous system of the oldest animals. The structures which are characterized by the presence of multimodal inputs (the spine of the peduncle lobe and the basal lobes) present the most obvious signs of degeneration.

### INTRODUCTION

The ontogenesis of the behaviour of cephalopods has been the subject of a few detailed studies. Among these, the evolution of predatory behaviour in the cuttlefish<sup>24,25</sup> and the evolution of its cognitive capacities should be emphasized<sup>18,25</sup>. For the latter, an improvement in learning experiments has been correlated with a large increase in the vertical system during the postembryonic period of the *Sepia officinalis*<sup>18,19,28</sup>. Contrary to this, no study has been carried out on the deleterious behavioural and structural processes linked to aging. Only one paper<sup>29</sup> has attributed an important role of the optic gland in the triggering-off of the senescent processes. Mollusca (and especially *Aplysia*) have already provided very interesting models for the study of aging mechanisms<sup>1</sup>.

The cephalopods are by far the most highly developed of the Molluscs; their central nervous system is extremely well-known<sup>20,31–35</sup>, their natural behaviour and in particular their predatory behaviour has been the subject of numerous studies<sup>4,5,11,17</sup>. The range of their learning capacities (for example: detour experiments<sup>26</sup>, improvement in reversal discriminative learning<sup>14</sup>) allows us to situate them alongside the vertebrates.

The life cycle of the common cuttlefish (*Sepia officinalis*) in the English Channel offers an interesting opportunity to study the biological phenomena linked to aging in this species. Indeed, given the climatic condi-

tions, we witness a succession of homogeneous population cohorts. The adults aged between 21 and 22 months (average length of the mantle: 24–25 cm) migrate towards coastal waters from the beginning of April to the beginning of May for reproduction. After this, these animals then have a very rapid phase of senescence which leads to their death at the end of June or the beginning of July. The maximum peak in egg-laying is at the beginning of May and hatching takes place at the end of July or the beginning of August given the considerable influence of the temperature of the water on the length of the period of embryonic development: 80–90 days at 15°C, 40–45 days at 20°C<sup>22</sup>. The young cuttlefishes then grow quickly and leave the cold coastal waters at the end of October. The following spring these cuttlefishes (average length of the mantle: 10–11 cm) return to the coastal waters at the end of May or the beginning of June and undergo a new phase of extremely rapid growth. Then, the animals will once again leave the coastal waters before the winter. At this stage they are aged on average 16 months, the males are all mature and the females are undergoing the period of sexual maturation (average length of the mantle: 16–17 cm). These animals will return to the coastal waters the following spring for reproduction. The life cycle of the cuttlefish in the English Channel was described for the first time a number of years ago<sup>22</sup> and over a period of 15 years our own observations have confirmed the results of this first

study. It may however be worth noting the existence, though rare, of a few sexually mature females at the end of the summer of the first year of the cycle. These females are probably responsible for the exceptional batches of eggs which can be found in nature. The survival of these eggs is uncertain: at best, the young cuttlefish will hatch when the coastal waters begin to cool; at worst, the embryonic development is abortive. Thus, according to the size of the animal and the date of its capture, an age can easily be assigned to it and in the cases of the older animal an estimation can be given of the likely date of its natural death.

The capture of prey by tentacle ejection implies very precise visuomotor coordinations. It therefore seemed interesting to study the evolution of capture performances during aging. For the cuttlefish a protocol of associative learning with negative reinforcement was developed<sup>18,19</sup>. We adopted but slightly modified this protocol in order to evaluate the possible cognitive defects linked to senescence. Together with these behavioural studies we will consider in this paper the main results of the localization of spontaneous degenerating fibres in the central nervous system in senescent animals.

