A biphasic memory curve in the chambered nautilus, *Nautilus pompilius* L. (Cephalopoda: Nautiloidea)

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Accepted 2 April 2008

SUMMARY

Cephalopods are an exceptional taxon for examining the competing influences of ecology and evolutionary history on brain and behaviour. Coleoid cephalopods (octopuses, cuttlefishes and squids) have evolved specialised brains containing dedicated learning and memory centres, and rely on plastic behaviours to hunt prey effectively and communicate intricate visual displays. Their closest living relative, the primitive nautilus, is the sole remnant of an ancient lineage that has persisted since the Cambrian. Nautilus brains are the simplest among the extant cephalopods, and the absence of dedicated learning and memory regions may represent an ancestral condition. It is assumed that the absence of these regions should limit memory storage and recall in nautilus, but this assumption has never been tested. Here we describe the first evidence of learning and memory in chambered nautilus (*Nautilus pompilius*). Using a Pavlovian conditioning paradigm, we demonstrate that chambered nautilus exhibits temporally separated short- and long-term memory stores, producing a characteristic biphasic memory curve similar to that of cuttlefishes. Short-term memory persisted for less than 1 h post-training, whereas long-term memory was expressed between 6 and 24 h after training. Despite lacking the dedicated neural regions that support learning and memory in all other extant cephalopods, nautilus expressed a similar memory profile to coleoids. Thus the absence of these regions in the nautilus brain does not appear to limit memory expression, as hypothesised. Our results provide valuable insights into the evolution of neural structures supporting memory.

Key words: Nautilus, cephalopod, learning, memory, classical conditioning.

INTRODUCTION

The chambered nautilus, *Nautilus pompilius* (Linnaeus 1758) is one of a handful of surviving species of externally shelled cephalopods that flourished between 450 and 150 million years ago (Dzik, 1981; Teichert, 1988). The apparent similarity between living nautiluses and ancestral cephalopods, coupled with the more recent diversification of soft-bodied cephalopods from externally shelled ancestors, suggest that nautilus may provide important insights into the evolution of complexity in invertebrate nervous systems. Investigations of the putative primitive state of the nautilus central nervous system (Young, 1965; Shigeno et al., 2007) are likely to be valuable to the fields of both evolutionary biology and neurobiology.

In many respects nautilus is an anomaly among modern cephalopods. It is long-lived, slow growing and largely sedentary, scavenging for its food rather than actively hunting prey (Saunders, 1985; Hanlon and Messenger, 1996). It lives predominately in deep, cold waters surrounding the coral reefs of the Indo-Pacific, and makes diurnal migrations from dark and deep water (>300 m) where it spends daylight hours to shallower, warmer waters (<75 m) to forage during darkness (Carlson et al., 1984; Ward et al., 1984). Learning and memory may be of use during these repetitive daily movements across familiar terrain.

Light penetration in the deep ocean habitat of nautilus is minimal, and it is likely that nautilus relies mostly on olfaction and touch to locate food sources, in contrast to the visually oriented hunting behaviours of many coleoids (Saunders, 1985). The structure and visual acuity of the primitive, lensless eye lends support to the hypothesis that vision is of limited use (Muntz, 1986; Muntz, 1987). Nautilus forages in darkness by tracking odour with its rhinophores and tentacles, which typically fan out and extend in a characteristic search posture (‘cone of search’) when the animal senses odours (Bidder, 1962; Basil et al., 2000; Basil et al., 2005). The efficiency with which nautiluses locate food sources suggests they are specialised for exploiting a patchy and changeable resource distribution, and may rely on local information to forage rather than investing in memory of feeding locations.

The central nervous system (CNS) of nautilus reflects the heavier reliance on olfactory rather than visual processing, but is similar in overall structure to the coleoid CNS. The thirteen main lobes are not clearly differentiated from the surrounding tissue (Young, 1965) and there appears to be little specialisation. The vertical and sub-frontal lobes, regions of the brain that have been implicated in tactile and visual learning and memory in coleoids (Young, 1960; Young, 1961; Hochner et al., 2003) are entirely absent from the nautilus CNS (Young, 1965), and there is some evidence that the structural simplicity of the brain may be representative of an ancestral condition (Young 1991; Shigeno et al., 2007).

