

## Developmental study of multiple memory stages in the cuttlefish, *Sepia officinalis*

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### Abstract

The inhibition of predatory behavior observed during the “prawn-in-the-tube” procedure has been extensively used in studies of cuttlefish learning. The present study examines the effect of age on the conditioning of this response in the cuttlefish, *Sepia officinalis*. Data show that a significant increase in acquisition performance occurs between 15 and 21 days of age. The retention curves in 8- and 15-day-old cuttlefish show a monotonic memory process, presumably reflecting the presence of only short-term memory. In 21-day-old cuttlefish, there are two distinct processes which could be a labile short-term memory, and a subsequent intermediate memory. These mnemonic systems seem to become more effective over the course of post-embryonic development. Moreover, the retention curves obtained in the oldest cuttlefish (30- and 90-day-old) bear a close resemblance to that observed in adults. These behavioral findings will allow further work on the cellular and molecular mechanisms of learning and memory in the cuttlefish.

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**Keywords:** Cephalopods; *Sepia officinalis*; Development; Associative learning; Short-, intermediate-, long-term memory

### 1. Introduction

Learning can be considered as the ability to acquire new information; this experience-dependent process allows living organisms to adopt adaptive behaviors according to environmental changes. Memory can be considered as the storage and recall of acquired experiences. Behavioral, cellular, molecular, and pharmacological studies have demonstrated that memory can be classically divided into three categories: short- (STM), intermediate- (ITM), and long-term memory (LTM) (Botzer, Markovich, & Susswein, 1998; Crow, Redell, Tian, Xue-Bian, & Dash, 2003; DeZazzo & Tully, 1995; Gibbs, Andrew, & Ng, 2003; Kane, Robichon, Dickinson, & Greenspan, 1997; Lechner, Squire, & Byrne, 1999; Lukowiak, Adatia, Krygier, & Syed, 2000; Ng et al., 1997; Rosenzweig,

Bennett, Colombo, Lee, & Serrano, 1993; Sanders & Barlow, 1971; Sangha, Scheibstock, McComb, & Lukowiak, 2003; Sutton, Ide, Masters, & Carew, 2002; Sutton, Masters, Bagnall, & Carew, 2001; Yin et al., 1994). STM persists for a short time, and is based on transient changes in synaptic morphology (Stork & Welzl, 1999), whereas ITM and LTM, the long-lasting forms, require new protein synthesis (Davis & Squire, 1984; Stork & Welzl, 1999).

Cuttlefish actively prey upon shrimp, capturing them by shooting out their two tentacles for a strike. This behavior, visually driven, has been extensively studied in laboratory conditions: a shrimp is placed in a glass tube in the middle of an experimental tank; the cuttlefish then attacks its prey but does not obtain it (Agin, Chichery, & Chichery, 2001; Agin, Chichery, Maubert, & Chichery, 2003; Bellanger, Dauphin, Chichery, & Chichery, 2003; Dickel, Boal, & Budelmann, 2000; Dickel, Chichery, & Chichery, 1997, 1998, 2001; Messenger, 1971). Under these conditions, *S. officinalis*

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promptly learns to inhibit the predatory motor pattern: the number of capture attempts (tentacle strikes) decreases with stimulus presentations. This learning has been recognized as a form of associative learning (Agin, Chichery, Dickel, & Chichery, 2006; Messenger, 1973a). Using a massed procedure (a single continuous 20-min training phase), with various retention times (between 2 min and 2 days), Messenger (1971) reported differential performance of memory recall in adult cuttlefish: they showed excellent retention of the task for between 2 and 8 min, with a recovery of the predatory response at around 22 min; then a very good level of retention beginning again 1 h after training. Messenger (1971) considered this retention curve to be a product of two memory stores: a labile STM lasting for a period of some minutes, and a LTM lasting at least 2 days, and probably more.