## MATERIALS AND METHODS

### *The evolution of predatory behaviour*

Adult cuttlefish aged between 21 and 22 months were caught off Luc-sur-Mer at the very beginning of the period of migration for reproduction. They were first placed for a few days in big circular tanks (1.75 m diameter × 0.7 m height) filled with seawater. After a period of adaptation to this environment, we discarded any animals showing external scars or not eating regularly. The remaining animals were selected and kept separately in experimental opaque plastic tanks (0.80 × 0.60 × 0.40 m) containing running and filtered seawater to a depth of 0.30 m. Fifteen animals were thus bred from May 1988 to the beginning of July 1988. The cuttlefish were fed every day with live shrimps (*Crangon crangon* 3–3.5 cm in length). The prey is distributed individually and the criterion adopted is the percentage of success at the first trial<sup>4,5,11,17</sup>.

### *The evolution of performances during associative learning*

A detailed description of the protocol used is given in a previous paper on the subject<sup>9</sup>. Three shrimps were enclosed in a glass tube (7 cm in diameter) with a basal water inlet. This inlet was connected to a small pump which provoked a water flow sufficient enough to cause the permanent movement of the shrimps throughout the experiment. The learning protocol includes 8 trials, lasting 3 min each, separated by 30 min and a retention test 24 h later. In this situation adult cuttlefish quickly learn to inhibit their predatory behaviour. For each trial we counted every capture attempt regardless of the strategy of attack used (tentacle strategy or jumping strategy).

In this paper, the performances of two groups are compared: young adults (about 11 months old,  $n = 14$ , group Y) and old animals (about 22 months,  $n = 15$ , group O), this last group having been tested one month before the average peak of mortality in this population cohort.

### *Analysis of degeneration*

This analysis was carried out on animals aged about two years

and then compared to the analysis of animals aged one year. Preliminary observations showed that when the animal stops feeding for several days its death is imminent. The oldest cuttlefish were therefore killed at this stage and the brains removed for neurohistological investigations. Different works<sup>3,21</sup> show the possibility of using Fink-Heimer's technique for the visualization of degeneration in *Octopus*.

We did not find the use of the technique entirely satisfactory for *Sepia*. We therefore modified the times and the concentration of all solutions indicated by the above authors. To confirm our results, we visualized the terminal degenerations of afferent fibres the precise location of which was determined previously by the centrifugal cobalt filling method. The afferent fibres of the anterior funnel nerve project up into the anterior magnocellular lobe<sup>6</sup>. The section of the latter nerve induces a very conspicuous terminal degeneration in the anterior magnocellular lobe. The concordance of the results obtained by both techniques (Figs. 1 and 2) validate the modifications we have brought to Fink-Heimer's technique.

The degeneration was much more visible on previously fixed material, since we trimmed excess cartilage and placed the brains in chilled, neutralized 10% (w/v) formalin in seawater. After a minimum period of 4 weeks of fixation the material was immersed in a solution of formalin in seawater with 30% sucrose added, until it sank to the bottom of the container (24–36 h). The brains were then quickly dried on absorbant paper, mounted on frozen OCT fluid (R. Lamb) and covered with more OCT fluid<sup>3</sup>. So as to avoid crystal forming, the freezing must take place quickly (in the case of very large material prefreezing in liquid nitrogen is necessary). At a temperature of -30°C, serial sections were cut at 25 µm in a cryostat and mounted on slides coated with a chrome alum/gelatin mixture.

The Fink-Heimer technique which was used was the following: the sections were immersed in 0.05% potassium permanganate for 18 min, rinsed in bidistilled water and placed in 1% oxalic acid: 1% hydroquinone for 1 min 30 s. The sections were then carefully rinsed in bidistilled water and transferred to a mixture of silver nitrate (3.75 g) and uranyl nitrate (0.5 g) in 250 ml of bidistilled water for 7 min. The sections were then rinsed with bidistilled water for 8 min. The slides were placed for 25 s in a solution of fresh ammoniacal silver nitrate which was prepared thus: 6 g of silver nitrate in 240 ml bidistilled water; 8.5 ml ammonia; 0.2 g sodium hydroxide in 8 ml bidistilled water. Without rinsing the slides were transferred into Nauta solution for 1 min: 227.5 ml bidistilled water; 22.5 ml 95% ethanol; 6.75 ml 10% formalin; 6.75 ml 1% citric acid. The slides were then rinsed in bidistilled water and placed in a solution of 0.5% sodium thiosulphate for 1 min, re-rinsed, dehydrated on a hotplate and mounted in glycerol gelatin (Sigma).