Learning and memory are well known in coleoids – cuttlefish and octopuses have yielded a wealth of information about physiological bases of invertebrate memory, and have demonstrated impressive learning abilities (for reviews, see Mather, 1995; Hanlon and Messenger, 1996; Hochner et al., 2006; Alves et al., 2007). By contrast, nautilus has been largely overlooked for behavioural...
Biphasic memory in *Nautilus* studies. Among the non-cephalopod molluscs there are numerous experimental examples of learning by conditioning (for reviews, see Benjamin et al., 2000; Balaban, 2002). Although the range of conditioned behaviours is somewhat more restricted than those shown by the coleoid cephalopods, gastropods, nudibranchs and opisthobranchs all learn through conditioning [e.g. *Helix* (Balaban, 2002); *Lymnaea* (Benjamin et al., 2000; Lukowiak et al., 2003); *Aplysia* (Kandel, 1979; Carew et al., 1981); *Hermissenda* (Crow, 2004)]. Long-term memory lasting from several days to several weeks after training (e.g. Lukowiak et al., 2000; Benjamin et al., 2000) shows that even ‘simple’ brains are capable of remembering conditioned behaviours for considerable periods – a vertical lobe is certainly not the only structure necessary for storing memory, and simply demonstrating memory in another genus of mollusc is not remarkable. To our knowledge there have been no successful attempts to demonstrate learning in any of the nautiloids. Here we describe a procedure for conditioning chambered nautilus (*Nautilus pompilius*) that elicited behavioural changes indicative of learning and memory. Using a Pavlovian paradigm that utilised the animals’ strong innate response to water-borne odours, we investigated memory expression across a period ranging from 3 min to 24 h after training.

**MATERIALS AND METHODS**

**Subjects**

Twelve wild-caught adult or sub-adult *Nautilus pompilius* (shell diameter 11–16 cm) of undetermined sex were obtained from a commercial supplier (Sea Dwelling Creatures™, Los Angeles, CA, USA). Animals were housed for the duration of the experiments in two darkened, cylindrical tanks (1.5 m high × 0.8 m diameter) in a 530 l, recirculating, artificial seawater system (Instant Ocean™, Mentor, OH, USA). Both tanks were connected in tandem to a 94.8 l biofilter that supplied aeration and filtration for the system. Water was maintained at 17°C by a chiller (Aqua Logic 1.3hp AE4, San Diego, CA, USA), and was kept sterile by two UV filters (Emperor Aquatics 80W model 02080; Pottstown, PA, USA) and two protein skimmers (Red Sea™, Houston, TX, USA), operating constantly. The 12 h:12 h light:dark cycle (06:00–18:00) alternated between very dim light inside the tanks (0.18 μW m⁻² s⁻¹) and complete darkness. Animals were maintained in the holding facility for at least 2 weeks before being used in any experimental procedure. We fed each animal by hand every 4 days on a 1.5 cm cube of frozen tilapia (*Oreochromis niloticus*) head. Animals taking part in conditioning procedures were not fed the day before or immediately prior to experimental procedures, but were always fed after the conclusion of testing.

**Stimuli**

We used a conditioned stimulus (CS) of a 0.5 s pulse of blue light (λmax 480 nm; Stylus Streamlight; Eagleville, PA, USA; model 3327547), positioned 20 cm above the water surface and 10 cm behind the animals’ shell. Nautilus eyes have many receptors tuned to this wavelength (Muntz, 1986) and it is similar to the light emitted
Six of the 12 animals were used for testing at each retention interval. Each animal was assigned randomly to participate in training and testing for three of the six retention intervals (+ symbols in each column). For each retention interval, the six animals that were used were assigned randomly to receive either the CS+ or the CS− training and its associated test procedure first. Two weeks after that procedure was completed the animals received the reverse training and testing procedure. At least 6 weeks passed between testing at each retention interval.

by bioluminescent bacteria found in their habitat (Haddock and Case, 1999). The lighting angle provided background illumination of the whole experimental arena that was measured at 0.42 μm m−2 s−1. Preliminary tests showed that this stimulus elicited no innate (unconditioned) response in our animals, which were accustomed to changes in light intensity that occurred during routine maintenance procedures. The unconditioned stimulus (US) was a 2% w/v infusion of the normal food substance, frozen tilapia head, infused in home-tank water. The solution was freshly made for each training procedure from a 2 cm cube of frozen meat blended with 100 ml of home-tank water, and then strained to remove particulate matter. Preliminary testing confirmed that animals showed a pronounced excitatory response (tentacle extension and rapid ventilation) when this solution was introduced into their home tank. In each CS+ training trial 2 ml of the US solution was included. The control US, used in CS− trials, was 2 ml of home-tank water, which was effectively odourless but identical otherwise.