The cuttlefish is an ideal model for developmental studies as there is neither a planktonic nor a larval stage. Hatchlings emerge as miniature replicas of adults, assume a necto-benthic mode of life, and use identical prey capture strategies (Boletzky, 2003; Darmoillacq, Chichery, Poirier, & Dickel, 2004). As a consequence, the inhibition of predatory behavior makes a single learning paradigm applicable in this species at each stage of post-embryonic development. Using the “prawn-in-the-tube” massed procedure in juvenile cuttlefish, Dickel, Chichery, and Chichery (1998) recorded good 5-min retention performance from the age of 8 days, but poor 60-min retention performance was observed until the age of 15 days. The emergence of these learning abilities was correlated with the late maturation of the vertical lobe complex (Dickel, Chichery, & Chichery, 2001). While these results are of great interest in themselves, they do not extend to a detailed consideration of the nature of the learning curve in the juvenile cuttlefish. The present series of experiments is therefore designed to make a more complete investigation of this area.

## 2. Materials and methods

### 2.1. Subjects

*Sepia officinalis* juveniles ( $n = 330$ ) used in this experiment came from three egg batches that were maintained in the laboratory over a period of  $35 \pm 3$  days until hatching occurred (water temperature  $20 \pm 1^\circ\text{C}$ ). In order not to include prematurely hatched cuttlefish in the experiments, they were collected from those hatched on the day with the highest hatching proportion. We collected hatchlings at 9 a.m. from eggs that had hatched during the night. From the day of hatching, cuttlefish were reared communally in opaque plastic tanks ( $300 \times 300 \times 100$  mm;  $n = 10$  in each tank) with well-oxygenated sea water sterilized by UV light. They were fed daily ad libitum with shrimp of suitable size. They were tested at 8, 15, 21, 30, and 90 days post-hatch (average dorsal mantle length of  $9.86 \pm 0.6$  mm,  $12.53 \pm 1.08$  mm,  $14.49 \pm 0.91$  mm,  $16.26 \pm 1.59$  mm, and  $43.57 \pm 5.46$  mm, respectively). Although, in the English Channel the cuttlefish reaches sexual maturity around 12–15 months, we selected the period from 8 to 90 days as previous studies have shown a significant improvement in learning and memory capacities during the first 3 months of post-embryonic life (Dickel et al., 2001). During this time, there is an important growth of associative nervous structures, which reach proportions similar to those found in adults (Dickel et al., 2001). Cuttlefish were transferred to individual tanks ( $120 \times 90 \times 60$  mm for 8- to 30-day-old animals;  $210 \times 90 \times 60$  mm for 90-day-old animals) 24 h prior to the start of the experiments. Each tested cuttlefish was naive at the outset and was used

only once. Good ethical standards have been followed in the care, housing, and use of the subjects in our study.

### 2.2. Procedure

The experimental apparatus is identical to that described by Messenger (1971). Briefly, five shrimp were enclosed in a transparent glass tube filled with seawater, of a diameter adapted to that of the young cuttlefish (15 mm diameter for 8- to 21-day-old animals; 30 mm diameter for 30- and 90-day-old ones). The glass tube was introduced into the tank 12 h prior to the start of the learning session, during the time the shrimp were concealed behind an opaque plastic cylinder placed around the tube. Before the experiment, cuttlefish were underfed for 12 h (only one prey per cuttlefish). The glass tube containing the prey was visible to the cuttlefish for a single session of 20 min (training phase). We used a 20 min duration for the training phase, as we demonstrated in a previous study (Agin, Dickel, Chichery, & Chichery, 1998) that a shorter duration (5 min) of initial exposure to the glass tube containing the prey does not allow sufficient learning in young cuttlefish. During this time, the number of strikes that the cuttlefish exhibited was counted. The exact time at which training was considered to have commenced was the moment of the first tentacle strike on the glass. At the end of the training phase, the opaque cylinder was again gently placed around the glass tube (this took 3–4 s) and left for 2, 4, 8, 10, 15, 20 or 60 min, during the time the cuttlefish were not fed (resting interval). The lengths of rest-intervals were chosen according to the literature regarding adult cuttlefish (Messenger, 1973a). At the end of the resting interval, the opaque cylinder was removed and the number of strikes during a single 3 min session (testing phase) was counted to determine the level of memory recall for the inhibition of predatory behavior. We demonstrated in a previous study (Agin et al., 1998) that the manipulations made between the training and testing phases to the opaque cylinder used to hide the prey do not impair the predatory behavior in young and adult cuttlefish.

