BEHAVIORAL CORRELATES OF LEARNING AND MEMORY IN CHAMBERED NAUTILUS, *NAUTILUS POMPILIUS*

By

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfilment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

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By

Robyn Crook

Advisor: Dr. Jennifer Basil

Cephalopod molluscs are among the most complex and highly derived invertebrates. In the early Cambrian cephalopods diverged into two parallel lineages that remained similar morphologically until the Tertiary, when coleoid cephalopods (octopuses, cuttlefish and squids) diversified into new forms and niches, but the remaining species of nautiloids retained mostly ancestral features and began to decline in diversity. The last remnants of the nautiloid lineage, the genera *Nautilus* and *Allonautilus*, have simple brains and a limited range of behaviour, and may represent an 'evolutionary snapshot' of ancient cephalopods. In contrast, the modern coleoid cephalopods demonstrate remarkable behavioural plasticity, and have developed specialised brains containing dedicated learning and memory centres. These features have made them valuable neurobiological models that help to inform general principles of complex brains and behaviours. In contrast, almost nothing is known about behaviour and neurobiology of nautiloids. Behavioural experiments on nautilus may provide insights into the evolution of the complex brains of modern cephalopods. This study investigated learning and memory in the chambered nautilus, *Nautilus pompilius.* A Pavlovian conditioning

procedure produced temporally separated short- and long-term memory stores, resulting in a biphasic memory curve. Short-term memory duration in *N. pompilius* was comparable to short-term memory in cuttlefish, but long-term memory was considerably shorter. In contrast, performance in a spatial memory task produced memory that was stable for at least three weeks, comparable to or exceeding observed retention times in octopuses. These results show that despite differences in neurobiology, behaviour and ecology between nautilus and the coleoids, at least some aspects of memory profile and duration are similar in both groups. In a final experiment investigating navigational strategy, results showed nautiluses were capable of encoding and recalling the relationships of multiple, visual cues to a goal location, and were able to navigate successfully using several different strategies. Overall this study demonstrates that despite their simple brain and limited behavioural range, nautiluses were adept at learning and remembering associations and visual features of their environment, and their performance was comparable to that of coleoids tested under similar conditions.

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Lastly, to the nautiluses, who worked much harder than I did to make this study a success.

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Continuo contemplare, omnia mutatione fieri, adsuesce intelligere, nihil neque diligere universitatis naturam atque ea, quae sunt, mutare et nova similia efficere: semen enim quodammodo quidquid est ejus, quod ex eo oriturum est.

- Marcus Aurelius Antoninus, Meditations ch. IV, 36

Ocean is more ancient than the mountains, and freighted with the memories and the dreams of Time.

- H. P. Lovecraft

Chapter 1 – Introduction

1.1 Background

Animals with common evolutionary origins may diversify to occupy vastly different ecological niches. Evolutionary theory predicts that in such cases the phenotypes of those divergent groups will be driven toward optimality in their new niche: as those niches become more distant, so too the phenotypes. Extant taxa whose fossil histories display evidence of dramatic niche shifts offer uncommon opportunities to examine the competing influences of phylogeny and ecology on the limits of divergence. The cephalopod molluscs exemplify such a pattern: the two reciprocally monophyletic subclasses have rich fossil records indicating a previously parallel evolutionary route (Jeletzky, 1965; Dzik, 1981; Clarke, 1988a,b; Engeser, 1988; Teichert, 1986, 1988; Mutvei and Donovan, 2006; Strugnell et al., 2005, 2006; Westermann, 2001) but are now strikingly different in their ecology, physiology and behaviour (Bidder, 1962; Barber and Wright, 1969; Saunders and Ward, 1987; Young, 1988, 1991; Wells, 1992; Hanlon and Messenger, 1996; Westermann and Schipp, 1998; Basil et al., 2000, 2005). The most recent common ancestor of the two subclasses appeared in the Cambrian (Dzik, 1981). Thus there has been a long period of independent evolution along the two branches, but the two groups remained nonetheless highly similar morphologically until the Tertiary. During this period, externally shelled Belemnitites gave rise to the modern coleoid cephalopods, while the parallel Nautilid line maintained their ancestral, externally shelled form (Teichert, 1988). The

pleisiomorphic Nautilids are represented today by only two genera, *Nautilus* and *Allonautilus* (Ward and Saunders, 1997), whereas the coleoids have radiated into three separate orders and hundreds of species. Nautilids appear to have changed little since the advent of the first undisputed cephalopod during the Cambrian explosion, retaining many ancestral characters, while the coleoids (octopuses, cuttlefishes and squids) have evolved into specialised hunters that retain few ancestral characters (Anderson, 2000; Bonnaud et al., 2006; Clarke, 1998a; Packard, 1972; von Boletzky, 1987).

Coleoids display a range of complex behaviours (e.g. Adamo et al., 2006; Boal and Golden, 1999; Boal and Marsh, 1998; Byrne et al., 2006a,b; Darmaillacq et al., 2006; Fiorito and Scotto, 1992) and possess heavily modified brains that include dedicated learning and memory centres (Boycott and Young, 1955; Young 1960a, 1965c, 1961, 1988, Dickel et al., 2001; Graindorge et al., 2006; Hochner et al., 2003). In contrast, nautilids have a pleisiomorphic, ring-shaped brain that shows little internal differentiation (Young, 1965b), and a restricted range of behaviour that appears stereotyped and rather fixed. However, the basic neural structure of nautilids is conserved, although greatly extended, in coleoids, making comparative studies of the two subclasses particularly informative for understanding evolution of complex neuroanatomy and behaviour. Coleoids can be considered ‘memory specialists’: their behaviour is highly plastic and behavioural changes that occur after learning are retained for long periods, particularly considering their typically brief lifespan (Agin et al., 1998; Alves et al., 2006; Boal, 1991; Boal et al., 2000; Darmaillacq et al., 2004; Hanlon and Messenger, 1996; Messenger 1973). Nautilid behaviour is rarely studied and little evidence has been uncovered of similar learning abilities in these ancient animals. The

behaviours associated with learning and memory are likely to differ considerably between the subclasses, providing a highly informative substrate for examining general principles underlying the evolution of complex neuroanatomies and behaviours.

1.2 Purpose

Currently, little is known about the behaviour of nautilus. Given its deep-water habitat it is a difficult species to study in its natural environment, and captive animals tend to be unresponsive in experimental situations. In contrast, coleoids demonstrate a range of somewhat naturalistic behaviours in captive settings and are typically responsive to behavioural testing (e.g., Agin et al., 2006; Alves et al., 2007; Boal, 1991, 2000; Karson et al., 2003; Sanders and Young, 1940; Wells and Young, 1970; Young 1961). Thus currently there is a relatively large body of work on the neurobiology and behaviour of the coleoid cephalopods (see Hanlon and Messenger, 1996, Hochner et al., 2006; Mather, 1995, 2007 for reviews), but very little on nautilus. The value of *Nautilus* as an evolutionary 'snapshot' genus has received little attention, despite its considerable promise for comparative studies of neural evolution. The wealth of knowledge about its closest relatives makes studies of nautilus behaviour particularly attractive, and arguably somewhat overdue given their long evolutionary history and unusual lifestyle among cephalopods.

This study is designed to establish a foundation for future comparative studies of cephalopod behaviour and neurobiology. There are a number of widely accepted

paradigms for studying behavioural and physiological correlates of learning and memory in coleoids (e.g. Agin et al., 1998; Boal, 1991; Boycott and Young, 1955; Mather, 1991; Messenger, 1971, 1973; Papini and Bitterman, 1991; Young, 1960a), however these are unlikely to be suitable for nautilus. One objective of this project was to identify and develop several different experimental paradigms that exploit the natural behavioural tendencies of nautilus, yet remain applicable to other species of cephalopods. Establishing such procedures in nautilus, a difficult and unusual cephalopod, will allow detailed comparative studies of behaviour and neurobiology that have previously not been possible.

The general objective of this study is to improve understanding of the behaviour of nautilus. This study should illuminate some aspects of behaviour that are currently understood poorly. For example, laboratory studies of movement and navigation may aid understanding of space-use in the wild. At present it is unknown whether wild animals maintain some home range or defined territory, or whether they instead drift passively with ocean currents across large areas of the reef-face (Carlson et al., 1984; Ward et al., 1984). Location fidelity would suggest that animals have some need to remember both the physical features of their environment, as well as, perhaps, the presence and specific location of high-value feeding areas and shelters, so demonstrating spatial learning in laboratory studies would support this hypothesis. Alternatively, the highly developed olfactory sense (Barber, 1987; Barber and Wright, 1969; Basil et al., 2000, 2002, 2005; Kier, 1987; Ruth et al., 2002) may attenuate the pressure to remember favourable locations, with animals instead being able to rely on orientation to odour plumes to navigate through their environment. Improved understanding of the way nautilus uses its

habitat will be of use in conservation and protection of the species in the wild, where it is vulnerable to threats from pollution, illegal collection and reef degradation.

This study is also aimed at improving understanding of the evolution of the complex cephalopod brain. The brains of coleoids are vastly more complex than those of other molluscs including nautilus, their closest living relative. The aim was to place the abilities of nautilus in context with other extant cephalopod species, by examining learning behaviours that have received detailed study in coleoids. Nautilus is considered by some to be a viable analogue of ancestral cephalopods (Jacobs and Landman, 1994; Shigeno et al., 2007, but see Saunders and Ward, 1994) with evidence that it is similar at least in gross morphology to the ancestors of both extant cephalopod lineages. This makes it particularly interesting from an evolutionary context – nautilids are a highly successful lineage that have changed little since their earliest history, whereas their coleoid relatives have changed markedly in a far shorter period. If the findings of this study suggest that nautilus is capable of relatively equivalent learning and memory processes as coleoids, the common hypothesis that its simple brain prevented it from competing for niches currently occupied by coleoids is in doubt (Packard, 1972). Such a finding would raise further questions about the selective forces that worked on the coleoid brain to drive the development of dedicated learning and memory centres, if indeed the nautilus brain can perform at a similar level in the absence of such regions. If instead the results show that the abilities of nautilus are less advanced than the modern coleoids, this suggests instead that the complex coleoid brain was an important factor in their radiation into niches that were not available to the nautilids. By identifying the limits of cognitive ability in nautilus, we can to some degree infer what role the simple

neuroanatomy of its ancestors had in shaping the highly divergent niches of the modern cephalopods, and shed new light on the evolution of the complex coleoid brain. Future areas of interest involve identifying the site of memory storage in the nautilus brain. Its brain lacks the known memory centres of coleoids so any memory must by necessity be stored elsewhere. Identifying the functional analogues of the vertical and subfrontal regions of the coleoid brain would therefore represent a considerable advance in understanding the evolution of these regions currently present in the coleoid brain.

1.3 Experiment Aims.

Experiment 1 - Classical Conditioning.

The objective of this experiment was to investigate the acquisition and memory profile of *N. pompilius*. This experiment described the profiles of both short- and long-term memory, and provided a point of comparison to other cephalopod species.

Experiment 2 – Extinction and Spontaneous Recovery of the Conditioned Response.

The aim of this experiment was to examine the behavioural response to extinction of a previously learned association, and to characterise the spontaneous recovery process at several time periods post-extinction.

Experiment 3 - Spatial Learning and Memory.