## RESULTS

### *Predatory behaviour*

Out of the 15 animals originally included in the experiments, 5 were discarded for the analysis of the results. Scars appeared very quickly on the mantles of the latter, thus causing them to float and consequently their predatory behaviour was greatly impaired. Moreover, these scars can cause parasite degeneration in the central nervous system which is independent of the aging process. The remaining 10 animals showed regular eating behaviour for about 6 weeks. No scars were apparent and the animals were killed 4 days after their predatory behaviour had completely ceased, as this indicates the imminent natural death of the cuttlefish.

The performances of these animals (Table I) showed

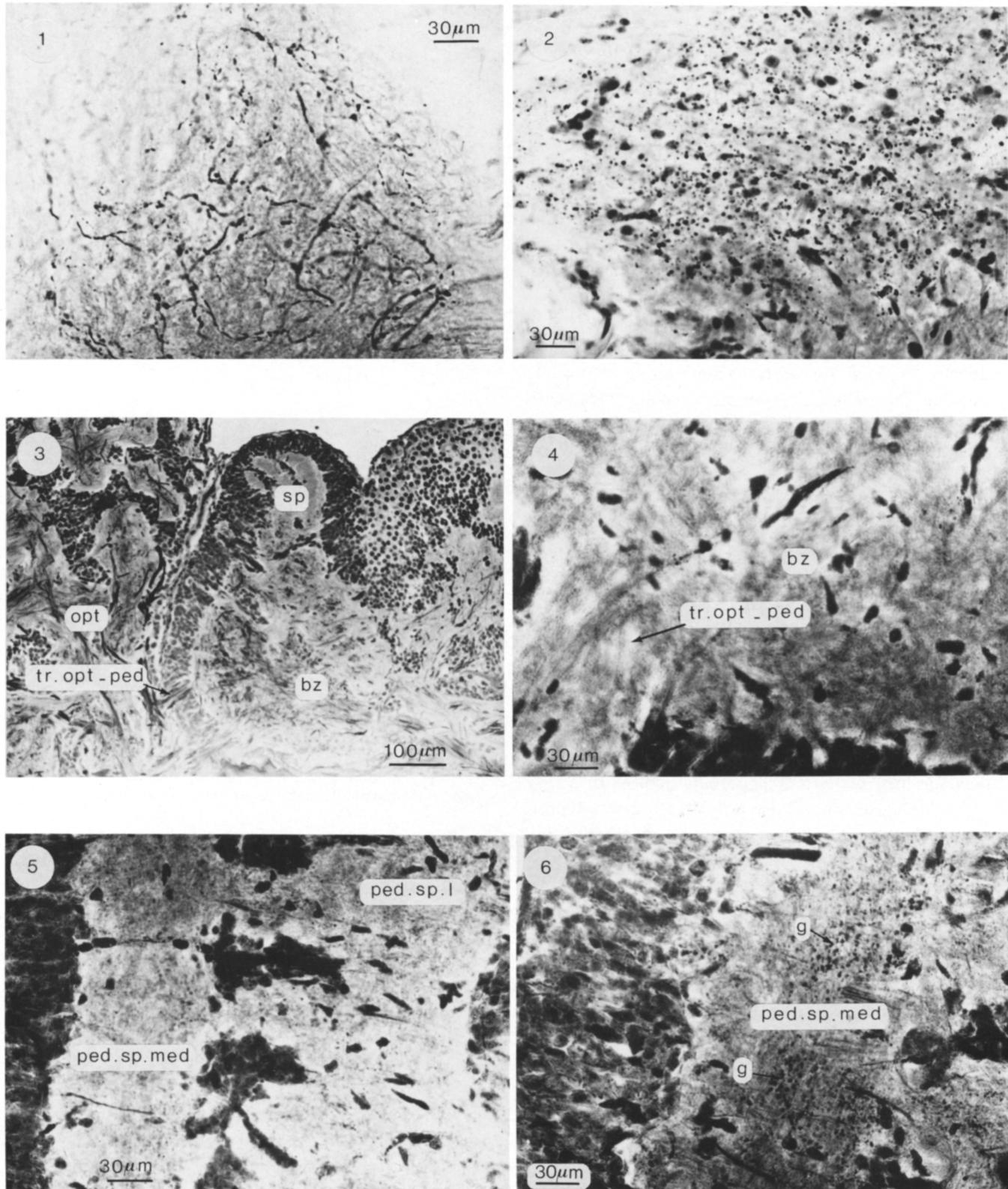


Fig. 1. Transverse section through the anterior magnocellular lobe: afferent fibres of the anterior funnel nerve. Centrifugal cobalt filling.  
 Fig. 2. Transverse section through the anterior magnocellular lobe: terminal degeneration after a section of the anterior funnel nerve. Fink-Heimer technique.

Fig. 3. Transverse section of the peduncle lobe. Cajal silver stain. bz, basal zone; opt, optic lobe; sp, spine; tr. opt.-ped, optic to peduncle tract.

Fig. 4. Horizontal section through the basal zone of the peduncle lobe. Old animal (aged about 23 months). Fink-Heimer technique.

Fig. 5. Horizontal section through the spine of the peduncle lobe. Young animal (aged about 12 months). No evident degenerating material. Fink-Heimer technique. ped.sp.med, median bank of peduncle spine; ped.sp.l, lateral bank of peduncle spine.

Fig. 6. Horizontal section through the spine of the peduncle lobe. Old animal (aged about 23 months). Terminal degeneration. Fink-Heimer technique. ped.sp.med, median bank of peduncle spine; g, degeneration granules.

TABLE I

*Success rate in percentage at the first capture attempt during the longitudinal study of senescent animals*

First 4 weeks	Second last week	Last week before complete stop of feeding behaviour