### 2.3. Data analyses

Acquisition and retention performances were analyzed within and between groups and subgroups. Parametric tests were performed to analyze acquisition performance (age groups). However, for analysis of retention performance (resting interval subgroups), data were typically not normally distributed, and thus were analyzed with non-parametric tests. Statistical analyses were conducted with the Systat software package (version 5.02). All  $P$  values are two-tailed. To assess acquisition performance within each age group, the number of tentacle strikes exhibited by the cuttlefish during the first 3 min of the training phase (A) was compared with the number observed during the last 3 min of the training phase (B; Paired Student's  $t$ -test). To compare acquisition between groups, it was necessary to correct for any potential differences in initial levels of predation in the cuttlefish. Thus, the number of tentacle strikes during B was expressed as a percentage of A. The statistical significance of differences between groups was evaluated using ANOVA followed by Tukey's HSD tests as post hoc tests. To assess retention performance within each subgroup (resting interval), the number of tentacle strikes exhibited during A was compared with the number observed during the 3 min of the testing phase (C; Wilcoxon matched pair tests). To compare retention performance between subgroups, it was necessary to correct for any potential differences in the initial levels of predation of the cuttlefish. Thus, numbers of tentacle strikes during C were expressed as a percentage of A. Kruskal–Wallis tests were used for multiple comparisons. When significant, Mann–Whitney  $U$ -tests were performed as post hoc tests.

## 3. Results

### 3.1. Acquisition performance

Cuttlefish from all age groups made significantly fewer tentacle strikes during the last 3 min of the training phase when compared to their initial scores (8-day-old cuttlefish:

$t = 15.239$ ,  $P < .001$ ; 15-day-old cuttlefish:  $t = 14.343$ ,  $P < .001$ ; 21-day-old cuttlefish:  $t = 13.909$ ,  $P < .001$ ; 30-day-old cuttlefish:  $t = 18.226$ ,  $P < .001$ ; 90-day-old cuttlefish:  $t = 13.670$ ,  $P < .001$ ; Fig. 1). Thus, the decrease in strike rate during the training phase clearly shows that young cuttlefish inhibit their predatory behavior towards the prey

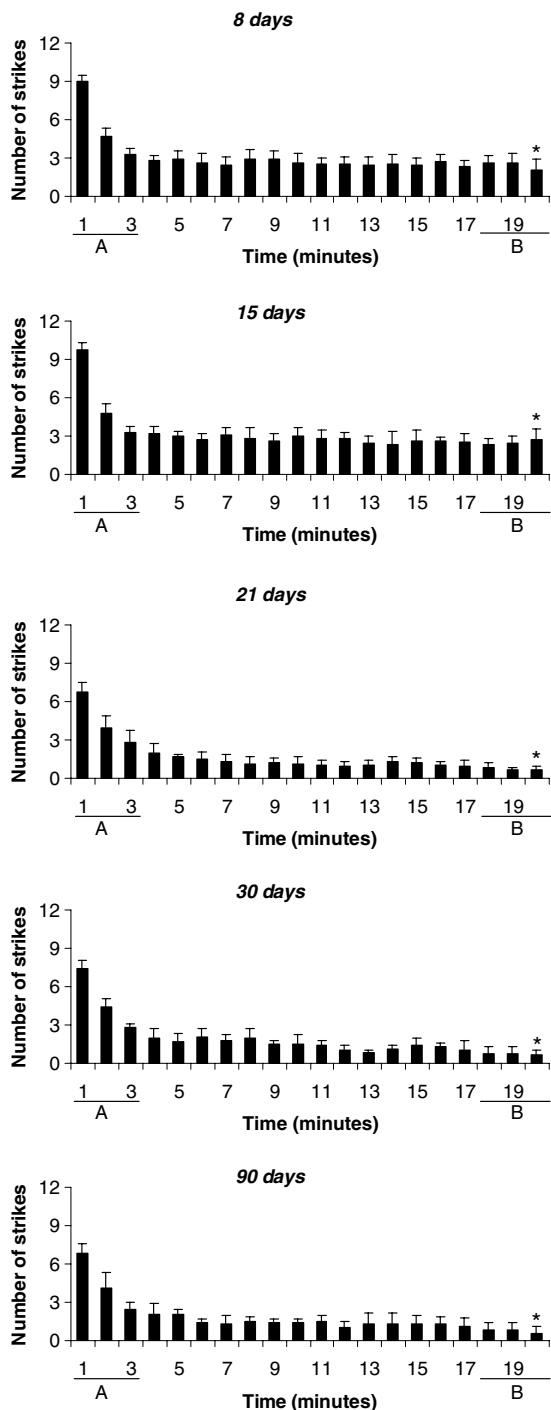


Fig. 1. The number of tentacle strikes during training phase. A and B: first and last 3 min of the training phase, respectively. Error bars indicate SEM. For all groups other than 90 days,  $n = 70$ ; for 90 days,  $n = 50$ . Asterisk indicates significant difference between B and A (Paired Student's  $t$ -test,  $*P < .001$ ).