The third experiment focussed on spatial memory. This type of learning is likely to be of most relevance to the species in its natural environment. Spatial memory in coleoids is relatively well understood - maze navigation in octopus and cuttlefish has

been studied extensively, providing scope for comparison with the results of this experiment. Nautiluses forage over large areas that they probably traverse repeatedly over both short and long timeframes. The objective of this experiment was to identify how well nautilus forms and retains memory of small- scale spatial representations.

Experiment 4 - Navigational Strategy in Spatial Learning.

This experiment was designed to identify the types of cues nautiluses use to navigate over a small spatial scale. Several different cue types and configurations were provided to animals in the same general set-up as in the spatial memory task. The aim of this experiment was to examine the relative contributions of egocentric and geocentric cue use in navigation.

1.4 List of abbreviations

CNS	Central nervous system
CS	Conditioned stimulus – A previously neutral stimulus that, after becoming associated with the unconditioned stimulus, eventually comes to trigger a conditioned response.
CS+	A reinforced presentation of the conditioned stimulus (CS coupled with US).
CS-	An un-reinforced presentation of the CS (CS coupled with control or no US).
CR	Conditioned response – a learned response to a previously neutral stimulus (the conditioned stimulus).
EES	Estimated standardised effect size – a standardised, scale-free measure of the relative size of the effect of a treatment on an outcome. Given by the

	equation effect size=mean (group 1) – mean (group 2) / pooled standard deviation.
ITM	Intermediate-term memory – a form of memory that is independent of protein transcription, and that lasts typically for hours, not days.
ITI	Inter-trial interval – the time between a single conditioning event (trial) and the next
K-T	Cretaceous-Tertiary boundary – the transition between the Cretaceous and Tertiary periods of geologic time characterized by a mass extinction of many forms of life, 65 million years before present.
LTM	Long-term memory – memory formation that is associated with gene expression, *de novo* protein synthesis, and the growth of new synaptic connections, and lasting days or weeks.
Mya	Million years ago
MRCA	Most recent common ancestor – the most recent individual or species from which all organisms in the group are directly descended.
NS	Navigational strategy
RI	Retention Interval
STM	Short-term memory – involves an increase in the efficiency with which nerve impulses pass across synapses, but is not dependent on protein synthesis, and persists for seconds or minutes.
TER	Tentacle extension response
US	Unconditioned stimulus – a stimulus that innately or automatically triggers a response, independent of training.
UR	Unconditioned response – the unlearned response that occurs naturally in response to the unconditioned stimulus.

Chapter 2 – Nautilus Biology

2.1 Overview.

Nautilus is an ancient lineage that at least in modern times, is an anomaly among cephalopods. Although nautilids were at one time amongst the most numerous of marine creatures, representing the typical cephalopod *bauplan,* the two surviving genera are the last remnants of the externally shelled cephalopods. *Nautilus pompilius* Linnaeus is the type species of the genus, and since its description in 1758, up to 11 species in the genus *Nautilus* have been described, mostly based on morphology of drifting, empty shells. The precise number of species remains unknown, although most scientists now accept five valid *Nautilus* species: *N. pompilius*, *N. macromphalus* Sowerby, 1849, *N. stenomphalus* Sowerby, 1849, *N. scrobiculatus* Lightfoot, 1786, *and N. belauensis* Saunders, 1981 (Bonnaud et al., 2002, 2004). The morphology of the soft parts of the different species is strikingly similar, with almost all the phylogentically informative characters coming from the shells (Willey, 1902). Elevation of *N. scrobiculatus* to the genus *Allonautilus* is supported by morphological (Ward and Saunders, 1997) and genetic (Bonnaud et al., 2004) evidence, although morphology of the internal structures is very similar in all species of nautilus.

2.1.1 Anatomy and Physiology

Nautiloids are the only extant cephalopods that secrete a permanent, external shell (Fig. 2.1a). The distinctive, zebra-striped phragmocone is divided into air-filled chambers, which are added and sealed off as the animal grows. Its body mass is

contained entirely within the outermost chamber, and is attached to the shell by thick muscle bands that migrate forward as the animal grows, and by the siphuncle, which aids in cameral emptying. In other cephalopods the shell has either been internalised and reduced (cuttlefish and squids) or lost entirely (octopuses). The anatomy of the soft parts is similar to other cephalopods, although nautilus has numerous thin tentacles instead of suckered arms like coleoids, and there are other fine differences. The digital tentacles are covered in transverse adhesive ridges but lack the strength and dexterity to handle live prey efficiently (Bidder, 1962; Kier, 1987; Ruth et al., 2002). When the animal senses odours it extends the digital tentacles in a characteristic search posture, but this response is slow and very different from the explosive prey-strike behaviour of its relatives. Paired post-ocular and pre-ocular tentacles project from just behind, and in front of the eye, respectively. The ocular tentacles are shorter and less mobile than the digital tentacles, and appear to be primarily chemosensory structures. Ocular tentacles are not used in food manipulation, although they do project as the animal extends its digital tentacles to search for food (Bidder, 1962; Basil et al., 2002, 2005).

The hood, which covers the anterior portion of the fleshy parts, is derived from modified tentacles (Young, 1965b; Shigeno et al., 2007) and can be closed over the external body parts to offer some protection from predation, since the hood is muscular and particularly tough. There is no inc-sac. The hyponome is constructed from a fold of tissue (Fig. 2.1b), rather than a closed tube as in coleoids (Willey, 1902, Shigeno et al., 2007) and the animal propels itself slowly through the water by expelling water through this siphon. The structure is highly flexible, allowing the animal a degree of control over the direction of water flow and consequent swimming direction, which is relatively

slow-paced. Ventilation rate can be measured by observing the contraction of the hyponome during expiration.

2.1.2 Vision

The two large, stalked eyes are located posterior to the digital tentacles. Unlike coleoid eyes, they lack an internal lens and are instead more reminiscent of a pinhole camera. The eye is filled with a jelly-like liquid, similar in refractance value to seawater, and the eye is open to the water via the pupil (Muntz, 1987 a, b; Muntz and Raj, 1984). The iris groove, which runs down vertically from the pupil, contains ciliated mucosal cells and may function to prevent foreign matter entering the eye through the opening of the pupil (Muntz, 1987a). The pupil has some dilatory and constriction capacity in response to changing to light levels, although this response is slow (in the order of 90 seconds, Muntz, 1986; Muntz and Raj, 1984) compared with that of other animals. The lack of an internal lens and the relatively primitive structure of the eye suggest that nautilus has limited visual acuity. The optomotor response is certainly less developed than that of *Octopus* (Muntz and Raj, 1984). It is likely that the eye can resolve changes in luminosity and is sensitive to movement, but probably not capable of the fine visual discrimination of coleoid cephalopods. The retinular pigment, rhodopsin, has its maximum absorption (λ_{max}) at 467 nm (Hara et al., 1995), which is a shorter wavelength than the maximum absorption found in most other cephalopods (Lange et al., 1979; Muntz, 1986). This wavelength, in the deep-blue section of the spectrum, persists at some level at depths where nautilus is known to exist and also is similar in wavelength to the light emitted by some bioluminescent organisms that may signal food-sources (Haddock and Case, 1999). Animals display positive phototaxis in lab-based tasks with a

constant light source (Muntz and Raj, 1984), and this behaviour may be important in food localisation in the wild.

The eyes show a compensatory response to rotational movement of the body (Muntz, 1987), suggesting that the animal maintains its visual orientation at a point that is constant even when the body moves. Neurons leading from the eye into the optic lobe do not converge in a chiasma as in other cephalopods, suggesting that the inverted images formed by the eye are not first rotated by a spiral arrangement of neurons through the optic chiasma (Young, 1965b). Emission of water through the hyponome during swimming produces a constant oscillation about the horizontal axis of the animal, making some compensatory response necessary if a fixed visual position is to be maintained. The thick eyestalks mediate rotational eye movement as the statocyst provides positional feedback, keeping the eyes in a constant position. Statocyst ablation affects this stabilisation response (Hartline et al., 1979; Neumeister and Budelmann, 1997). This complex response seems somewhat at odds with the otherwise limited capacity of the eye to resolve images, and suggests that the acuity of the eye may be greater than first hypothesised.

2.1.3 Olfaction

The primary sensory modality in food and conspecific location appears to be chemosensory. Each of the tentacles is covered in tastebud-like cells (Fukuda, 1980; Ruth et al., 2002), and the animal has specialised olfactory organs, the rhinophores, located just below each eye. Animals can detect and localise very dilute concentrations of odour in the water column (Basil et al., 2000), and have been shown to use odour to locate conspecifics (Basil et al., 2002; Westermann and Beuerline, 2005). Blocking the

rhinophores results in impaired localisation behaviour (Basil et al., 2000). It seems likely that olfaction is the primary means of food location in these animals, given their limited visual capabilities and the darkness of their natural environment.

2.1.4 The Central Nervous System

The nautilus CNS exemplifies the basic molluscan pattern of ring-shaped nerve cords encircling the oesophagus (Fig. 2.2). The supraoesophageal nerve cord contains the optic lobes, which are easily distinguishable from the surrounding nerve cord, and several other distinct regions. The optic lobe is large, although smaller and less complex than in coleoids. Although there is no defined optic gland, similar cells are present at the base of the lobe (Young, 1965b). The organization of the optic lobe suggests that it is not designed for detailed processing of visual input from the eyes, although the exact nature of their visual acuity remains somewhat uncertain. The optic lobe is more complex than that of gastropods, but less so than that of coleoids, so it seems likely that visual acuity is also intermediate between the two groups. The olfactory lobe is likewise a lateral extension of the supraoesophageal cord, and lies dorsal and lateral to the optic lobe. The olfactory lobe is more highly developed than in any other cephalopod, although it is not clearly differentiated from the surrounding cerebral cord. This different organization reflects perhaps the animal's heavy reliance on its olfactory rather than its visual sense.

In cross section the supraoesophageal cord shows several distinct regions, loosely radially symmetrical. The main regions described by Young (1965b) are the posterior buccal region, the plexiform zone posterior to the buccal region, the laminated zone, lying internal to the plexiform zone, and a central region in the middle of the cord. The plexiform region appears to receive input from all the receptor systems in the CNS,

and is somewhat similar in appearance to the inferior and superior frontal lobes of *Octopus* (Young, 1965a). This region may be the precursor of a differentiated region controlling motivation, reward and memory systems (Young, 1965b). The presence of such a region is of considerable interest to this study.

The magnocellular lobe is located to the side, as in other cephalopods, but is smaller, and includes nerves from the optic lobe and rhinophore. Its anterior end is contiguous with the anterior suboesophageal cord, although internally it is distinct. The three nerve cords all converge close to the magnocellular lobe, and afferent fibres from the receptor regions all enter here. Thus it is likely that the magnocellular lobe is important in coordination of movement and action (Young, 1965b), and may also function as a decision-making centre.

The anterior suboesophageal nerve cord innervates the tentacles, hood and funnel, and consists of the brachial lobe and the pedal lobe. The posterior suboesophageal nerve cord is analogous to the palliovisceral region in coleoids. Nerves from the statocyst enter here, and it is probably important in coordination of movement. The anterior suboesophageal cord and the supraoesophageal nerve cords are continuous, whereas the posterior suboesophageal nerve cord continues as the magnocellular lobe and shares some connectives with the anterior cord.