### Apparatus

All experiments were run in a light-proof room that was illuminated by a dim red light (60 W; Satco, Brentwood, NY, USA; model no. 4984) in one corner of the room, which provided a background light level of 0.04 μm m−2 s−1. Although it is probable that the red light was undetectable to N. pompilius (Muntz, 1986) blinds were placed around the experimental arena and the camera such that the animal was shielded from the red light for the whole procedure. The experimental arena (a glass aquarium, 36 cm × 20.5 cm × 25 cm; Fig. 1) was filled with water taken from the animals’ home tank, to avoid cueing the animal with novel odours present in clean seawater. An air-stone was fixed into one corner of the tank and remained on for the whole procedure, providing background noise, oxygenation and water mixing.

Animals were held within the tank in an adjustable harness designed specifically to immobilise the shell, but to allow free movement of the eyestalks, tentacles and hyponome (Fig. 1A,B). The harness was constructed from a cross section of PVC pipe and clear plexiglass sheeting. A PVC pipe attached to the harness guided a graduated pipette containing the US (odour) into a consistent position (Fig. 1A,B). This arrangement ensured that the pipette released odour directly onto the tentacles and rhinophore of the restrained animal. The distance from the pipette tip to the rhinophore was 2–3 cm, depending on the size of the animal and its posture within the harness. Preliminary dye tests showed that this distance resulted in an inter-stimulus interval (ISI) of about 1 s. The guide tube also functioned to minimise any visual and tactile disturbance created when the pipette entered the water, and avoided accidental contact with the animal during stimulus delivery.

Trials were videotaped with a 0 lx Hi-8 camcorder (Sony, model CCD-TRV67) positioned 80 cm from the side of the tank (Fig. 1C). Recording began at the start of the acclimation periods and continued until 3 min after the final stimulus presentation. Submerged parts of the experimental apparatus, tank and equipment were washed thoroughly with a mild detergent and hot water between trials.

### Training and testing procedures

Experiments were conducted between June 2005 and February 2006, primarily during the dark period of the day:night cycle between 20:00 h and 04:00 h. We used a within-subjects, counterbalanced design, such that each animal received either CS+ or CS− training first, then the alternative procedure was conducted 2 weeks later. All animals received both CS+ and CS− training at different stages of the experiment (see Myers and Well, 2003). The order in which subjects were tested was re-randomised before testing of a new retention or recovery interval began.

We tested animals at six different retention intervals in random order over the course of the experiment: 3 min, 30 min, 1 h, 6 h, 12 h and 24 h post training. Six animals were tested at each retention interval, a small number but sufficient to provide statistically significant results. Because we aimed to minimise the number of animals we required for the experiment, each subject was used to test three different retention intervals (Table 1). Animals

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**Table 1. Testing regime for the 12 animals used for the experiment**

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*Fig. 2. The scoring system for tentacle extension response (TER) in chambered nautilus. TER was graded every 5 s from a minimum score of 0 to a maximum score of 3. Each level corresponds to a range of percentage extension relative to the length of the animal’s hood. Zero is recorded when all tentacles are retracted into their sheaths. A score of 1 corresponds to an extension of <33% of the hood length. A score of 2 corresponds to extension between 34% and 66%, and 3 is given when tentacles are extended beyond 67% of hood length.*
were allowed at least 6 weeks without participating in any procedures before being used to test another retention interval. We considered this ample time for any lasting effects of handling and training during the previous procedure to decay.

Procedure

Approximately 20 min before the start of the conditioning procedure, an animal was collected from the home tank and transported in a dark bucket to the experiment room. The subject was transferred to the arena and allowed to swim about freely while the empty harness was positioned in the tank. The animal was then placed in the harness and the restraints were adjusted to provide minimal interference with the eyes, tentacles or funnel. So the experimenter (R.C.) remained invisible to the animal, blinds were secured around the tank such that only the lens of the camera protruded into the potential visual field of the animal (Fig. 1C). Tape recording began as soon as the blinds were erected and the animal was allowed 15 min to acclimatise to the arena and the harness.