enclosed in the glass tube. In other words, cuttlefish learned the task even at 8 days of age. However, ANOVA yielded a significant age effect on training performance ( $F(4, 325) = 25.363$ ,  $P < .001$ ). Post hoc tests revealed that acquisition performance was significantly better for 21-, 30-, and 90-day-old cuttlefish than for 8- and 15-day-old cuttlefish (8- versus 21-, 30- or 90-day-old cuttlefish,  $P < .001$ ; 15- versus 21-, 30- or 90-day-old cuttlefish,  $P < .001$ ; Fig. 2). Furthermore, the acquisition performance did not differ significantly between the youngest cuttlefish (8- versus 15-day-old cuttlefish,  $P = .999$ ; Fig. 2) or between the oldest cuttlefish (21- versus 30-day-old cuttlefish,  $P = .985$ ; 21- versus 90-day-old cuttlefish,  $P = .945$ ; 30- versus 90-day-old cuttlefish,  $P = .999$ ; Fig. 2). These results show that a significant increase in acquisition performance occurs between 15 and 21 days of age.

### 3.2. Retention performance

8-, 15-, 21-, and 30-day-old cuttlefish made significantly fewer tentacle strikes during the testing phase, performed after a 2-, 4-, 8-, 10-, 15- or 20-min resting interval, when compared to their initial scores (Table 1). These results show retention of the task. Ninety-day-old cuttlefish also exhibited significant retention after a 2-, 4-, 8-, 10- or 20-min resting interval (Table 1); though, surprisingly, they showed poor retention performance after a 15-min resting interval (Table 1). The response at 60 min did not differ significantly when compared to the initial response in 8-day-old cuttlefish (Table 1). This signifies a poor retention of the task at 60 min in these cuttlefish. However, the retention after a 60-min resting interval became significant in 15-day-old cuttlefish although the performances were again low (Table 1). From 21 days on, retention performance at 60 min showed a strong improvement (Table 1).

To simplify a comparison between our data and those of Messenger (1973a) experimenting with adult cuttlefish, the results are represented in the form of a curve (Fig. 3). Since the strike-level is a measure of learning (the fewer the strikes the better the memory), this complex curve rep-

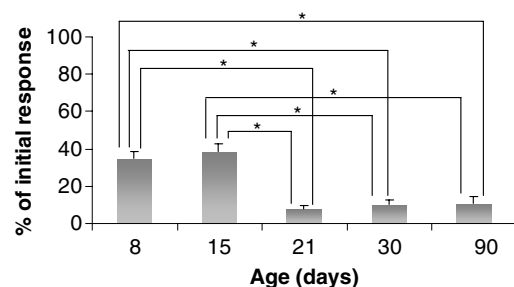


Fig. 2. The number of tentacle strikes during the last 3 min of the training phase expressed as a percentage of the number of tentacle strikes during the first 3 min of the training phase. Error bars indicate SEM. For all groups other than 90 days,  $n = 70$ ; for 90 days,  $n = 50$ . Asterisk indicates significant difference between groups (Tukey's HSD test,  $*P < .001$ ).