Of crucial importance to this study is the apparent absence of any structures clearly analogous to the vertical or subfrontal lobe complexes of coleoids, which are implicated strongly in memory processing and formation (e.g., Boycott and Young, 1955; Young, 1960a). The laminated area of the olfactory lobe and surrounding cerebral nerve cord is located in a similar region in nautilus as the vertical lobe in coleoids. The

laminated area receives impulses from the plexiform region, which is similar structurally to the frontal lobe of coleoids. It seems possible that these areas are the antecedents of the specialised higher processing regions that characterise the modern coleoid brain. The basic neural architecture present in nautilus is essentially preserved, although much modified and extended, in the modern coleoids. The possibility that nautilus retains features of its ancestors' neurology preserved largely unchanged, as suggested by recent embryological evidence (Shigeno et al., 2007), makes the study of their learning and memory processes interesting both in terms of neurobiology and evolution. Of the novel regions present in the coleoid CNS, at least two have been implicated in learning and in memory storage (Boycott and Young, 1955; Young, 1960a,b, 1988, 1991), suggesting that perhaps the present neural arrangement in nautilus is a limiting factor in memory formation.

2.2 Behaviour

There are relatively few studies of nautilus in its natural habitat, as the depths at which it spends most of its time make in-situ studies essentially impossible. Most of what is known about behaviour in the wild is inferential, and based on a small number of radio tracking and trapping studies (e.g., Ward et al., 1984; Carlson et al., 1984). Nautilus makes large, daily migrations from very deep water during daylight hours to the warmer shallows during darkness (Fig. 2.3). Their daily vertical migration typically takes them up beside the cliff-face to the shallower reef environment, tracking close to

the bottom in a cyclical offshore-onshore migration (Ward et al., 1984). The vast majority of their life is spent in complete darkness, although in shallow water their visual sense may be of some use in navigation or predator avoidance. They are tolerant of large pressure variations, and in a single day they may migrate from shallow water (<50m), down to 300m or more at their deepest observed level (Carlson et al., 1984; Ward et al., 1984). The vertical migration has been proposed as a mechanism for avoiding predators that hunt in shallow water during daylight hours, or as a means of covering a large area in the search for food that has fallen into crevices along the reef wall (Saunders, 1985; Ward et al., 1984).

Behavioural observation of the animal in its natural habitat is extremely difficult, as animals typically descend to depths below those reachable by divers, are neither numerous nor apparently social, and difficult to find without providing some artificial attractant. Thus observations of wild animals are possibly unrepresentative of natural behaviour. Saunders and Ward (1987) suggest that the increasing success rate at long term trapping locations in Palau (from 375 specimens in 1977 to almost 1000 in 1982), and the high incidence of recaptured animals in the trap (around 30%) toward the end of the five-year program may be due to animals congregating around the 'feeding station' of the baited trap, and suggests that providing a food attractant certainly produces some alteration of normal behavioural patterns. Future studies involving observation only may yield recordings of more natural behaviour.

Using a food-bait demonstrates at least that long-distance food searching is highly efficient in nautilus. How animals find their food over long distances remains somewhat uncertain, although their highly developed olfactory sense coupled with daily

vertical migrations probably allows them to locate food with a high degree of efficiency. Their intra-specific interactions in the wild are also largely unstudied, although there have been occasional successful captive breeding efforts. It is assumed that animals are solitary, only coming together to breed or congregating opportunistically around shared feeding resources. Copulation in captivity appears to be reasonably common. Recent work has demonstrated that females are attracted to the scent of males (Basil et al., 2002; Westermann and Beuerline, 2005), suggesting a mechanism by which mates may be located in the wild.

Animals in captivity tend to remain sedentary for long periods. When aroused by a novel stimulus, animals typically detach from the wall where they remain held usually by a single tentacle, and proceed to swim slowly around the tank. If they sense food or other foreign odours tentacles are extended in a stereotyped search pattern, fanned wide on either side of the siphon, which is directed forward in a 'cone of search' or 'cat's whiskers' (Bidder, 1962) display. This behaviour is indicative of arousal. If an animal becomes alarmed or distressed it produces a characteristic rocking motion, sometimes turning a full circle, by rapid expulsion of water from the hyponome. Alarmed animals may also withdraw their tentacles and close their hoods, either hanging motionless in the water column or attaching tightly to the wall of the tank. Animals seem to become progressively less excitable as their time in captivity increases (pers. obs.).

The repertoire of behaviour associated with food search and arousal in captivity has been described previously in some detail (Bidder 1962; Basil et al., 2000, 2005). Of particular note is the consistency with which the digital tentacles are extended whenever animals sense odour. Although there are several different postures employed for food

search, each involves noticeable extension of the digital tentacles. Typically more tentacles are extended as food motivation increases (Basil et al., 2005), and this is a consistent and robust behaviour.

2.3 Roles for learning and memory

All species of nautilus are slow-growing and long-lived, possibly surviving in the wild for more than ten years (Landman and Cochran, 1987, Landman et al., 1989), a strategy that is very different to the typical coleoid 'live-fast, die-young' pattern (Packard, 1972). Nautilus inhabits a complex reef environment, where large-scale daily migrations take it over a large area of ground. Within this environment it must find food, shelter and occasionally mates, and then return to a deep sheltered area each day to avoid predators. This cyclical pattern of behaviour suggests that animals that are able to localise appropriate shelter areas quickly, and can locate high value foraging areas accurately may be more likely to survive. Although nautiluses are most likely not social, and probably do not need to remember individuals over long periods, it is unknown whether strong site fidelity is important in their behaviour. If this is case, it is likely that they may encounter the same individuals inhabiting neighbouring areas on a number of occasions. Memory of previously encountered individuals may be advantageous in this situation.

Nautilus is relatively defenceless against predation – it lacks the ink-sac used by its relatives to produce a deterrent or distraction to predators, is slow moving, non-toxic

and protected only by a soft hood. Beaked reef fish will remove animals from their shells with relative ease (Saunders et al., 1987), and octopuses can drill through the shell and consume the soft tissue. Any mechanism of predator recognition or avoidance would be highly advantageous, given nautilus' vulnerability to predation. The adaptive value of learned predator avoidance promotes rapid learning and long memory of encounters with predators. It is unknown whether nautilus has any specific anti-predator behaviours or is capable of learned recognition of potential predators.

Nautilus feeds only intermittently, and must abandon feeding in shallow waters each day once light levels increase. Being able to return to a feeding station on successive nights without having to expend energy on costly search behaviour is likely to be highly advantageous to animals with limited feeding time. Alternatively, it is possible that their highly developed food location abilities render the memory of feeding locations redundant.

It is currently unknown whether nautilus maintains some home-range, or returns to the same shelter on successive occasions, as some octopuses do. Central place foraging is plausible although unlikely in nautilus, with animals perhaps returning to known shelters between foraging episodes. Tracking studies on *Nautilus belauensis* showed that one individual travelled a large distance over seven days (Saunders and Spinosa, 1979), apparently drifting passively with the water current, suggesting a pelagic habit and no defined home range. There is currently only limited knowledge of movement in the wild, and it is possible that some animals have a restricted range of habitat whereas other may be truly pelagic.

The combination of longevity, inhabiting a complex environment, covering a large foraging range and susceptibility to predation is likely to promote memory and enhance the value of learned experiences. The competing aspects of life history characteristics that appear to promote relatively little need for memory, their phylogenetic relationship to the most neurologically complex invertebrates and a neurological structure that seems to show the rudiments of higher processing centres so evident in their close relatives, make the study of cognitive processes in *Nautilus* particularly interesting.

2.4 Evolutionary history of nautilus

The evolutionary history of the cephalopods is relatively well known, thanks largely to the extraordinary prevalence of externally shelled cephalopods from the late Cambrian to the present.

The earliest cephalopods appeared in the middle or late Cambrian, and were probably derived from monoplacophorans (Teichert, 1988; Dzik, 1981). The earliest cephalopod, *Plectonoceras,* had a gently curved shell and was rather snail-like. The characteristic spiral shells of the modern nautilids and many ancestral cephalopods arose for the first time in the late Cambrian, when the large family Ellesmeroceratidae diverged into straight shelled (orthoconic) and spiral shelled forms. Teichert (1988) suggests that the planispiral shelled groups gave rise to ammonoids and, later, coleoids, while the orthoconic lineage gave rise to the nautilids, which almost certainly had

straight-shelled ancestors. Under this hypothesis, coleoids and nautilids most recently shared a common ancestor some 500 million years ago, and coleoids are more closely related to ammonoids than to nautiloids. The ammonoids and nautiloids follow a somewhat convergent path until the final extinction of the ammonoids at the Cretaceous-Tertiary boundary. Interestingly, despite their similar morphologies and ancestry, the two groups were differently affected by each of the major extinction events occurring throughout their history (Dzik, 1981; Teichert, 1986, 1988; Ward, 1987), and the rise and fall of each seems largely disconnected to the success of the other.

The earliest appearance of the modern coleoid ancestors appears to be in the Cambrian and Ordovician, where the earliest cephalopods radiated into a number of orders. In the Ordovician the Ellesmeroceratidae were joined by several other orders, most of which were extinct either by the end of the Silurian or the Carboniferous. Of the surviving orders, the Oncocertatidae and the Bactritidae flourished throughout the Ordovician and Silurian. The Oncoceratidae gave rise to four families with exogastric coiling, among which are the Acleistoceratidae, the probable ancestor of the Order Nautilida. The Bactritidae probably gave rise to the ammonoid-coleoid lineage. The carboniferous family Belemnitidae are descended from the Bactritidae, and are distinguished from the bactritids by mantle covering the shell, placing the earliest appearance of coleoids in the Mississippian (Clarke, 1988b; Dzik, 1981).

By the Devonian the Orders Nautilida and Ammonoidea were present (Kroger and Mutvei, 2005), although ammonoids remained rare until the late Devonian when their numbers exploded and their forms diversified (Dzik 1981; House, 1985; Teichert and Matsumoto, 1987; Ward, 1987). Throughout the Carboniferous the order Nautilida

radiated to around 50 genera, the height of its diversity. During this period the ammonoids showed increasing sutural complexity (Henderson et al., 2002; Lewy, 2002), which probably increased the structural strength of their shells and allowed them to withstand greater hydrostatic pressures, although their shell morphology suggests they never achieved the range of depths inhabited by the nautilids (Ward, 1987). At the end of the Mississippian, ammonoids suffered a major crisis and only a single family, the Prolecanitida, survived. The nautilid lineage was not affected by this event, and continued to radiate until the Permian-Triassic Boundary. In the Permian the oldest undisputed coleoid, a Teuthid, appears (Clarke, 1988a).

The Permian-Triassic boundary saw the extinction of the Bactritidae. At least six cephalopod orders survived into the Triassic, among them the Nautilida, three Ammonoid orders and the coleoid order Aulacocerida. In the Triassic there were three main super-families of Nautilida, the Tainoceratidae, Trigonoceratidae and Clydonautilidae. The Clydonautilidae gave rise to the late-Triassic family Liroceratidae, which survived into the Jurassic and gave rise to the Syringonautilidae. The Syringonautilidae contained the genus *Cenoceras,* the only nautilid to survive into the Jurassic. *Cenoceras* is therefore the ancestor of all Mesozoic and Cenozoic nautilids (Teichert, 1988).