85.3 ± 7.1	81.7 ± 6.8	70.2 ± 15.6

no changes during the first 4 weeks of the longitudinal study. The average success rate at the first capture attempt remained at the same levels as previously described<sup>4,11,17</sup>. In contrast, the average success rate dramatically dropped for each animal during the week preceding the moment when their feeding behaviour stopped. The increase of S.D. clearly shows the variability of the aging behaviour: some animals are scarcely affected, others greatly so.

Statistical analysis showed a very significant difference in the performances of the animals between the second last and last week preceding the complete stop in feeding behaviour ( $t = 1$ ,  $P = 0.002$ , Wilcoxon test). The analysis of failed attacks showed that the latter were almost always the result of an underestimation of the distance of tentacle ejection. Normal tentacle ejection is always overestimated<sup>17</sup> and in these senescent animals the under-estimation is often of several cm. After several unsuccessful attempts the cuttlefish recalibrated its behaviour and the shrimp was captured normally. Feeding behaviour then underwent important changes in a few days. In young starving adults, prey detection was almost always followed by an active pursuit on the part of the predator and a capture attempt. At this stage, the senescent cuttlefish showed a particular behaviour: the shrimps were detected as normal but were not pursued when they left the frontal visual field of the predator. At a later stage the natural behaviour was more seriously affected: prey detection continued as normal, the beginning of positioning together with an ocular convergence was observed when the shrimp remained in the frontal visual field, then suddenly ocular convergence stopped, the animal projected water between its arms and lost all interest in the prey. The ultimate stage foreshadowing the imminent death of the animal was characterized by a total disinterest in the prey.