Training trials began as soon as the acclimatisation period was over. We trained animals with a single block of ten training trials, using an inter-trial interval (ITI) of 3 min. In CS+ trials, 2 ml of fish-infused home-tank water (US) were delivered via the guide tube in each presentation. At the same time as the odour was released, a single 500 ms light pulse was flashed into the arena, illuminating the tank and the surroundings. In CS– trials, the light pulse was paired with 2 ml of home-tank water, but the procedure was otherwise identical. To control for the presence of fish odour in the tank during the conditioning procedure, in CS– trials 20 ml of fish-head infusion was added to the arena at the beginning of the acclimation stage of the control training. After the training phase (both CS+ and CS–) was complete the animal was maintained in a small holding tank in the experimental room during the 30 min and 1 h retention intervals, or was transported back to the home tank for the longer (6 h, 12 h, 24 h) retention intervals. In the 3 min tests animals remained in the experimental arena as handling would almost certainly have proved disruptive. Test procedures involved a single unrewarded presentation of the CS at one of the six retention intervals, and the responses were taped from the beginning of the 15 min acclimation period, except in the case of the 3 min interval when the camera continued recording after training until 3 min after delivery of the test light-pulse.

Data analysis and statistical procedures

Data from each of the six testing sessions were scored by three observers (R.C., J.B. and one assistant). Tapes were numbered and mixed to minimise bias in scoring. We analysed behavioural data within the 30–60 s period after the test presentation of the CS (light pulse only). This 30 s interval was divided into 5 s bins for behavioural scoring, and ventilation rate and tentacle extension response (TER, described below) were recorded for each bin. Nautiluses increase ventilation and extend their tentacles during food searches but not in response to exposure to blue light, therefore we considered these behaviours as appropriate measures of a conditioned response. We scored ventilation rates by counting the opening and closing of the funnel during each breath, or by monitoring the rhythmic beating of the funnel wings. We graded tentacle extension from zero to a maximum value of three (Fig. 2), based on a proportional measure of tentacle length to hood length, since animals were of different sizes. Hood length was measured from the point of contact of the hood with the shell behind the eye to the distal edge of the hood. The highest TER and ventilation rate observed from each 5 s bin were recorded and cross-checked double-blind by two of the three observers. For each animal, a mean ventilation rate and mean TER score from the six, 5 s bins were calculated to avoid pseudo-replication of behavioural scores. A grand mean was computed from each animal’s mean in CS+ and CS– groups for that retention interval. We compared ventilation rates and TER scores between CS+ and CS– conditions, and also across the retention intervals within each condition. We used non-parametric statistics as our small sample sizes meant that meeting the assumption of a normal distribution of errors was difficult to confirm. A Wilcoxon signed-ranks test for paired samples was used to compare behaviour between CS+ and CS– conditions at each retention interval. For comparisons of behaviours across the time intervals we used a Kruskall–Wallis test to identify overall differences in behaviours among the six retention intervals, and post-hoc Mann–Whitney U-tests for pair-wise comparisons if an overall effect of time was detected.

RESULTS

Comparisons of CS+ and CS– groups at each retention interval

In conditioned animals there was a pronounced response latency of around 30 s before animals responded to the presentation of the light-
pulse during testing (Fig. 3A,B). We therefore conducted our statistical analyses on data from the period between 30 and 60 s after the light-pulse was presented. For the test period of each retention interval, we compared the tentacle extension responses (Fig. 4A) and the ventilation rates (Fig. 4B) between conditioned (CS+, N=6) and control (CS–, N=6) animals. Results are expressed as means ± 1 s.e.m.

At 3 min post-training, conditioned animals showed significantly higher TER scores (Z=2.32, P=0.02; Fig. 4A) and higher ventilation rates (Z=2.21, P=0.03; Fig. 4B) than CS– animals. At 30 min post-training there was a similar pattern, although the differences were less pronounced (TER: Z=2.02, P=0.05; ventilation rate: Z=2.27, P=0.03). By 1 h post-training neither behavioural measure reflected a difference between the treatment groups. TER scores were not significantly different in CS– and CS+ animals (Z=–0.27, P=0.78), nor were ventilation rates (Z=–0.42, P=0.67). At 6 h post-training both behaviours were significantly higher among conditioned versus control animals (TER: Z=–2.21, P=0.02; ventilation rate: Z=–2.21, P=0.02). This difference was also apparent at 12 h post-training, although there was only a marginal difference in TER scores (Z=–2.03, P=0.05). Higher ventilation rates persisted in the conditioned group (Z=–2.20, P=0.02). By 24 h after training there was no difference between TER scores (Z=–0.14, P=0.89), or ventilation rates (Z=–0.21, P=0.83).