During the Triassic the ammonoids were hugely successful, and the Coleoids continued to diverge (von Boletzky, 2004). Jeletzky (1965) proposes the early Jurassic as the point of octopod and teuthid divergence. Both fossil octopods and teuthids were present in the fossil record from the mid-Jurassic on (Engeser, 1988), with Vampyromorpha appearing by the late-Jurassic. By the Cretaceous sepiids were present,

and ammonites had reached giant proportions, with some up to 3.5m in diameter (House, 1985).

The Cretaceous-Tertiary boundary marks a mass-extinction event that destroyed some 95% of all living species, and up to 52% of marine families (Raup and Sepkoski, 1982). Whilst nautilids seemed virtually unaffected by the wave of extinctions, with at least 3 families of Nautilida crossing the Cretaceous-Tertiary Boundary, ammonites disappeared completely. By this stage it is likely that nautilids existed at a far greater average depth than ammonoids, which probably remained shallow-water inhabitants (Kroger, 2002; Wani, 2004; Ward, 1987; Westermann, 2001). Deeper water certainly affords improved buffering against environmental change, and it is unsurprising that nautilid generalist scavengers would survive better than the predatory ammonoids. Contrary to this hypothesis, the predominately shallow-water coleoids also survived the mass extinction event with seemingly little effect on their diversity.

With the disappearance of the ammonoids at the start of the Tertiary, nautilids radiated rapidly into the vacant niches and enjoyed considerable diversity. Changes in shell morphology after the Tertiary suggest that nautilids converged somewhat on the typically ammonoid morphology of strong lateral compression, which promotes more streamlined and faster swimming. Large adductor muscle scars on the shells of tertiary nautilids suggest they were strong swimmers (Kroger and Mutvei, 2005; Ward, 1987). The flourishing diversity of Nautilida after the Cretaceous-Tertiary boundary declined slowly through the Tertiary to the Holocene, where again, only two nautilid genera survive.

Thus the evolutionary histories of the extant groups are very different. Nautilids have existed virtually unchanged since the Permian-Triassic Boundary, when the Family Nautilidae first appeared. The order Nautilida is far older, probably first arising in the Silurian or Devonian. Their evolutionary history is characterised by gradual radiation and then sudden and drastic reductions in diversity. This pattern appears to have been repeated in the Late Devonian, Late Permian and End Triassic, where in each case the Nautilida reached diversity peaks just before each crisis (House, 1985). Only the pattern of decline in the Holocene is gradual. By contrast the ammonoids show a long period of slow decline before their eventual extinction at the K-T boundary. The coleoids are a younger lineage, which can be traced to the Belemnitites of the Carboniferous, some 100 million years after the advent of the order Nautilida, and their evolutionary history is characterised by gradual radiation into the different coleoid families present in the Holocene. Like the nautilids, they survived the K-T mass extinction with seemingly little effect, although they almost certainly occupied very different niches to the nautiloids by that time, and continued to diverge into predator niches throughout the Holocene (Packard, 1972; Aronson, 1991).

The internal structures of cephalopods are rarely preserved, thus the relationships and affinities of different groups are usually based on shell morphology and calcified structures such as radulae and jaw structure. The evolution of the soft parts such as tentacles can occasionally be inferred from fossilised remains, but there is of course no way of definitively identifying differences in neurology between the groups. Inferences drawn from extant groups are reliable only if the evolution of complexity is assumed to be uni-directional. If, for example, all nautilids had similar brains to

Nautilus, and there has been no secondary simplification of the ancestral condition, such that *Nautilus* is somehow less complex than its ancestors, then useful comparisons between coleoids and *Nautilus* can be drawn, and reliable interpolation to extinct forms may be made.

Evidence from the neuroanatomy of the modern nautiluses is equivocal - the large oesophagus causes the nerve cords to be separated broadly and limits the regions of interconnectivity between the supra- and sub-oesophageal cords. As ancestral nautiloids were probably active hunters, and a large oesophageal tract may be an apomorphy related to the recent development of a scavenging lifestyle. Ancestral nautiloids may have possessed a more centralised and compact brain, more similar in appearance to modern coleoids. Ammonoids were almost certainly predators, and are more closely related to modern coleoids than to nautilus, thus they probably had brains quite dissimilar to nautilus, and more like coleoids.

The solitary, scavenging lifestyle of nautilus places fewer demands on the brain than active hunting. They have not the need for the advanced visual processing regions of coleoids, lacking both their excellent vision and cell-mediated camouflage and communication system. Despite this the basic neural architecture of extant cephalopods is strikingly similar. Although the nautilus brain is less complex, the additions and extensions of the coleoids are traced relatively easily to their antecedent regions still present in the nautilus CNS (Young 1965a, 1965b, 1991).

Thus there are three plausible paths to the nautilus brain: firstly, that the common ancestor of the two lineages had a brain similar to nautilus, which has been maintained largely unchanged throughout the nautilid lineage but has been much

modified through ammonoids to coleoids. Secondly, that the common ancestor possessed a brain similar to modern coleoids, and that nautilids have become simplified as their brain has adapted to their unusual scavenging, slow-paced lifestyle and dark environment, or, thirdly, that the common ancestor possessed a brain more rudimentary than both groups, and the nautilid and coleoid lineages have diverged in different directions from the ancestral condition. Of these three the second is the least likely, being both the least parsimonious and requiring the somewhat extraordinary assumption that the Cambrian common ancestor had developed a brain similar to the most specialised and complex of all extant invertebrates.

The first hypothesis is the most parsimonious of the three, requiring only changes of state along a single evolutionary branch. This hypothesis is additionally attractive for its consistency with the accepted evolution of the external anatomy of nautiloids, which has remained similar since the advent of the first exogastrically-coiled shells appeared in the Ordovician. Under this hypothesis, the neurobiology of *Nautilus* is a valid model for even the earliest cephalopods, and may be used to identify which features of brain function were present in the ancestors of the three major groups of cephalopods, and which of these were extended and modified along the ammonoid-coleoid lineage.

The third hypothesis requires slightly more state changes than the first, and thus is less parsimonious, although more intuitively appropriate. It proposes that the common ancestor possessed a basic neural architecture that was modified in different directions as the two major cephalopod lineages diverged. Thus the common ancestor must have possessed the basic neural plan of both nautiloids and Coleoids, with the ability for each

to diverge to their current states. Under this hypothesis it is possible that ammonoids and nautiloids shared a similar neuroanatomy, essentially derived from a common plan, which was subsequently highly modified and extended in the coleoids. As ammonoids were mostly shallow-water predators, it is probable that their brains were optimised for a visually oriented hunting lifestyle, similar to coleoids, but probably did not show the extensive apomorphies present in modern coleoids. This hypothesis is appealing for number of reasons. Firstly, it does not require that the common ancestor of the three main cephalopod groups possessed any kind of remarkable brain - indeed, the basic plan is so similar across the phylum that there is evidently great commonality among the ancestors of modern molluscs, and some considerable constraint on the degree of divergence possible. However, there must have existed the *potential* for great modification in various directions in the cephalopod ancestor that was not present in those other groups, since this hypothesis implies that both Nautilids and Coleoids have diverged substantially from the ancestral condition. Since evolution cannot create adaptations *ex nihil,* the basic neural architecture must have been in place before the two groups diverged. Thus their common ancestor and its immediate descendents by necessity had brains that contained the basic elements present in both nautiloids and the coleoids, and were sufficiently plastic as to be able to optimise in two separate directions as the ecological niches of the two groups diverged. This presents a number of interesting questions. Why, for example, if nautilids and ammonoids were similar structurally, did nautilids remain scavengers as ammonites evolved into sophisticated predators? Obviously many features of their physiologies underlie the differences, but it is plausible that neurology had some role to play as well. Certainly the close relationship

of ammonites and coleoids suggests that ammonoid brains were already more complex and differentiated than nautilids, and possibly that ammonite behaviour was somewhat similar to coleoids. Whatever the relationships between the brains of extinct and modern cephalopods, closer examination of the neurophysiology of nautilus should contribute to ongoing debate.

2.5 Phylogeny of the Modern Cephalopods

In the past ten years there has been considerable interest and a large number of papers published on the phylogeny of coleoid cephalopods (Akasaki et al., 2006; Bonnaud et al., 1997, 2002, 2006; Carlini et al., 2001; Guzik et al., 2005; Lindgren et al., 2004, 2005; Passamaneck et al., 2004; Pernice et al., 2006; Steiner and Dreyer, 2003; Strugnell et al., 2005; Strugnell et al., 2006) although less so on nautilus. Historically, nautiluses were placed within the subclass Tetrabranchiata, along with all ancestral forms, with the fossil and modern coleoids placed in the subclass Dibranchiata. This overly simple classification scheme has been rejected by modern taxonomists (e.g. Teichert, 1988; Clarke, 1988a,b; Young and Vecchione, 2002), as it is essentially impossible to determine the number of gills possessed by fossilised forms often known only from shells or shell fragments. Other classification schemes based on morphological characters (e.g., Naef, 1916, 1921-1923) have been superseded by more recent molecular studies that split the taxa more definitively (e.g., Lindgren et al., 2004). It is clear that nautilids and coleoids have been evolving along separate lineages since

the earliest Ordovician (Dzik, 1981; Teichert, 1988) so the phylogenetic distance between the extant groups is large regardless of the order of appearance of the extant coleoid taxa. Bonnaud et al. (2004) suggest that the nautilid-octopod split occurred first, with the sepiida and teuthida branching more recently from the octopod lineage, although in some respects this is an unusual result, given that sepiids and teuthids have maintained several ancestral features lost by the octopods. However, more recent studies (Strugnell et al., 2005, Strugnell et al., 2006) combining molecular data with fossil date constraints suggest that octopods are phylogenetically younger than the two decapod lineages. The oldest octopod in their combined molecular and morphological analysis was a vampyromorph. The authors place its origin in the lower Jurassic, considerably older than previously thought, with the divergence of sepiids and teuthids occurring in the later Carboniferous, around 295 mya.

Within the living species of nautilus, there appears to be little phylogenetic signal within the few sequences that have been analysed to date (Bergmann et al., 2006; Bonnaud et al., 2002, 2004), and the designation of most of the generally-accepted species remains unclear. It is, however, now accepted widely that the genera *Nautilus* and *Allonautilus* are indeed separate (Ward and Saunders, 1997, but see Harvey et al., 1999), although there is ongoing debate about which of these genera is phylogenetically older (Ward and Saunders, 1994). However as the internal structures of living nautilids, including the brains, are highly conserved *N. pompilius* is likely to be representative of all taxa within the nautilids in terms of behaviour and neurophysiology.

2.6 Summary

Despite the paucity of knowledge of the living nautilids, they represent a hugely valuable clade for understanding general principles of the evolution of complex brains and behaviours. Their life history suggests conflicting pressures on the advent of advanced learning and memory structures that are found in their closest relatives. Unlike most coleoids, nautiluses are long lived, making the adaptive value of long-term memory stores considerable. They live in a complex environment, suggesting a role for memory of spatial features, however their daily vertical movement is mediated by pressure- and light-gradients that are almost certainly independent of any learning. In contrast to coleoids, they appear not to rely on complex hunting behaviour that might require a measure of behavioural plasticity, instead scavenging for food as they move up and down the reef-face. Additionally, their evolutionary history and phylogenetic relationships suggest that studies of nautilus may yield understanding of some of the fundamental principles that underlie the evolution of complex brains and behaviour.