#### Learning experiments

Our protocol of associative learning was easily controlled by all the animals tested; each animal behaved identically during the last trials (6, 7 and 8). During the 4th and 5th trials a significant difference was noted between the animals aged one year and those aged two

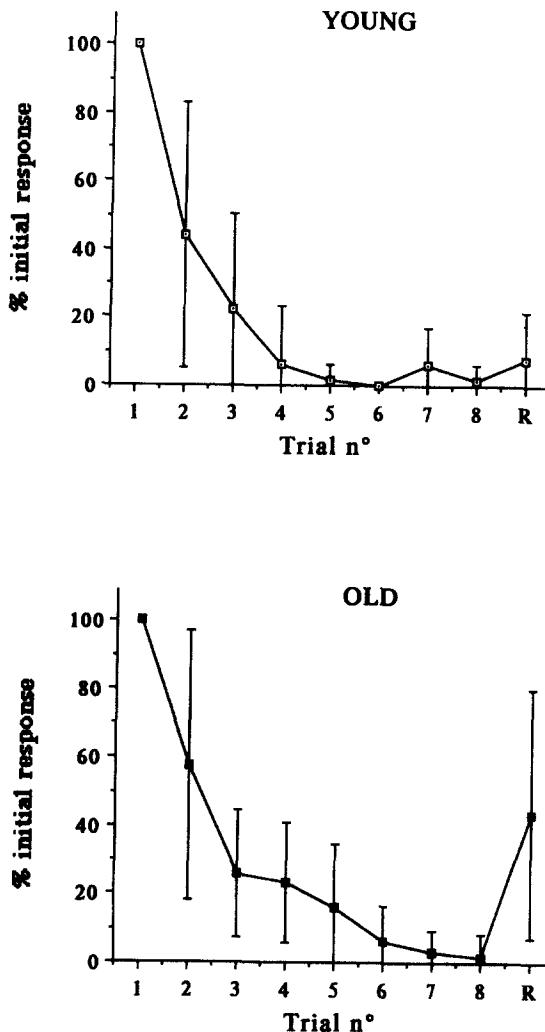


Fig. 7. Average performance curves of the two experimental groups.

years ( $U = 41$ ,  $U = 44.5$ ,  $P < 0.01$ , Mann-Whitney test). The retention test showed a significant difference ( $U = 38$ ,  $0.001 < P < 0.01$ , Mann-Whitney test) in the performances of animals aged one and two years and a greater dispersion in the scores of the older animals (Fig. 7). The use of Kolmogorov-Smirnov two-sample test allows a better comparison of the acquisition curves by each cohort; no significant differences are apparent.

#### Neurohistological analysis of spontaneous degeneration

The brains used for the neurohistological analysis were taken from the 10 remaining senescent animals which showed no scars and were used in the longitudinal study of predatory behaviour. The animals were killed in the phase during which feeding behaviour stops, i.e. very little time before their natural death. These brains were compared to those of 5 control animals aged one year. Depending on the size of the brain, between 200 and 300 serial sections were cut from the supra-oesophageal mass and then examined as well as about 50 sections cut from