Table 1

Comparisons of the number of tentacle strikes exhibited during the first 3 min of the training phase (A) with the number observed during the three minutes of the testing phase (C)

	Group				
	8-day-old cuttlefish	15-day-old cuttlefish	21-day-old cuttlefish	30-day-old cuttlefish	90-day-old cuttlefish
<i>Subgroup</i>					
2 min	$Z = -2.809; P < .01^*$	$Z = -2.670; P < .01^*$	$Z = -2.805; P < .01^*$	$Z = -2.812; P < .01^*$	$Z = -2.366; P < .05^*$
4 min	$Z = -2.659; P < .01^*$	$Z = -2.668; P < .01^*$	$Z = -2.812; P < .01^*$	$Z = -2.805; P < .01^*$	$Z = -2.521; P < .05^*$
8 min	$Z = -2.812; P < .01^*$	$Z = -2.397; P < .05^*$	$Z = -2.807; P < .01^*$	$Z = -2.809; P < .01^*$	$Z = -2.533; P < .05^*$
10 min	$Z = -2.803; P < .01^*$	$Z = -2.703; P < .01^*$	$Z = -2.812; P < .01^*$	$Z = -2.505; P < .05^*$	$Z = -2.366; P < .05^*$
15 min	$Z = -2.803; P < .01^*$	$Z = -2.652; P < .01^*$	$Z = -2.814; P < .01^*$	$Z = -2.347; P < .05^*$	$Z = -1.472; P = .141$
20 min	$Z = -2.383; P < .05^*$	$Z = -2.703; P < .01^*$	$Z = -2.675; P < .01^*$	$Z = -2.553; P < .05^*$	$Z = -2.207; P < .05^*$
60 min	$Z = -1.686; P = .092$	$Z = -2.041; P < .05^*$	$Z = -2.705; P < .01^*$	$Z = -2.805; P < .01^*$	$Z = -2.375; P < .05^*$

Asterisk indicates significant difference between A and C (Wilcoxon matched pair tests).

resents a type of “forgetting” curve. In the youngest cuttlefish (8-day-old), retention, good after a 2-min resting interval (18% of the initial response), promptly deteriorated after a 4-min resting interval (40% of the initial response), towards bad performance at 60 min (61% of the initial response). Similar results were observed in 15-day-old cuttlefish. In 21-day-old cuttlefish, a Kruskal–Wallis test revealed a significant resting interval effect on retention performance ( $H(6) = 17.867, P < .01$ ). Post hoc tests confirmed that the good retention performance obtained at 2 and 4 min (13 and 25%, respectively, of the initial response) decreased markedly around 8 min (38% of the initial response; 2 versus 8 min,  $U = 20.5, P < .05$ ). Thereafter, they improved until 15 min (14% of the initial response; 8 versus 10 min,  $U = 88.5, P < .01$ ; 8 versus 15 min,  $U = 79, P < .05$ ). At 20 min, retention had again decreased (39% of the initial response; 10 versus 20 min,  $U = 15, P < .01$ ), but had then increased at 60 min (20% of the initial response; 20 versus 60 min,  $U = 76.5, P < .05$ ). These paradoxical results indicate that cuttlefish showed better retention of the stimulus-situation after 10 min than after 8 min, and after 60 min than after 20 min. In 30-day-old cuttlefish, a Kruskal–Wallis test yielded a significant difference in retention performance between subgroups ( $H(6) = 17.231, P < .01$ ). Post hoc tests confirmed that the poorest retention performance was observed after a 15-min resting interval (2 versus 15 min,  $U = 2, P < .001$ ; 4 versus 15 min,  $U = 18, P < .05$ ). A recovery of the response was indeed observed after 15 min (66% of the initial response), and thereafter retention of the task gradually improved, and an excellent score was reached at 60 min (17% of the initial response; 15 versus 60 min,  $U = 84.5, P < .01$ ). In 90-day-old cuttlefish, a Kruskal–Wallis test again revealed a significant resting interval effect on retention performance ( $H(6) = 17.072, P < .01$ ). The oldest cuttlefish exhibited a retention curve similar to that of the 30-day-old cuttlefish, with, however, a stronger recovery of the response at 15 min (70% of the initial response, 2 versus 15 min,  $U = 3, P < .01$ ; 4 versus 15 min,  $U = 5, P < .01$ ; 10 versus 15 min,  $U = 5, P < .05$ ). Afterwards, retention performance improved again to finish significantly higher at 60 min (9% of the initial response, 15 versus 60 min,  $U = 46, P < .01$ ).

#### 4. Discussion

Our data are consistent with earlier studies in showing that, from 8 days of age, all behavioral components of predatory behavior are present (Boletzky, 2003; Darmaillacq et al., 2004). Moreover, the results show a marked improvement in acquisition performance between 15 and 21 days of age.