Chapter 3 – Initial Investigations and Preliminary Attempts at Conditioning

3.1 Overview

In the early stages of this study I tested a range of experimental paradigms that were designed to demonstrate learning in *N. pompilius.* In these initial investigations I used a number of variations on a Pavlovian conditioning procedure, in stimulus type, intensity and timing in an attempt to produce conditioning (Table 3.1). Most of these initial approaches were unsuccessful or showed equivocal evidence of learning, and were tested only briefly before being modified further until a successful procedure was identified. Some procedures were abandoned before large data sets were collected, while the most promising procedures were tested and analysed more fully. Data from each of these experiments were analysed and are discussed below.

3.2 Background

To date there are no published records of conditioning or learning in nautilus. Although there have been several highly effective methods developed for conditioning cuttlefish (Cole and Adamo, 2005; Messenger, 1971, 1973) and octopuses (eg. Boal, 1991; Boal et al., 2000; Boycott and Young, 1955; Wells and Young, 1970), the likelihood of these approaches being successful when applied to nautilus were considered to be slim. Most methods reported in the literature for *Sepia* involve conditioning prey-strike behaviour (the 'prawn-in-the-tube' procedure, Messenger,

1971), and octopuses have been trained in a number of tactile and visual discrimination tasks that typically take advantage of the animals' natural tendencies to explore and handle novel objects (e.g., Sutherland, 1963). Nautilus lacks the strong food motivation typical of both cuttlefish and octopuses, will not strike reliably or manipulate food or objects, and does not react readily to changes in its environment under experimental conditions.

The initial attempts at conditioning nautiluses were not encouraging. I attempted Pavlovian conditioning in two broad categories, 1. Appetitive conditioning involving a light stimulus paired with food odour, and 2. Aversive conditioning involving a light stimulus paired with a tap on the animals' hood. In preliminary investigations the light pulse was the only stimulus that elicited no innate response in freely moving animals when they were in their home tank. Thus it appeared that this was likely to be a suitable conditioned stimulus (more complete definitions of stimuli and responses is given in Chapter 4). The unconditioned stimulus (food odour) in the appetitive conditioning procedures produced a response in unrestrained and normally behaving animals, which was apparent shortly (~30 seconds) after food was introduced into the tanks during normal feeding procedures. Feeding of animals usually involved brief immersion of a piece of fish into the tanks then removal after several seconds. This allowed an odour plume to circulate through the tanks while the remaining food was prepared and encouraged animals to engage in normal food-search behaviour before being fed by hand. Observations of this period suggested that animals became more active once the odour stimulus was detectable, swimming and respiring more quickly than when at rest, and extending their tentacles in a search behaviour described in detail by Bidder (1962)

and Basil et al. (2005). These behaviours appeared to be coupled reliably with food odour, thus they formed the basis of the behavioural assays in conditioning experiments, and are described in detail in Chapter 4. The methods and results of selected preliminary investigations are presented in brief below.

3.3 Appetitive Conditioning Procedures

3.3.1 Unrestrained Animals

The first protocol used food odour as the US and a blue spotlight as the CS (see sections 1.4 and 4.1 for definitions). The ITI and duration of the CS were chosen arbitrarily as 15 minutes and 5 seconds respectively. Five presentations of the CS/US pairing were delivered over a period of one hour and 15 minutes. Animals were placed into the trial tank and remained unrestrained for the duration of the procedure. Retention was tested after 15 minutes but there was no apparent effect on ventilation rate or tentacle extension.

This procedure appeared to be hampered by a number of factors. The small size of the tank and small water volume limited the period of time for running the trial, as water temperature rose quickly after one hour. The small tank also appeared to distress the animal, as it made frequent collisions with the walls and bottom of the tank as it swam about during the trial. The unrestrained animal also posed a number of problems for delivery of stimuli. Octopuses display lateralisation of eye use (Bryne et al., 2004, but see Boal and Fenwick, 2007), suggesting that a CS should be delivered ideally to the

same eye to convey the same information. Although nothing is known about Nautilus eye use, delivery of the CS to varying aspects of the animal as its position changed may have hindered conditioning. The same problem presumably affected the US delivery. Nautilus senses odour via the rhinophore and tentacles, thus the maximum stimulus intensity should occur if odour is delivered directly onto these structures. Delivery of the odour onto different areas of the body would result in decreased odour concentration arriving at the chemosensory structures, and also affect the timing of the US detection by the animal. It seems likely that conditioning would be affected by both these factors, as an inconsistent US concentration over the course of the presentations would affect the animals' perception of the reliability of the CS as a predictor, and the changing degree of temporal coupling of the CS and US also degrades the reliability of the CS (Hilgard and Marquis, 1940). The small number of presentations per training session was presumed to be a limiting factor in this procedure, however later results suggest that in fact five training trials may be sufficient to produce a conditioned response if other factors are closer to optimal.

As the main problems in the first setup related to the free movement of the animal, the following procedures all involve the animal restrained in a custom-designed and built harness (Fig. 3.1). This eliminated the stress caused to the animal by repeated collisions with the walls of the tank and also allowed far greater control over the placement of the stimulus-delivery devices. The 15-minute ITI also limited the number of presentations so in all other procedures it was reduced by varying amounts.

3.3.2 Restrained Animals

1. Restrained Animal (Focused CS, Focused US): In the next paradigm, the stimuli and number of presentations remained the same but the ITI was reduced to 5 minutes, allowing the entire training block to be completed in a shorter period. The CS (light pulse) was delivered directly at the eye of the restrained animal, and when paired with the food odour directed at the rhinophore of the animal, elicited a clear increase in ventilation and TER, which persisted in testing at 24 hours and 7 days post-training. However the control animals (CS-, light pulse paired with tank-water) also demonstrated a strong response during testing (TER: Fig. 3.2; ventilation rate, Fig. 3.3). Closer examination of the video revealed that during light exposure the hyponome was directed toward the front of the tank, indicating that animals were attempting to escape from the stimulus. This result was consistent in CS+ and CS- trials, suggesting that if conditioning had occurred in the CS+ animals its expression was subserved by the highly aversive nature of the 5-second light delivery into the eye of the animal.

2. Restrained Animal (Dispersed Strobe CS/Focused US): The delivery point of the CS (light) appeared to be the main cause of failure of conditioning, and in subsequent attempts the projection position was moved to a point on the back wall of the tank, posterior to the animal. This resulted in strong background illumination of the tank but was essentially non-directional from the animals' perspective. The duration of the stimulus also seemed to promote unconditioned tentacle extension. In the next procedure, the period of constant illumination that had been used previously, was substituted with five, half-second light flashes. This strobe effect lasted for the same total time but the illumination was not constant. The light flashes seemed to have a

cumulative effect on unconditioned tentacle extension, with little response apparent until the 3rd, 4th or 5th pulse. Again, when this procedure was tested, the unconditioned response to the light in CS- trials interfered with the observation of conditioning in CS+ trials. Alternatively, the separation of the light pulses may have interrupted the encoding of the temporal coupling required for conditioning to occur. This procedure failed to produce evidence of learning in the preliminary stages and no detailed results were collected.

3.4 Aversive Conditioning

The aversive conditioning attempts were brief, and were abandoned after a single, unsuccessful attempt. The method for this procedure involved a 5-second light pulse of the CS coupled with tapping on the animal's hood with a rounded glass rod (US). The stimulus was designed to approximate a predator attack on the soft tissue of the hood and tentacles. In preliminary testing this stimulus caused animals to retract the tentacles, close their hood and reduce their ventilation rates. As nautilus has no active defence mechanisms and is a comparably slow swimmer, it is plausible that these are normal anti-predator behaviours. Animals were restrained in the harness and given 10 presentations of the light/tap pairing every 30 seconds. In this setup the effect of massed training trials was also tested by reducing the ITI from 5 minutes to 30 seconds. Animals responded by closing their hoods and suppressing ventilation for the entire trial. During test periods animals remained 'closed' at 30 s and 5min, and response the CS was very

low (TER: Fig. 3.4; ventilation: Fig. 3.5). Nautilus can suppress ventilation and activity for long periods, and it appeared that this conditioning technique promoted that behaviour, making measurements of change in response to the CS delivery difficult to observe. As with several other procedures, it is possible that conditioning did occur, but that the learned response to the stimulus made observation of learning and memory difficult.

3.5 Successful Procedure

Restrained Animal (Dispersed Uniform CS/Focused US): The final and ultimately most successful procedure involved a single 500ms light pulse, followed by the food odour with an ISI of approximately one second. The ITI was reduced to three minutes, based upon the observation from previous trials that animals returned to baseline behavioural levels approximately 60 to 90 seconds after delivery of the US. When animals received 10 training trials under this paradigm, the CS+ animals demonstrated a clear conditioned response to the light pulse, whereas CS- animals appeared to produce no response when tested. Although this may not be the optimal procedure for conditioning nautilus, in preliminary testing it produced a replicable and quantifiable difference between control and conditioned animals. This paradigm formed the basis of the conditioning experiments in Chapter 4.

3.6 Summary

The failure of several paradigms to produce a measurable effect on behaviour may have been caused by a number of factors. Animals may either have failed to learn the association, or other behaviours may have masked the expression of learning and memory. In at least two of the paradigms this appeared to be a factor in the failure of the procedures. In the appetitive conditioning procedure that involved a sustained light pulse aimed at the animal's eye, the increase in both tentacle extension and ventilation appeared to be an innate aversive reaction to sudden change in light intensity, and occurred in animals that had been trained in CS+ and CS- conditions. It is possible that the animals did learn the association between light and food in this paradigm, but the behavioural response to the light pulse overshadowed any measurable difference between the treatment groups. In the aversive conditioning procedure, hood closure occurred both in animals that had received paired CS/US training, and animals that received US alone. Again, this untrained response prevented observation of any conditioned response. In both cases, differences between control and conditioned animals were obscured by innate responses to the conditioning stimuli and context.

In all of the unsuccessful paradigms that were attempted, and in the single case where conditioning occurred and was expressed in behaviour, the procedures involved cross-modal stimulus pairings, with either a visual or tactile stimulus being paired with an olfactory stimulus. In some animals, single modality or similar CS/US pairs are learned more easily than cross modal pairs, (the 'Garcia effect' in rats, Garcia and

Koelling, 1966). Cross-modal CS/US pairs were used in the development stages of this study simply because nautilus has a limited behavioural repertoire in the laboratory. Identifying distinct behavioural differences between conditioned and unconditioned animals was judged to be more feasible when the behaviours associated with the CS and with the US in the untrained state were very different.

The success of at least one of the appetitive conditioning procedures indicated that the ITI and number of presentations were the major determining factors in the formation and encoding of the association, not the types or the combinations of stimuli themselves. The nautilus brain lacks known integrative regions analogous to the palliovisceral and vertical lobes in coleoids (Young 1965b, 1987), and as such it may have been reasonable to expect that there would be some limitations on the modalities of stimuli that can be paired to create an association. However at least one cross-modal pairing (dispersed blue-light and concentrated food odour) was successful at producing conditioning, although several slight variations on this same combination failed. This suggests that other types and combinations of stimuli, including those that failed in these attempts, might also be successful if the ideal interval length and number of repetitions could be identified.

