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Research report

The "prawn-in-the-tube" procedure: What do cuttlefish learn and memorize?

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HIGHLIGHTS

- ► In cuttlefish neurobiology of learning is studied with the prawn in the tube procedure.
- ► The nature of cues and reinforcement involved in this learning remains unclear.
- ► The main reinforcement of this learning is the absence of food intake.
- ► Cuttlefish can perceive the tube because of light polarization.
- ► Cuttlefish rely on both tactile and visual cues to memorize the presence of the tube.

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ABSTRACT

For several decades the "prawn-in-the-tube" procedure has been extensively used in the exploration of behavioral plasticity and its neural correlates in cuttlefish. Although the nature of the task has been characterized, the effect of reinforcement and the extent of different cues cuttlefish can use to solve and memorize the task remain unclear. To determine whether cuttlefish learned to inhibit predatory behavior because of pain incurred when the tentacles hit the glass tube, the shrimp prey (typically attacked with a tentacle strike) was replaced by crabs (normally caught by a jumping strategy, using all eight arms together, which is thought less likely to be painful). We showed that the cuttlefish is still capable of learning inhibition of predatory behavior when it adopts another catching strategy, which suggests that pain from the tentacles hitting the tube has little effect on the learning process. The two latest experiments have shown that cuttlefish do not learn to inhibit predatory behavior towards a specific type of prey, but rather learn and memorize visual (light polarization) and tactile information from the glass tube.

The "prawn-in-the-tube" procedure is a powerful and user-friendly tool in the investigation of the processing and retention of multisensory information in invertebrates. Our recent findings now open up new areas of investigation into the neural correlates of learning and memory processes in cuttlefish.

1. Introduction

Learning is generally considered as the ability to acquire new information [1–3]. This experience-dependent process allows living organisms to adopt adaptive behaviors according to environmental changes. Memory can be defined as the storage and recall of acquired experiences [1,2]. For decades, the reductionist approach has recommended the use of simple organisms and basic learning (associative and nonassociative) with easily identifiable stimuli or association [4]. Among marine invertebrates, Aplysia is a powerful tool extensively used to study the cellular and molecular bases of simple learning (e.g. habituation, sensitization or pavlovian conditioning) and memory [5]. However, the simplicity of this system does not allow an integrative approach with a higher level of analysis (behavior, information processing, etc.). Cephalopods have developed the most sophisticated CNS of all invertebrates (although relatively simpler than that of vertebrates) and exhibit unexpected behavioral abilities that are comparable to those of vertebrates [6–9], and so they appear to be a good compromise between more and less complex systems. For this reason, they have been extensively studied for their learning abilities [10–13] as well as for the plasticity of their predatory behavior [12–18].

The cuttlefish, *Sepia officinalis*, preys upon various crustaceans and fishes using two different strategies of attack [19–21]. Fast moving prey such as shrimp and fish are attacked by rapidly striking out with both tentacles (hereafter called strike/striking strategy; 15 ms in adult, [22]); slow moving prey such as crabs are preferentially seized quickly from behind with all eight arms together (hereafter called jumping attack/jumping strategy; 248 ms in adult, [23]). The "prawn-in-the-tube" procedure has made extensive use of the striking strategy to study learning and memory abilities in the cuttlefish [24–32]. In this procedure cuttlefish, presented with a glass tube containing moving shrimp, strikes at the prey but can

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never obtain it [26]. Under these conditions, the number of tentacle strikes decreases with time demonstrating that the cuttlefish has learned to inhibit its predatory behavior. For this learning to pass into long-term memory, consolidation processes based on de novo protein synthesis are required [33]. This learning is apparently very simple and practical, but to be reliably used in functional neurobiology, it is of crucial importance to characterize more clearly the nature of (i) learning, (ii) reinforcement, (iii) the different cues cuttlefish can rely on to solve and memorize the task. If the first point has been clarified, and there is now a consensus that the inhibition of predatory behavior in the cuttlefish is the result of associative learning [26,28,29,34,35], the last two points remain unclear. Indeed, it has often been claimed that when the tentacle club hit the glass tube, the cuttlefish experienced pain, resulting in the inhibition of the predatory behavior. However, a cuttlefish with its tentacles cut, so that it cannot strike the tube, can still learn to inhibit its predatory behavior, even if this takes longer. Moreover, Dickel et al. [31] showed that the higher the number of tentacle strikes at the beginning of the learning process, the longer the time needed for the animal's predatory behavior to be inhibited. Thus, although the "pain hypothesis" might suggest otherwise, a high number of tentacle impacts on the glass tube does not make the animal learn faster. Lastly, the glass tube is probably not as "invisible" to the cuttlefish as was previously thought. They are sensitive to the linear polarization of light, this sensitivity has been shown to help them detect transparent prey [36,37]. Therefore it seems likely that cuttlefish might perceive other transparent objects, like a glass or a Perspex tube. Interestingly, some authors reported that cuttlefish readily caught shrimp or crab presented outside the tube after learning had been achieved [31,32].

In this study we examined whether the learned inhibition of predatory behavior was due to the painful impact of the tentacles on the glass tube. To this end, in a first experiment, we used crabs instead of shrimp in the tube because they are caught by the jumping strategy rather than by tentacle strikes. In the other two experiments, we examined whether this learning was species-specific (do they learn that a given prey species is unreachable?) or contextual (do they learn to inhibit their predatory behavior when they see the glass tube?). To address the former question we used different types of prey for the training phase and for the retention phase, and for the latter question we used a depolarizing filter to attenuate the visual cues from the tube itself.