In all groups, good performance was recorded during the short-term retention tests (2-, 4-, and 8-min resting interval). We have previously demonstrated in young and adult cuttlefish that the low rate of capture attempts during the testing phase correspond to a specific learning effect and not to some non-specific such as “contextual fear” in experimental conditions (Agin et al., 1998). It appears that, at as early as 8 days of age, a continuous 20-min presentation of living prey in a glass tube produces sufficient inhibition of predatory behavior to cause good retention performance. However, 8-day-old cuttlefish showed poor performance after a 60-min resting interval. Despite a slight improvement, performance was only moderate at 15 days of age. Taken together, our data concur with earlier observations using the “prawn-in-the-tube” massed procedure in showing good short-term memory capacities, and low 60-min retention performance during the first days of post-embryonic life (Dickel et al., 1998). Furthermore, earlier studies of learning during development (Dickel et al., 2001; Messenger, 1973b) have reported poor acquisition performance in 8-day-old cuttlefish with a “prawn-in-the-tube” spaced procedure (eight successive presentations of the glass tube, lasting 3 min each, separated by 30 min). Our results are in accordance with these studies since, in spaced procedure, the interval of time between sessions (30 min) was longer than was the cuttlefish’s memory capacity required to obtain good training performance.

The retention curves merit a closer analysis. Performance in 8- and 15-day-old cuttlefish, good after a 2-min resting interval, progressively decreased as the resting interval approached 60 min. Recovery seems to be a single process, increasing in a linear fashion with time. However, having limited the analysis of retention period to 60 min, it is difficult to definitely conclude the absence of polyphasic retention curves in the youngest cuttlefish. In the oldest cuttlefish, the retention curves are neither simple



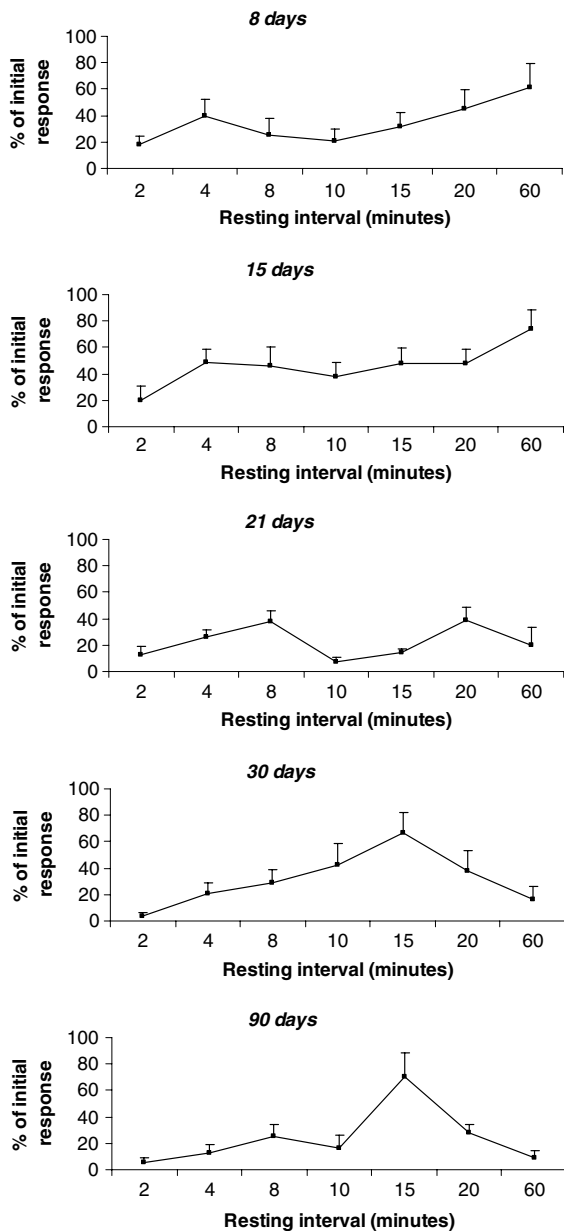


Fig. 3. The number of tentacle strikes during the 3 min of the testing phase expressed as a percentage of the number of tentacle strikes during the first 3 min of the training phase. Error bars indicate SEM. For all groups other than 90 days,  $n = 10$  for 2, 4, 8, 10, 15, 20, and 60 min; for 90 days,  $n = 6$  for 20 min,  $n = 7$  for 2, 10, 15, and 60 min,  $n = 8$  for 4, and 8 min.