Chapter 4 – Classical Conditioning: Characterisation of Acquisition, Memory and Extinction of a Conditioned Response

4.1 Introduction.

4.1.1 Learning, Memory and Classical Conditioning.

Learning and memory are somewhat nebulous concepts. 'Learning', in its broadest terms, can be considered as a "more than transient modification of behaviour which presumably results from past experience" (Wegner, 1956). Memory may be defined practically as the retention of a changed behaviour, expressed in the absence of the factor originally causing that change, or "experience dependent internal representations…..acquired models of the world, encoded in the spatiotemporal activity of brain circuits" (Dudai, 2002). Memory as a physiological process has been studied extensively in both vertebrate and invertebrate models. Different configurations of memory are defined not by their duration but rather by their physiological underpinnings. Short-term memory (STM) involves transient changes of synaptic morphology (Stork and Welzl, 1999), whereas intermediate-term (ITM) and long-term memory (LTM) require changes in gene activity (Davis and Squire, 1984; Rosenweig et al., 1993). ITM and LTM are distinct processes – LTM requires transcription whereas ITM requires only translation (Sangha et al., 2003).

Classical conditioning is one of the simplest forms of associative learning. It represents one of the lowest levels of cognition on Thomas' scale of cognitive abilities (Thomas, 1980, 1996), only superior to habituation and sensitisation. Conditioning

entails the creation of an association between two or more events, which may either be expressed through behaviour or represented internally by changes in neural activity (Gallistel, 2003). Classical, or Pavlovian, conditioning is defined by a stimulus-reinforcement contingency - the reinforcement follows the stimulus with some likelihood that is fixed and irrespective of the subject's response (Pavlov 1927). In standard Pavlovian conditioning procedures some previously neutral stimulus is paired with a meaningful stimulus (one that elicits a response from the subject in normal circumstances). The meaningful stimulus is termed the 'unconditioned stimulus' (US, food odour in this procedure), as it requires no new learning to produce a response, and the subject's response is the 'unconditioned response' (UR, food search behaviour). The neutral stimulus with which the US is paired is the 'conditioned stimulus' (CS, in this procedure, a pulse of blue light). After training, the CS comes to be associated reliably with the US such that the CS in isolation will elicit a response where none was present before training. Thus the unconditioned response becomes the conditioned response (CR). Although the UR and the CR are generally similar they need not be identical to demonstrate conditioning has occurred (e.g., Levy and Susswein, 1999). The CR may differ in strength, timing or character yet still demonstrate that an association between the CS and US has been formed.

Within conditioning paradigms there are other variables that affect the strength of the formed association. The reliability of the CS as a predictor of the US is paramount – as the CS becomes a less reliable predictor of the US, the associative strength is altered. The temporal coupling of the CS and the US is also vitally important – if the US precedes the CS (backward conditioning), associations are extremely difficult to

produce. In forward conditioning, where the CS precedes the US, there is an optimal interval between the two (Hilgard and Marquis, 1940), and progressively longer or shorter intervals tend to produce less and less robust associations. The interval between the onset of the CS and the onset of the US is termed the inter-stimulus interval (ISI). The inter-trial interval (ITI) is the time between each paired presentation of the CS and US. Short ITIs produce 'massed conditioning', and typically promote STM over LTM, whereas longer ITIs, 'spaced conditioning', tend to promote longer-term memory. The schedule under which the reinforcement is delivered also produces a characteristic effect on behaviour. Reinforcement may be given on every trial or on a fraction of trials. Partial reinforcement produces a conditioned response particularly resistant to extinction.

4.1.2 Extinction and Spontaneous Recovery.

Extinction of a response is characterised by a gradual degradation of performance of a previously learned behaviour. Extinction in a classical conditioning paradigm occurs when the CS is given repeatedly with no reinforcement, and the conditioned response degrades accordingly in the absence of feedback. Thus extinction provides information both about the strength of the original association and its stability in the presence of new information (Hilgard and Marquis, 1940). Extinction does not so much promote forgetting of the association but rather subjugates the original CR while a new behavioural response is expressed in its place. This distinction is emphasised further by the reappearance of the original CR at a later time, although typically the CR strength has declined somewhat. Spontaneous recovery is the reappearance of the conditioned response that occurs with the passage of time following extinction, as described by

Pavlov (1927). The exact mechanism underlying recovery remains poorly understood, and various explanations have been proposed for its occurrence (see Brooks and Bouton, 1993, for review). Spontaneous recovery suggests that the original association was not forgotten but simply suppressed while a more relevant response took precedence. During extinction training there are therefore two competing processes at work – the overlay of a new CS–no US representation onto the existing CS–US memory trace, and a potential reconsolidation of the original CS–US trace. This reconsolidation of the original CS–US memory is perhaps facilitated by contextual cues that remain the same during the original training and extinction, and thus reminds the animal of the original situation as it processes the difference in the new CS–no US condition. This comparison may in and of itself serve to reconsolidate the original memory. The respective strengths of the two associations have been shown to be dependent on several factors, including duration of the CS exposure, the age of the original memory trace, and the strength of the memory (Suzuki et al., 2004). Examining extinction, reconsolidation and spontaneous recovery in nautilus should provide some insight into the processes underlying expression of the conditioned response, as well as its strength, duration and vulnerability to disturbance.

The following experiments were designed to provide an initial characterisation of the learning and memory profile in *Nautilus pompilius*. The classical conditioning procedure that was developed was used first to examine acquisition and retention of the conditioned response. Memory was tested at both short-term (three minutes, thirty minutes and one hour) and long-term intervals (six, twelve and twenty-four hours) after the initial training procedure where animals learned the association. In the second experiment (extinction and recovery) the procedure was modified slightly to focus on

extinction of the conditioned response at a short interval (one hour) after training, and recovery of the conditioned response at six, twelve and twenty-four hours after extinction. Taken together, the results from these two experiments provide the first demonstration of acquisition, duration and stability of learning and memory in nautilus.

4.2 Methods

4.2.1 Animals

Wild-caught adult or sub-adult *Nautilus pompilius* were obtained from a commercial supplier (Sea Dwelling Creatures ™, California) and air-freighted to the holding facility. In all cases animals were cage-trapped in the Philippines and shipped within a week of capture. Animals were maintained in their home tanks for at least two weeks before being used in any experimental procedure. During this period animals were offered food every three days and their health was monitored closely. Animals that had not begun to accept food after two weeks were not used in any experimental procedure until they began to feed normally, although in most cases animals fed normally within one to two days after arrival. Prior observations suggest this acclimation period is ample to ensure that animals are in good health before they are used. Several animals displayed abnormal buoyancy regulation behaviour after arrival, which may have been caused either by the trapping procedure or by the shipping process. Floating did not appear to affect other aspects of behaviour such as feeding and activity, and these animals were included in experiments where buoyancy was not critical to the procedure.

Animals were maintained throughout the experimental period in two covered holding tanks (Fig. 4.1), containing re-circulating artificial seawater (Artificial Ocean ^{TM}) at 17 degrees Celsius and pH range 8.0 to 8.4. Water was kept sterile by 2 40W UV filters, and solid waste was removed every day either by hand or by protein skimmers attached to each tank. The 12hr light/dark cycle alternated between very dim light and complete darkness to minimise stress caused by bright light. The tank covers, which only allowed light into the tanks in one location, also prevented undue exposure to light stimuli which potentially could have affected the salience of the proposed CS in later experiments. However, as the tank covers were lifted during feeding and for tank maintenance, it is possible that some shaping of the tentacle extension response to a light stimulus occurred, but as control and conditioned animals were kept in the same conditions, and all subjects received both CS+ and CS- training, any effect of autoshaping on the results would have been minimal. Animals were fed every four days on a 2cm cube of frozen Tilapia (*Oreochromis niloticus*) head. A restricted feeding regime ensured that animals remained food-motivated throughout the experimental procedure. This husbandry procedure has previously been used to keep animals for up to one year in the holding tanks, with minimal observable ill-effects.

Feeding times were altered to accommodate the experimental schedule, ensuring that animals were not fed either the day before or on the day of a scheduled training procedure, and were always fed after being tested for retention.

4.2.2 Apparatus

All experiments were run in a blacked-out room, lit by a dim red light. Although it is likely that red light in undetectable to nautilus, animals were shielded from the light

and from the experimenter by blinds placed around the experimental arena and the camera.

The experimental tank (glass aquarium, 36 x 20.5 x 25cm) was placed on thick Styrofoam and filled with water taken from the animals' home tank (Fig. 4.2). Home-tank water was used to avoid cueing the animal with novel odours present in clean seawater. The Styrofoam mat absorbed ambient vibration and provided a stable base for the tank. Within the tank, an air-stone was fixed into one corner of the tank and remained on for the whole procedure, providing both background noise and constant water movement.

Animals were held within the tank in a harness designed specifically to immobilise the shell, but allow free movement of the tentacles and hyponome. A pipe attached to the harness guided the pipette containing the US into the correct place, alongside the tentacles and rhinophore (Fig. 4.2, inset). This arrangement ensured that the pipette was guided to the same position each time, releasing odour directly into the gill current that brings odour across the rhinophores. The distance from the pipette tip to the rhinophore was approximately 2 cm. The guide tube minimised the visual and tactile disturbance created when the pipette was not concealed within it, and provided a guide for the experimenter as there was minimal visibility in the experimental room.

All trials were videotaped from the start of acclimation to the end of the trial, using a 0-lux Hi-8 (Sony, model CCD-TRV67) camcorder positioned one metre in front of the tank, providing a lateral recording view. All submerged parts of the experimental apparatus were washed thoroughly with a mild detergent and hot water between trials.

4.2.3 Stimuli

Several combinations of stimuli were tested in preliminary investigations for this procedure. Details of these approaches and their results were given in Chapter 3. The approach that produced the most robust results during preliminary testing was an appetitive-conditioning paradigm involving a light pulse and a reward of food-odour (Fig. 4.3). The light pulse served as the conditioned stimulus (CS), and consisted of a ~500ms flash (480nm), from a blue pen-light (Stylus Streamlight, model 3327547). The CS was aimed at the same point in the back of the tank (behind the restrained animal), where it produced a spot of light 4 inches in diameter on the tank backing, and reflected illumination through the whole arena. This CS was paired with an unconditioned stimulus (US) of food solution, made from the normal food substance, frozen heads of Tilapia (*O. niloticus*). A 2 cm cube of meat was mashed into 200 ml of home-tank water, then strained, producing a concentrated odour solution that elicited a clear unconditioned response (UR). The two stimuli were delivered with an inter-stimulus interval (ISI) of approximately 1 second.

4.2.4 Procedure for Classical Conditioning Tasks

Animals (n=12) were assigned randomly to receive either the CS+ training or CS- training first, for each retention interval. The reverse procedure was conducted two weeks after the first. Retention intervals (RI) were tested on separate occasions, in the order 24h, 6h, 1h, 3 min, 12h and 30 min. Six animals were used to test each RI (Table 4.2)

Each trial began when the animal was removed from its home tank. An animal was collected by hand then transferred immediately to a darkened bucket containing home tank water. Animals were transported in darkness to the experiment room, which

was illuminated with a dim red light. The experimental arena was half-filled with water before the animal was placed into the tank, then the tank was filled to the top with water from the transport bucket. An air-stone was secured into the tank corner and the harness positioned while the animal was free to swim about in the tank. Once the animal had either attached to the wall of the tank or had slowed its swimming activity, it was secured carefully in the harness. The restraints were positioned to provide minimal interference with the eyes, tentacles or funnel, but ensure the animal remained fastened for the duration of the experiment. Once the animal was fixed into the harness, blinds were secured around the tank such that only the camera lens was visible from the tank. The experimenter thus remained invisible to animals once the blinds were erected.