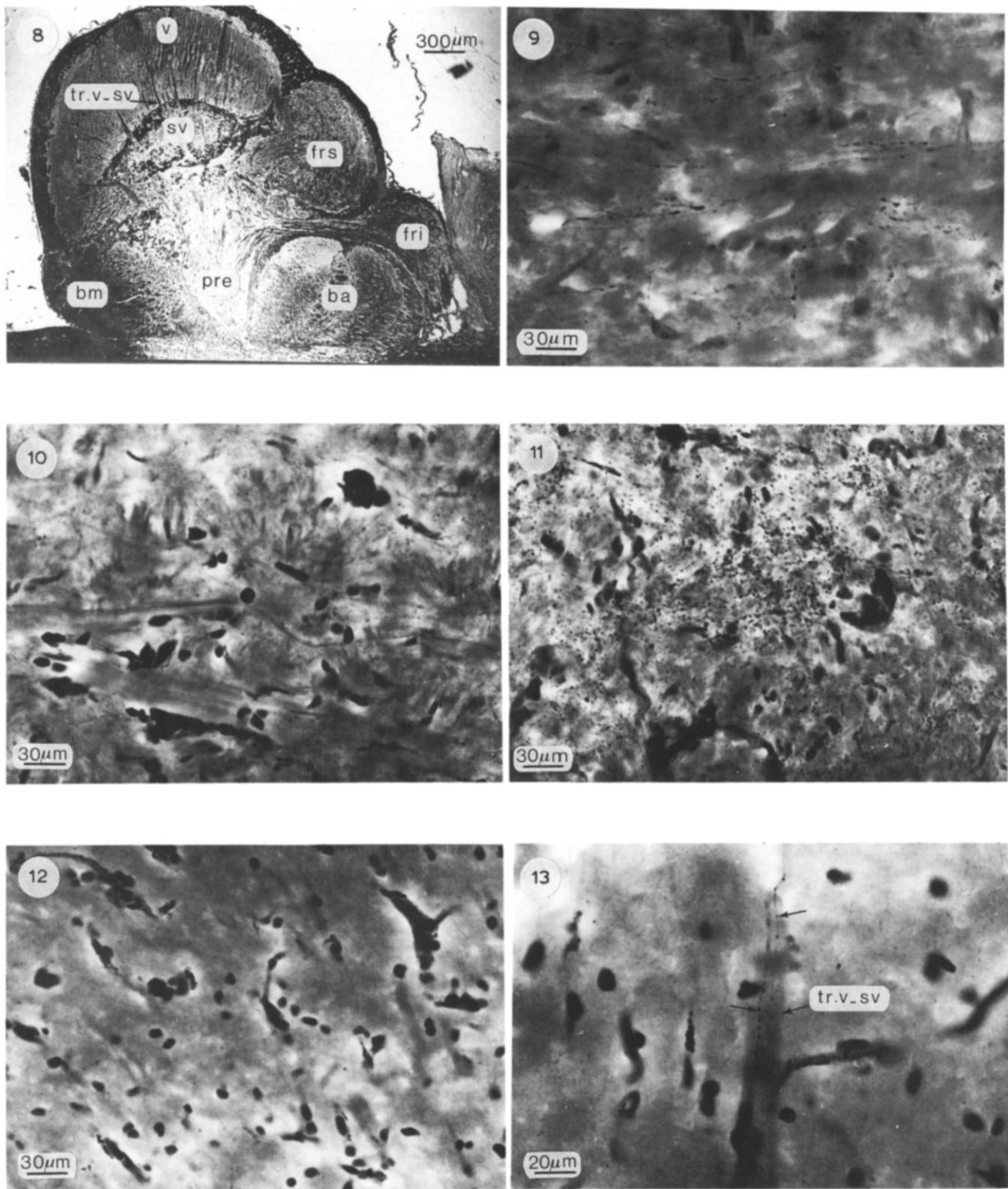


Fig. 8. Sagittal section of the supra-oesophageal mass. Cajal silver stain. ba, anterior basal lobe; bm, median basal lobe; fri, inferior frontal lobe; frs, superior frontal lobe; pre, precommissural lobe; sv, subvertical lobe; tr. v-sv, vertical to subvertical tract; v, vertical lobe.

Fig. 9. Transverse section of the anterior basal lobe. Detail. Degenerating fibres. Old animal (aged about 23 months). Fink-Heimer technique.

Fig. 10. Transverse section of the lateral part of the median basal lobe. Young animal (aged about 12 months). Fink-Heimer technique.

Fig. 11. Transverse section of the lateral part of the median basal lobe. Old animal (aged about 23 months). Terminal degeneration. Fink-Heimer technique.

Fig. 12. Transverse section of the vertical lobe. Old animal (aged about 23 months). Fink-Heimer technique.

Fig. 13. Transverse section of the vertical lobe. Detail. Old animal (aged about 23 months). Degenerating fibres in the vertical to subvertical lobe tract. Fink-Heimer technique. tr.v.-sv, vertical to subvertical lobe tract.

the dorsal part of the optic lobe, including all of the peduncle and olfactory lobes.

A detailed cartography of spontaneous degenerations in the central nervous system of *Sepia* and their evolution during aging will be the subject of a future publication. In this paper, we will limit ourselves to the most obvious localizations in senescent animals.