**Comparisons of behaviours across the six retention intervals**

Kruskall–Wallis tests on TER and ventilation rates among the six retention intervals revealed no significant effect of time intervals on behaviours in the CS– group (TER: H=1.60, d.f.=5, P=0.90; ventilation rate: H=9.55, d.f.=5, P=0.09). There was a significant effect of time interval on TER (H=17.38, d.f.=5, P=0.004) and on ventilation (H=13.816, d.f.=5, P=0.017) in the CS+ treatment group, reflecting the expression of the two distinct memory peaks in CS+ animals. Significant pair-wise comparisons (Mann–Whitney U-tests) for TER and ventilation rates are shown in Fig. 4A and B respectively. TER in the 3 min retention interval was significantly higher than TER at 1 h (Z=–2.28, P=0.02) and 24 h (Z=–2.42, P=0.01). At the 6 h retention interval was significantly higher than TER scores at 30 min (Z=–2.25, P=0.02), 1 h (Z=–2.58, P=0.01) and 24 h (Z=–2.53, P=0.01). TER at the 12 h retention interval was higher than TER at 1 h (Z=–2.12, P=0.03) and 24 h (Z=–2.01, P=0.04). There were fewer differences among ventilation rates across time. Only comparisons between 30 min and 1 h (Z=–2.59, P=0.01), 30 min and 24 h (Z=–2.42, P=0.02) and 1 h and 12 h (Z=–2.11, P=0.03) were significant.

**DISCUSSION**

Our study provides the first evidence of learning and memory in this ancient genus. The memory profile of *N. pompilius* was biphasic, a very similar pattern to that expressed by some coleoid cephalopods (Agin et al., 2003; Agin et al., 2006). Like cuttlefish (Messenger, 1971; Agin et al., 2003; Agin et al., 2006), *N. pompilius* exhibits temporally separated short- and long-term memory stores. This is a somewhat surprising finding given the absence of the vertical lobe complex in nautiloids. We stress that the characterisation of ‘short-term’ and ‘long-term’ memory here is descriptive only, and at this stage there is no physiological confirmation of these states. For the sake of clarity, the memory expressed in the first peak will be referred to as STM and that of the later peak as LTM, but this awaits confirmation in future physiological studies.

The duration of the short-term memory profile in nautilus was comparable to other cephalopods, however, long-term memory was considerably shorter. In adult cuttlefish (*Sepia officinalis*), Messenger (Messenger, 1971) found a response recovery period at 22 min post-training, indicating that memory of the aversive ‘prawn-in-the-tube’ procedure had decayed by that time. In *N. pompilius* there was some memory apparent at least 30 min post-training and probably slightly beyond, but minimal accessible memory at 1 h post-training. The apex of the response curve is also similar – in cuttlefish STM is expressed strongly between 2 and 8 min post-training in adults (Agin et al., 1998) before declining to baseline levels over the next 10 to 12 min (Messenger, 1971). In *N. pompilius*
there was a strong behavioural response in CS+ trials at 3 min post-training, suggesting rapid memory formation consistent with STM, and an STM persistence that is only slightly longer that that expressed by adult cuttlefish.

In contrast to the result for STM, the duration of the LTM curve was surprisingly short: there was no evidence of memory present at 24 h post-training. In measurements taken while developing our procedure there was no memory expressed at either 36, 48 or 72 h using the same conditioning paradigm, suggesting that LTM does indeed degrade very early in N. pompilius, at least under these training conditions. Interestingly the advent of LTM is consistent with LTM appearance in other cephalopods, suggesting consolidation occurs on a comparable schedule but decay is accelerated in nautilus. The duration of LTM observed in both octopuses and cuttlefish is considerably longer – certainly beyond 24 h and possibly lasting weeks in octopus (Young, 1961; Sutherland, 1963; Boal et al., 2000). If LTM genuinely does not persist beyond 24 h in nautilus, the mechanisms underlying such a short retention period are worthy of consideration.

The simplest explanation is that our conditioning procedure was not optimal to produce and sustain LTM. We were unable to train to a performance criterion using this paradigm, as the very brief (500 ms) light pulse that served as the CS and the short ISI (~1 s) did not permit clear determination of which animals demonstrated acquisition of the task during the 10 training trials they received.