Tape recording began once the blinds were secured and the animal was allowed 15 minutes to acclimate to the arena and the harness. The experimenter left the room after recording began and re-entered at ten minutes, when five minutes of acclimation time remained. This ensured that any disturbance detected by the subject did not occur concurrently or closely coupled with the presentation of the conditioning procedure.

Training trials began as soon as the acclimation period was over. In CS+ trials, two millilitres of home-tank water infused with fish odour (US) were drawn into a pipette, and the pipette was threaded into the guide tube. Pipette insertion occurred 20 seconds before odour release to decouple any effect of water disturbance from the US presentation. The light was flashed once into a predetermined spot on the back of the tank behind the restrained animal, and at the same time the pipette was depressed to release odour into the water (Fig. 4.3). Because of the distance between the tentacles and the pipette tip, it is likely that the animal sensed the presence of the odour shortly after

the light flash occurred (probably in the order of 1 second later), but evidently this coupling was sufficient to induce a conditioned response. The pipette was removed once the US was delivered. Although it would have been preferable to have the delivery tube remaining in place throughout the procedure, the sensitivity of nautilus to even minute concentrations of odour meant that animals may have sensed a continuous stream of odour diffusing out of the end of the tube, thus eliminating the tight temporal coupling necessary for production of a conditioned response. Given this possibility, it seemed preferable to remove the pipette between each training presentation.

After completion of the training phase the animal was released immediately from the harness, returned to the bucket, and either maintained in a small holding tank in the experimental room during the 30-minute and one-hour retention intervals, or transported back to the home tank for the longer (6h, 12h, 24h) retention intervals. Animals remained in the experimental arena during the retention interval for three-minute tests, as handling would almost certainly have proved disruptive. All test procedures at each retention interval were identical, and involved a single unrewarded presentation of the CS, and were taped from the beginning of the 15-minute acclimation period to three minutes after the test was complete. Animals typically showed no sign of distress once returned to their home tank, and usually accepted food and ate normally, suggesting that any stress caused by the restraint or the conditioning procedure was minimal.

The control procedure (CS- trials) followed an identical format, except that the CS was paired instead with 2 ml of home-tank water that had been removed at the same time as the animal, providing a cue with minimal detectable odour but identical otherwise. To control for the presence of fish odour in the tank during the initial

conditioning procedure in CS+ training, 20 ml of odour solution was added to the tank at the beginning of the acclimation stage of the control (CS-) training. Preliminary experiments showed that animals did not react to any water current or other disturbances caused by the introduction of the control stimulus, suggesting that any observable conditioned response is a result of the US odour only, and not some other unaccounted-for stimulus.

A second control procedure was included to investigate the effect of the arena and the harness on animals. Subjects (n=3) were placed into the tank and restrained in an identical manner to the CS+ and CS- trials, but no stimuli were delivered. Sham test procedures (no light-pulse was delivered) occurred at 3 minutes, 30 minutes one, six, 12 and 24 hours after the sham-training period. Data were collected from the six 'test' periods, for 60 seconds each time. Thus these data mirrored those collected during testing procedures, but controlled for the effects of stimulus presentation and restraint procedures on the subjects' behaviour.

4.2.5 Procedure for Extinction Testing

This experiment used the same set of stimuli and response measures as Experiment 1, and involved an identical training procedure for CS+ and CS- animals (n=6). After training was complete, animals were released from the restraint and maintained in a darkened bucket for 45 minutes. Subjects were placed back into the experimental arena and acclimated for 15 minutes before extinction training began. The total time between training and extinction was one hour. Ten extinction trials were delivered with a three-minute ITI. Extinction trials in both CS+ and CS- groups used un-reinforced presentations of the conditioning stimulus only (light pulse, 480nm, ~500 ms

duration). There was no US delivered during extinction training. Once extinction training was complete animals were returned to their home tanks. Spontaneous recovery of the CR was tested at three intervals, each on separate occasions. The same 6 animals were used to test recovery at 6h, 12h and 24h post-extinction.

Recovery was tested with 10 unrewarded CS presentations, using a procedure identical to extinction training. Each animal was trained on three separate occasions, and tested at one of the three recovery intervals after each different training session. All animals were tested in the order 6h, 24h then 12h. Within each recovery interval animals were assigned randomly to receive either CS+ or CS- training first, then were given the alternate training two weeks after the first. Testing of each recovery interval was separated by 6 weeks (Table 4.2).

4.3. Data Analysis and Statistical Methods

4.3.1 Data Acquisition

All trials were taped from the beginning of the acclimation period to three minutes after the final presentation of the stimulus. Data from the testing session were analysed once each retention interval had been completed. Trials were divided into 5-second bins, and ventilation rate and tentacle extension rank (TER) were recorded for each bin.

These behavioural variables were judged the most appropriate given that both are robust and associated with food arousal. Ventilation rate was scored by either observing

the opening and closing of the funnel during each breath, or when the hyponome was not directly visible, by observing the slight opening and closing cycle of the hood that occurred upon each respiration. Mantle opening and closing could also been seen at the point behind the eye where the hood meets the shell. Each of these measures provides an unambiguous and consistent measure of ventilation.

Tentacle extension was ranked from 0 to a maximum value of 3, based on a proportional measure of tentacle length to hood length. 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, as visible on video recordings (see Figs 4.4 and 4.5 for details). Given that there is some size variation among the experimental animals, this proportional measure is more appropriate than a simple measure of tentacle length. For each 5-second bin, the highest TER observed within that bin was recorded. A detailed description and representative diagram of each score is also given in Table 4.1.

Preliminary testing showed that most animals produced an unconditioned response to fish odour most strongly in the first 60 seconds after it was delivered, and the intensity of the response waned over the following 30 seconds.

4.3.2 Statistical Analysis

Ventilation and TER data met the assumptions of normality and thus were compared between CS+ and CS- groups using a blocked ANOVA design. For each animal tested at each retention interval, a mean of TER and of ventilation rate for the 12 bins was computed for CS+ and CS- treatments, then grand means (across animals) of these individual means (within animals) were used for analysis. This avoided pseudo-replication of data that would have occurred if all bins were treated in the analysis as

independent observations. Additionally, means and 95% confidence intervals (CI) of means were calculated for effect-size estimates. Genuine effects are demonstrated by 95% CIs not overlapping zero. This approach does not encompass standard Fisherian hypothesis testing and thus does not include associated p-values with effect size estimates.

For Experiment 2, the behavioural variables and tape analysis procedures were identical to those used in Experiment 1. In this experiment I recorded data for the 60 seconds after every stimulus presentation (training, extinction and recovery testing). As each animal was trained and extinguished in an identical procedure there are essentially three replicates for each animal in the first two stages of the procedure, then one replicate per animal at 6 hours, 12 hours and 24 hours post-extinction.

4.3.3 Within Subject Design.

Nautiluses are highly sensitive to poor water quality and require specialised housing conditions. Although the housing system was monitored carefully and all animals were given close and dedicated care, maintaining them for long periods in captivity has proven to be very difficult. It appeared that animals that remained for long periods in captivity showed some abnormal growth patterns and became less responsive to food and other stimuli as they became habituated to sedentary tank life. Obtaining animals from the wild in good condition and in large numbers was not always possible, and was ethically questionable given our limited knowledge of their population status. There was also a considerable amount of behavioural and response variability present among individuals even before any experimental procedures were initiated. For these reasons, a within-subject design using six animals in each group was proposed as an

appropriate solution to each of these concerns. Six animals were sufficient to provide a statistically robust result even allowing for considerable variations in behaviour among animals, and was not considered excessive in terms of removal of animals from the wild.

In this way each animal served as its own control, as each was subject to both the conditioning procedure with paired CS/US (CS+) presentations, and the control procedure where the CS is unrewarded (CS-). Thus if inter-individual variation was high, animals could be analysed separately to examine intra-individual responses. This design does however require that animals were allowed sufficient 'forgetting' time between each classical conditioning procedure, such that the remnant memory of learned associations did not impact on control procedures, and vice versa. It was proposed that at least one week, preferably two, should elapse before any animal was tested under a new condition. A list of animals used and the sequence of their training and testing is given in Table 4.2.

Although the use of a within-subject design extended the running time of each classical conditioning experiment considerably, it was preferable to using more animals than was strictly necessary to obtain statistical validity.

4.4 Results

4.4.1 Experiment 1: Classical Conditioning.

During acclimation, animals explored the harness with their tentacles and ventilated strongly for a brief period. This may have been an attempt to remove themselves from the restraint. This behaviour usually ceased after several minutes, and by the end of the acclimation period most animals were respiring at typical resting rates, with their tentacles either withdrawn completely or extended slightly in front of the hood, but not actively exploring the restraint. Dilation of the pupil increased noticeably during the acclimation period, suggesting that the red light used in the experiment room was not as visible to the animals as the dim white-light level in their home tanks.

Initial data analysis showed that when conditioned (CS+) animals responded to the test presentation of the CS, there was a latency of approximately 30 seconds before a change in behaviour was apparent (Figs 4.6 and 4.7). There appeared to be no clear pattern of increase in the behaviour of control (CS-) animals. Data were recorded for the first 60 seconds after presentation of the CS, which was partitioned into two 30s periods for further analysis. Thus results are presented for the full 60s test interval (all bins), as well as for the first 30 seconds only (early bins), and the last 30 seconds only (late bins). Means, standard errors of means (s.e.m) and *p* values for each comparison are listed in Table 4.3.

Three-minute retention interval

At three minutes after training, conditioned animals showed elevated but not significantly higher TER and ventilation rates across the full 60-second recording interval (Fig. 4.8a). Animals in the CS- group showed mean TER scores of 0.45±0.24,

compared with 1.62±0.37 for conditioned animals (F=5.69, p=0.063). Animals in the control group ventilated an average of 3.30±0.27 times per five seconds, compared with 3.79±0.16 for conditioned animals (Fig. 4.9a, F=5.13, p=0.073). There were also no significant differences in behaviour in the first 30 seconds of the test period only. Animals that received CS- training had mean TER scores of 0.55±0.29, compared with 1.55±0.39 in animals that received CS+ training (Fig 4.8b, F=3.12, p=0.137). Mean ventilation rate in CS- animals was 3.30±0.33, and in CS+ animals was 3.77±0.20 (Fig 4.9b, F=2.76, p=0.157). In the final 30 seconds of the test period, conditioned animals showed significantly higher TER scores and ventilation rates than CS- animals. Animals in the control group showed mean TER scores of 0.36±0.21, compared with 1.69±0.39 for animals in the CS+ group (F=9.80, p=0.026; Fig. 4.8c). Mean ventilation rate in CS- animals was 3.27±0.23, and in CS+ animals was 3.80±0.17 (F=4.08, p=0.044; Fig. 4.9c).