The signs of degeneration described in this paper were confirmed with a similar intensity in all the senescent animals studied. Generally accepted criteria<sup>10</sup> of definite signs of degeneration were noted: (i) impregnation of a large number of more or less spheroidal particles varying in diameter between 0.5 and 2  $\mu\text{m}$ . The heavily argyrophilic particles represent (classically speaking) degenerating terminal axon ramifications often referred to as 'terminal degeneration'.

(ii) fragmented fibers showing highly irregular shapes (globular, fusiform, droplike...) and an important increase in argyrophilia of the affected fibres.

*The optic lobe.* The optic lobes represent large nervous masses laterally situated between the eye and the supra-oesophageal mass. These lobes exhibit cortical differentiation (deep retina) and a complex neuropile with dispersed cellular areas. The optic lobes are involved in higher-order visual processing and in control of motor programs<sup>2,8,32</sup>. In the oldest animals, we found that the neuropilar areas showed definite signs of degeneration: degeneration granules and the beading of some fibres.

*The peduncle and basal lobes.* The peduncle and basal lobes present a similar plan of structure: a basal part with various sized neurons and a dorsal part or spine with only very small neurons and parallel fibres in the neuropile. The peduncle lobe is a small structure lying horizontally and dorsally on the optic tracts (Fig. 3).

The histological structure of this lobe has previously been studied<sup>3,12,20,30</sup>.

The outputs of this lobe mainly go to the ipsilateral optic lobe, to the contralateral peduncle lobe, to the basal lobes and to the lateral-pedal lobes (oculomotor centres).

By using the Fink-Heimer technique<sup>3</sup> studies have shown that the inputs of this structure in *Octopus* are characterized by a multimodal convergence particularly in the lateral bank of the peduncle spine. These authors also found that the visual inputs maintained the retinotopy in the basal part of this lobe, in contrast, spread among the whole median bank of the peduncle spine. In our experiment, few degenerating materials were found in the basal zone of the peduncle lobe of the older animals (Fig. 4), on the other hand very numerous degeneration granules were clearly visible in the median and lateral banks of the peduncle spine (Figs. 5 and 6).

The anterior basal lobe is a large structure of the su-

pra-oesophageal mass (Fig. 8). This structure is involved in higher control of the movements of the animal<sup>2,4,7,34</sup>.

Numerous degenerating materials (terminals and fibres) were observed especially in the anteromedian lobe, in the ventral part of the neuropile of the anterior basal lobe (Fig. 9) and in the spine of this structure.

The median basal lobe and associated structures are very large lobes in the posterior part of the supra-oesophageal mass. It was in these lobes that the most obvious degenerating material was localized, especially in the lateral parts of the median basal, lateral basal, dorso-basal and subpedunculate lobes (Figs. 10 and 11).

*The 'vertical lobe system'.* This system includes the vertical, superior frontal, inferior frontal, subvertical and precommissural lobes (Fig. 8). In cuttlefish, these structures are not directly affected by motor control<sup>2</sup> but play an important role in the learning processes<sup>18,23,35</sup>. We observed clear degenerating material in the neuropile of the subvertical, precommissural, superior frontal and inferior frontal lobes. In contrast hardly any or no degeneration granules were visible in the vertical lobe (Fig. 12).

However we must emphasize the presence of numerous degenerating fibres in the vertical to subvertical tracts (Fig. 13).

## DISCUSSION

### *The peduncle and basal lobes and the control of predatory behaviour*

The various connections of these structures situate them as visuomotor centres. Stimulation and lesion experiments<sup>2,4,15,16</sup> have shown the importance of these structures in the precise control of the movements of these animals. Several authors have suggested a functional role similar to the cerebellum of vertebrates for the peduncle lobe<sup>12,20,21,30</sup>.