Although procedural artefacts may explain the short retention times and behavioural variability we observed, it is probable that the primitive neuroanatomy of nautilus may also influence memory expression. In octopus, Young (Young, 1960) found no substantial differences in performance of normal octopuses (O. vulgaris) trained in an operant procedure with either a 5 min or 1 h ITI, but found a considerable difference in performance in animals that had their vertical lobes removed. This finding is particularly interesting given the absence of a vertical lobe complex in nautilus, and suggests that both procedural artefacts and the particular neuroanatomical structure of nautilus may have combined to produce the short retention times we observed. In coleoids the vertical and subfrontal lobe complexes are necessary for visual and tactile memory, respectively (Boycott and Young, 1955; Sutherland, 1963; Maddock and Young, 1987; Young, 1961; Young, 1991; Fiorito and Chichery, 1995; Robertson et al., 1996). Nautilus lacks both these deductive regions (Young, 1965) and it seems likely that this plesiomorphic neuroanatomy retained by N. pompilius would affect its capacity for memory storage. Future studies examining learning in different contexts may provide clarification.

Certainly there are numerous examples of memory expression beyond 24 h in non-cephalopod molluscs; the presence of a vertical lobe is not a necessity for long-term storage and recall of conditioned behaviours. Gastropods and nudibranchs have simple brains yet clearly are capable of learning and remembering through conditioning (e.g. Kandel, 1979; Balaban, 2002; Chase, 2002). Our results provide more than a demonstration of conditioning in another genus of mollusc; however. Given the differences between the coleoid and nautilus brain, and the even greater differences between the cephalopod and the brains of other molluscs, our results highlight the role that disparate selective pressures can play in driving the development of unique neural structures dedicated to learning.

There are remarkable, if superficial, similarities between modern nautiluses and the externally shelled ancestors of the coleoids (Teichert, 1988; Clarke, 1998). Both lineages of cephalopods remained strikingly similar in appearance until relatively recently, when the coleoid descendants of the belemnite lineage internalised or lost their shells and radiated into predator niches, presumably exerting considerable selective pressure on neuroanatomy and behaviour (Packard, 1972; Aronson, 1991; Hanlon and Messenger, 1996). The resulting differences in lifestyle probably promoted corresponding changes in the neural architecture of the two lineages as they optimised in different directions. During the Mesozoic and onward, many coleoids adopted a fast, visual and predatory lifestyle geared toward avoiding bony-fish predators (Aronson, 1991; Packard, 1972), and their complex brains have been considered a vital requirement for such a niche shift (Packard, 1972). This implies that behavioural plasticity, expressed as rapid learning and stable memory expression, would have been selectively advantageous during this period of competition, and the absence of such regions in nautiloid brains limited their ability to compete equally. However, our results suggest that among modern cephalopods, nautiloids may perform comparably at simple cognitive tasks.

The limited information we have relating to this ancient genus provides us with both a great opportunity and some considerable difficulties. Although it is a potentially valuable taxon for the fields of neuroscience, ethology and evolutionary biology, using nautilus as a study organism poses a number of problems. We know little about its ecology and population structure, making the capture of large numbers of individuals ethically dubious. It spends most of its time at depths below those reachable by divers and thus field-based behavioural studies are extremely difficult. Conversely, destructive neurobiological techniques focused on proximate mechanisms are equally untenable; we certainly do not advocate nautilus as a common model organism for neurobiology. However, carefully targeted behavioural assays can provide us with unique insights into the competing roles that a close evolutionary relationship and widely divergent ecology have played in shaping neuroanatomy of modern cephalopods. Improving our understanding of nautilus behaviour will provide a more complete picture of cognition in cephalopods and other molluscs, complementing the rich existing literature on the evolution of learning and memory, as well as adding to our growing understanding of this ancient species.

We thank Tina Kuroiwa and Christian Soucier for their valuable input into the planning stages of this study and their assistance with animal care. Thank you to Martin Schreibman and the members of our Aquatic Facility (AREAC) for help with animal care and maintenance of our animal housing system. Kevin Kleiner helped with video analysis and conditioning procedures. We gratefully acknowledge the assistance that Roger Hanlon, Robert Rockwell, Peter Moller, Michael Kuba and Frank Grasso provided with statistical procedures, experiment design and interpretation of our results. The manuscript was improved greatly by the comments of two anonymous reviewers.

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The Journal of Experimental Biology