2. Methods

2.1. Animals

Cuttlefish eggs, *Sepia officinalis*, were obtained from fishing in the vicinity of Luc-sur-Mer, France. Forty cuttlefish were reared from hatching to 2 months in laboratory conditions with running oxygenated seawater at 15 ± 1 °C at the Centre de Recherches en Environnement Côtier (CREC, Luc-sur-Mer, France). They were housed in groups in enriched (or "semi natural") tanks (80 × 60 × 40 cm) following the procedure established by Dickel et al. [31], who showed that an enriched environment has positive effects on growth rates as well as learning and retention of information. They were fed daily with live shrimp (*Crangon crangon*) and crabs (*Carcinus maenas*) of suitable size. Twenty-four hours before behavioral experiments began; they were housed individually in an experimental circular tank (30 cm in diameter × 8 cm deep) with a shelter. At the beginning of the experiments, the mean dorsal mantle length of the cuttlefish was 5.6 ± 0.8 cm (mean ± sem).

2.2. General procedure

A glass tube (3 cm in diameter and 8 cm in high) containing 5 living prey items was introduced into the experimental tank 30 min prior to the start of the training session. It was placed opposite the entrance to the shelter where the cuttlefish has settled, and covered by an opaque plastic cylinder so that the cuttlefish cannot see the prey. After 30 min, the plastic cylinder was gently removed to allow the cuttlefish to see the unreachable prey. The latency of the first attack by the cuttlefish on the prey in the tube was measured and the training session began with this first attack. To ensure that the same criterion of learning was used for all cuttlefish, the glass container was presented continuously to each cuttlefish until only one attack was made in three consecutive minutes after the 18th minute (i.e., minimum duration of initial training was 21 min [31]). The number of attacks (strikes and jumps) was counted in 3 min time blocks (called T1, T2, etc.). At the end of the training session, the opaque cover was replaced onto the tube. The retention session was performed after 60 min to test the long-term memory capabilities of the cuttlefish. At this time the cover was removed from the glass tube so that the cuttlefish could see the prey again. The latency of first attack was recorded. The retention session began with this first attack and the number of subsequent attacks was counted. The retention session was limited to 6 min, resulting in two time blocks R1 and R2.

2.3. Experiments

Three sets of experiments were conducted. In the first experiment, the glass tube contained crabs (1 cm of carapace width) instead of shrimp during training and retention sessions (Cr–Cr group, $n = 10$). In the second experiment, one group was tested with crabs during training and shrimp during retention (Cr–Sh group, $n = 10$), a second group (Sh–Cr group, $n = 10$) with shrimp during training and crabs during retention. In the third experiment, cuttlefish were trained against crabs contained in a glass tube (with no filter–NF) and tested for their retention performances with crabs in the same glass tube depolarized thanks to a transparent depolarizer (NF–DF group).

2.4. Statistical analysis

Data were analyzed using StatXact 7 (Cytel Studio software) and Statview. All analyses used a significance threshold of $\alpha = 0.05$.

Friedman tests were used to compare the evolution of the number of striking and jumping attacks over training time in the Cr–Cr group. If the null hypothesis was rejected, Permutation tests were used for post hoc pairwise comparisons between time blocks of training and retention and Bonferroni corrections were applied.

Permutation tests for paired samples were used to compare the latency of first attack between training and retention in a same group. Kruskal–Wallis tests were used to compare the latency of first attack among groups. If the null hypothesis was rejected, Permutation tests were used for post hoc pairwise comparisons between groups. The same tests were used to compare the number of attacks between the end of training (T6) and the beginning of the retention (R1) and between groups.

3. Results

3.1. Nature of the reinforcement involved in associative learning

To assess whether cuttlefish learned to inhibit predatory behavior because of pain incurred when the tentacles hit the glass tube, we used crabs too big to be caught by tentacles alone, thus encouraging the cuttlefish to use the jumping strategy involving all eight arms. The jumping strategy is considered to be less painful than tentacle strikes. Under these conditions, we observed that the cuttlefish sometimes tried to catch the crabs with their tentacles (12% of the total number of attacks). However, the number of strikes remained small and did not change over the course of training ($P = 0.0987$) (Fig. 1). On the contrary, the number of jumping attacks

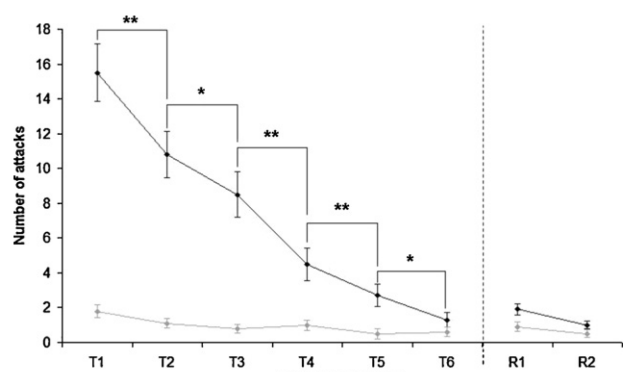

Fig. 1. Mean number (±sem) of attacks on the glass tube containing crabs during training (T1–T6) and retention (R1–R2). A delay of 1 h with an opaque cover on the tube was applied between training and retention. Two types of predatory behaviors are represented: — jumping attacks; ▬ striking attacks. Asterisks indicates significant difference using a Permutation test for related samples (* $P < 0.05$; ** $P < .01$).