Selective lesions of the anterior basal lobe<sup>4</sup> provoked, among other defects, an underestimation of the distance of tentacle ejection. This defect was also discovered in the older animals. The observation of numerous degenerating materials in the anterior basal lobe reinforced the hypothesis that this structure plays an important part in the control of this phase of predatory behaviour. A double system of visual input exists in the peduncle lobe of *Octopus*<sup>3</sup>, such is most probably also true for *Sepia*. Our histological results seem to indicate that only the divergent visual inputs were affected during the aging process. The functional significance of such a neural network remains problematic. The preserved retinotopy in the basal part of the lobe perhaps enables this structure to play an organizing role of the movements of the animal within

the visual space (e.g. collicular structures in vertebrates). On the other hand, regardless of the position of the prey in the animal's visual field, a stimulation of the median bank of the spine is possibly maintained. The projections of such a system on the basal part of the lobe may have an arousal effect on the attention mechanisms and on the maintenance of the predator's visual tracking behaviour. The defects in pursuit and the disinterest in the prey shown by the oldest cuttlefishes may be correlated to the deterioration of this diffused visual input.

The numerous degeneration granules observed in the lateral bank of the peduncle spine may signify a great deterioration of multiconvergent inputs (from statocysts, skin, the muscles of the mantle and the arms...) which are characteristics of this area.

Such systems, which are capable of dealing with sensorial information stemming from various sources, may play an important part in the programming of motor activities, especially in the case of feed-forward control<sup>13</sup>. It should however be noted at this point that final stages of the predatory behaviour in the cuttlefish are organized in open-loop control<sup>17</sup>.

The interpretation of dramatic degeneration in the neuropiles of the median basal, subpedunculate, lateral basal, dorsobasal and dorsolateral basal lobes is trickier. The functional significance of these structures still remains very obscure. Acute experiments<sup>2</sup> succeeded in setting off various motor effects by electrical stimulation of the median and lateral basal lobes.

In chronic experiments<sup>7</sup>, we only observed very limited motor responses, mainly affecting breathing and patterns of coloration.

One part of these structures (subpedunculate and dorsolateral lobes) adds to the innervation of the optic gland<sup>20</sup>. The latter would seem to be an endocrine organ and most usually has been attributed a role in the control of sexual maturation<sup>22,27</sup>.

Another function of this structure has been suggested<sup>29</sup>: it would seem to play a greatly inhibiting role on the feeding behaviour and may be responsible for triggering off the senescent processes. The highly conspicuous degeneration viewed in the structures of the posterior part of the supra-oesophageal mass would seem

to provide proof of the death of numerous neurons. It is perhaps this death which removes a central inhibition from the optic gland.

#### *The vertical system and memory process*

Our results on the oldest animals show significant poor scores in the retention test thus revealing a deterioration in the long-term memory process. We thus discover in the cephalopods a standard datum found in various animal species. The visual learning process has been the subject of numerous studies. Each study has shown that the vertical lobe and its associated structures play an important role. Its exact functional significance remains problematic: a privileged zone of treatment of the 'reading-into or reading-out-of' mechanisms of the memory store<sup>35</sup>.

In this context the low number of signs of degeneration in the neuropile of the vertical lobe is surprising. On the other hand, the input and output structures (superior frontal, subvertical and precommissural lobes) of this lobe show numerous signs of degeneration in the older animals. In newly hatched cuttlefish the very great postembryonic development of these structures (the vertical lobe in particular) was correlated with the improvement of performances of animals in learning experiments<sup>19</sup>. The baby cuttlefish also abandons its visual tracking behaviour when the prey moves out of its frontal visual field<sup>19</sup>. This phenomenon may be linked to the slight volume of the vertical system at birth and to the short-term memory processes which are as yet not fully developed. The defects, or absence of chase behaviour in the oldest animals may also be related to lesions which are linked to the senescence of the vertical system. This can be compared to the case of adult cuttlefishes with a lesion of the vertical system who did not pursue their prey once it had left the anterior visual field<sup>23</sup>.

**Acknowledgements.** We are grateful to Mrs Lesley Sourbe, English tutor at University of Caen for her assistance with the English translation. We also wish to thank Dr. N. Bons (E.P.H.E., Montpellier, France) and Dr. J. Armand for their advice on the Fink-Heimer technique. This research was supported by grants from the 'Ministère de la Recherche et de la Technologie' and the 'Fondation pour la Recherche Médicale'.

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