nor monotonic: instead, there are temporary reversals in retention. Thus, our results may support the hypothesis that cuttlefish, around the age of 21 days, go through a “sensitive” period concerning the development of mnemonic mechanisms, as retention appears to depend on two distinct processes. The first reaches its peak within minutes of the end of stimulation, and decays as the resting interval approaches 8 min, while the second reaches its peak 10 min after stimulation. The critical point at 8 min may equate with that recorded by Messenger (1971) at 22 min in adult cuttlefish. We have noticed an evolution of this retention curve in cuttlefish over 30 days old. The two

processes seem then to become more effective, since they persist until 10 and 60 min, respectively. The cuttlefish also display a strong recovery of the response, but with a temporal displacement at 15 min. These data would suggest an extension of the STM in the course of the post-embryonic development. It is interesting that the retention curve exhibited by 21- to 90-day-old cuttlefish bears a close resemblance to that obtained in adults by Messenger (1971), whose data suggest the operation of two different memory systems, a STM and a LTM. The STM appears to build up rapidly (2 min), and to decay fairly quickly as the resting interval approaches 8 or 15 min depending on the age of cuttlefish. It is interesting to note that the critical point at 15 min that we obtained in 30- and 90-day-old cuttlefish is close to the point at 22 min obtained in adults by Messenger (1971). The LTM, on the other hand, appears to develop more slowly, at least under this experimental design. LTM would seem to become fully operative about 10 min after stimulus-presentation, and then to decline rapidly in 21-day-old cuttlefish; from 30-day-old cuttlefish it appears to persist until 60 min after stimulus-presentation. These results suggest a time lag in short- and long-term retention in the course of post-embryonic development. However, the hypothesis of a functional LTM as early as 10 min after stimulation seems difficult to conceive. Studies of memory formation in various species have identified multiple stages that can be differentiated on the basis of the relative contribution of signal transduction pathways, protein synthesis, and gene induction. For example, in the chick, three sequentially dependent stages have been identified: a STM stage lasting approximately 10 min after avoidance learning; an ITM stage accessible between 20 and 50 min post-learning, and a LTM stage accessible from 60 min after learning (Ng & Gibbs, 1991). With regard to the retention curve obtained in adult cuttlefish by Messenger (1971), memory may be built according to three successive stages, identified as STM, ITM, and LTM. Thus, the STM stage would build up rapidly during training but would decay fairly quickly (so that by 22 min or so it has disappeared); the ITM stage would follow, becoming fully effective at 60 min, even though the LTM stage would be available beginning at 60 min after learning, then diminishing slightly but persisting at about the same level for at least two days, and possibly much longer. Our results in 21- to 90-day-old cuttlefish would be consistent with this assumption: a labile STM (2–4 min at 21 days of age; 2–10 min at 30 and 90 days of age), and a subsequent ITM (10–15 min at 21 days of age; 20–60 min at 30 and 90 days of age). We think that the LTM would be very likely to build up afterwards. Our recent work using a protein synthesis inhibitor (cycloheximide) is consistent with this assumption: we have established that *de novo* protein synthesis between 1 and 4 h post-training is an essential event in the formation of long-lasting memory (24 h after stimulus-presentation) of this learning task (Agin et al., 2003).

Further experiments will be needed to confirm the existence of multiple forms of memory in the cuttlefish. Previous studies have shown that LTM requires transcription processes, while ITM requires only translation processes (McGaugh, 2000; Sangha et al., 2003). Injections of cycloheximide (translation blocker), and actinomycin D (transcription blocker), with testing phases at different times after training could help us to gain more knowledge of the different forms of long-lasting memory in the cuttlefish. A complementary approach for identifying the multiple forms of memory could be to examine how the brain circuitry underlying information storage is reorganized over time. Using cytochrome oxidase (CO) histochemistry in cuttlefish, we provide the first metabolic evidence for the involvement of the superior frontal lobe in memory processes (Agin et al., 2001). Future works using functional brain imaging could also focus on other cerebral regions at different times for retention of the passive avoidance learning in an attempt to gain more knowledge of the cerebral systems involved in different forms of memory.

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