Thirty-minute retention interval

Animals tested at 30 minutes post-training showed no difference in mean TER between CS+ and CS- groups, when the 60-second test-period was analysed combined. Mean TER for control-group animals was 0.59±0.21, and 0.92±0.26 in conditioned animals (Fig. 4.8a; F=1.08, p=0.345). Mean ventilation rate was significantly higher in CS+ than CS- animals (Fig 4.9a; CS-, 3.77±0.21; CS+, 4.44±0.26; F=5.35, p=0.023). In the first 30 seconds of the test period, conditioned and control animals showed the same TER scores (Fig 4.8b; CS-, 0.63±0.20; CS+, 0.63±0.31; F=0.00, p=1.00). There was also no difference between ventilation rates of control and conditioned animals (Fig 4.9b; CS-, 3.83±0.20; CS+, 4.33±0.33; F=2.87, p=0.151). In the final 30 seconds of the test

period, conditioned animals extended their tentacles more than control animals, although this difference was not significant (Fig. 4.8c, CS-, 0.55±0.25; CS+ 1.38±0.31; F=5.95, p=0.058) Conditioned animals ventilated at higher rates than control animals in the last 30 seconds of the test (Fig 4.9c, CS-, 3.72±0.23; CS+ 4.55±0.20; F=19.74, p=0.006).

One-hour retention interval

At one hour post-training, control animals showed TER scores of 0.77±0.26, compared with 0.75±0.21 in conditioned animals, when the 60-second test period was analysed as a whole (F=0.01, p=0.93, Fig. 4.8a). Mean ventilation rates were also not significantly different between control animals and conditioned animals (control, 3.51± 0.15; conditioned, 3.76±0.17; F=1.08, p=0.3463; Fig. 4.9a). This trend did not change when the data were partitioned into early and late bins. In the first 30 seconds of the test period, TER scores of conditioned animals were 0.77±0.26, compared with 0.80±0.22 for animals in the control group (F=0.01, p= 0.942; Fig. 4.8b). There was also no difference in ventilation between the two groups in the early bins (CS-, 3.38±0.14; CS+ 3.94±0.28; F=2.94, p=0.147; Fig 4.9b). In the latter half of the test period, TER scores did not differ between CS- and CS+ animals (CS-, 0.77±0.26; CS+ 0.69±0.22; F=1.91, p= 0.771, Fig 4.8c), and ventilation rates did not differ either (CS-, 3.63±0.21; CS+ 3.58±0.15; F=0.06, p=0.822, Fig 4.9c).

Six-hour retention interval

Conditioned animals tested at six hours post-training showed higher mean TER scores across the whole 60s test period than control animals tested at the same interval, but the change was not significant (CS-, 0.98±0.50; CS+, 2.09±0.38, F=5.17, p=0.072).

Ventilation rates between CS+ and CS- animals also did not differ (CS-, 3.15±0.15; CS+, 3.63±0.32; F=1.64, p=0.256). When the data were partitioned into early and late bins, a difference in behaviour was apparent in the second half of the test period, but not in the first. In the first 30 seconds, animals showed no higher tentacle extension scores when given CS+ training than when they received CS- training (CS-, 1.00±0.51; CS+ 1.66± 0.38; F=1.98, p=0.281). Conditioned animals also ventilated at the same rate as control animals in the first 30 seconds (CS-, 3.13±0.13; CS+ 3.50±0.32; F=1.32, p=0.322). A difference between CS+ and CS- animals appeared in the latter half of the test period. Conditioned animals extended their tentacles significantly further than control animals (CS-, 0.97±0.49; CS+ 2.52±0.40; F=8.85, p=0.031). Ventilation rates were also significantly higher among conditioned animals than control animals in the last half of the test (CS-, 3.00±0.16; CS+ 4.66±0.28; F=17.09, p=0.009).

Twelve-hour retention interval

At 12 hours post-training there was no difference between conditioned and control animals' TER scores. Mean TER across the full 60s test period was 0.59±0.33 in animals given CS- training, and 1.15±0.42 in animals that received CS+ training (F=1.81, p=0.236). Ventilation rates remained significantly higher across the full test period (CS-, 3.33±0.12; CS+, 3.88±0.19, F=7.52, p=0.04), but when behaviours in the first half of the test period were compared there were no differences apparent between control and conditioned animals. Mean TER in animals given CS- training was 0.69± 0.35, and 1.00±0.43 in animals given CS+ training (F=0.80, p=0.413). Mean ventilation rate in control animals was 3.31±0.12, and 3.67±0.22 in conditioned animals (F=3.01,

p=0.143). Again, in the last half of the test period, animals given CS+ training showed greater mean tentacle extension than animals given CS- training (CS-, 0.38±0.21; CS+, 1.58±0.39; F=10.08, *p*=0.024). Ventilation rates were also higher among conditioned animals (CS-, 3.36±0.14; CS+, 4.11±0.21; F=9.27, *p*=0.028).

24-hour retention interval

In the final retention interval tests at 24 hours post-training there were no significant differences in either TER or ventilation rate in any comparison. When all the data were pooled across the 12 bins, control animals extended their tentacles for an average score of 0.80±0.43, compared with 0.72±0.21 for conditioned animals (*F*=0.06, *p*=0.809). Animals also ventilated at the same rate in both control and conditioned groups (CS-, 3.54±0.36; CS+, 3.47±0.27; *F*=0.03, *p*=0.859). Partitioning the data did not produce a difference between the two treatment groups. In the first 30 seconds, mean TER in control animals was 0.86±0.46, compared with 0.72±0.22 in conditioned animals (F=0.18, *p*=0.69)). Ventilation rates were also the same in both groups. Animals that received CS- training ventilated at 3.47±0.40 breaths per 5s bin, while animals given CS+ training had mean ventilation rates of 3.47±0.27 breaths per 5s (F=0.00, *p*=1.00). The results were the same in the second half of the test interval. Control animals had mean TER scores of 0.75±0.40, while mean TER in conditioned animals was 0.72±0.22 (F=0.01, *p*=0.93). Ventilation rate in control animals was 3.61±0.34, and 3.47±0.29 in conditioned animals (F=0.10, *p*=0.766).

Effect size estimates

The computations of effect sizes showed very similar trends to the results of the ANOVAs from each test period. For each retention interval I computed the size of the effect of conditioning on TER (Fig. 4.10) and ventilation rates (Fig. 4.11), using data from the last 30 seconds of the test period only.

At three minutes post-training, the estimated effect size (EES) of training on TER was 1.56, with an upper confidence limit (CL) at 2.86 and a lower confidence limit at 0.27. As these CLs do not overlap zero, there was a positive effect of training on TER. For ventilation rate, the estimated effect size (EES) was 0.97, with upper and lower CL bounds at 2.16 and -0.23 respectively. Thus there was no effect on ventilation, as the lower CL is below zero.

At 30 minutes post-training, there was a weaker, but still genuine effect of training on TER, with an estimated effect size of 1.32, upper CL 2.57 and lower CL 0.07. For ventilation rate, there was also a small effect of training remaining, with the effect size estimate of 1.44, with upper CL of 2.71, and lower CL of 0.17.

At one hour post-training the effects disappeared, reflecting the result of the ANOVA above. TER effect size was close to zero, estimated at -0.12, with upper and lower CLs of 1.01 and -1.25 respectively. The effect of training on ventilation also disappeared, with the EES of -0.08, and upper and lower CLs of 1.05 and -1.22 respectively.

At six hours post-training, there was a small positive effect on both TER and ventilation rate. The effect size estimate on TER was 1.25, with upper and lower CLs at 2.48 and 0.01 respectively, and for ventilation rate the EES was 1.66, with upper and lower CLs of 2.97 and 0.35.

At twelve hours post-training, the positive effect on TER had disappeared again, as the EES on TER was 1.13 with upper and lower bounds of 2.33 and -0.09. For ventilation rate the EES was 0.69 with upper and lower bounds of 1.85 and -0.48.

The effect of training on both TER and ventilation had completely dissipated at 24 hours post-training, with CLs for both measures overlapping zero. For TER, the EES was 0.00, with CLs of 1.13 and -1.13. For ventilation rate, the EES was -0.10, with the upper and lower bounds at 1.03 and -1.23. Thus the results of the two different analyses were largely identical.

4.4.2 Experiment 2: Extinction and Recovery of the Conditioned Response.

Overall the results of this experiment were less informative than those of Experiment 1. Although it appeared that both behavioural measures (TER and ventilation rate) reflected changes over the course of training, extinction and recovery, the variation in responses between animals was large and the trends are not clear. Following the results of Experiment 1, data from both the full 60-second test period and the latter 30 seconds only are presented in figures 4.12a and b (TER), and 4.13a and b (ventilation rates). Statistical analysis was conducted on the final 30 seconds of the test period only. Results of each stage of the procedure are presented below. Means, standard errors of means (s.e.m) and *p* values for selected comparisons are listed in Table 4.4.

Training trials (Presentations 1-10)

Over the course of ten training trials, animals given CS+ training showed similar response levels to animals receiving CS- training. Mean TER in the CS+ treatment was 1.04±0.24 in the first trial, compared with 1.02±0.22 for animals given CS- training

(F=0.00, p=0.94, Fig. 4.12). Mean ventilation rate in the first trial was 4.20±0.26 in conditioned animals and 3.74±0.28 in controls, a non-significant difference (F=1.47, p=0.23, Fig. 4.13). In the second training trial, TER for conditioned (CS+) animals increased to 1.60±0.19, whereas TER in animals given CS- training decreased to 0.85± 0.19, and the difference between the two treatments was significant (F=7.31, p=0.01). There was no difference in ventilation in the second trial, although animals given CS+ training did show a slight increase in ventilation rate from trial 1, but animals given CS- training showed no change. Mean ventilation in conditioned animals was 4.35±0.23, and 3.74±0.26 in control animals (F=3.09, p=0.09). The subsequent training trials did not differ between CS+ and CS- conditions in either tentacle extension response or ventilation rates (Table 4.4, Figs 4.12, 4.13).

Although there was a trend toward decreasing responses across the course of training within both CS+ and CS- conditions, these changes were not significant. Tentacle extension responses in conditioned animals compared between the first training trial and the tenth training trial in conditioned animals were not significantly different (mean TER in trial 1, 1.04±0.24; mean TER in trial 10, 1.53±0.27; p=0.13). In animals given CS- training, mean TER in trial 1 was 1.02±0.22, and 1.09±0.19 in the tenth (p=0.91). This was the same for ventilation rates. Conditioned (CS+) animals ventilated an average of 4.20±0.26 breaths per five seconds in the first trial, and 4.03±0.25 in the tenth (F=0.03, p=0.85). In the control procedure there was also no change from the first to the last training trial. Mean ventilation rate in trial 1 was 3.74±0.28, and 3.75±0.19 in the tenth (F=1.04, p=0.38).

Extinction trials (Presentations 11-20).

In the first extinction training presentation, animals that received CS- training showed very low tentacle extension in response to the light pulse. Animals that had received CS+ training showed responses roughly equal to those of training. Mean TER in CS- animals was 0.32±0.11, compared with 1.03±0.21 in conditioned animals ($F=9.76$, $p=0.0062$). This difference was not apparent in ventilation rates, as conditioned animals showed mean ventilation rates of 3.87±0.27, and control animals ventilated at 3.54±0.20 breaths per five seconds ($F=0.98$, $p=0.33$).

By the final extinction trial, the difference in TER scores between control and conditioned animals had disappeared (CS-, 0.74±0.15; CS+, 0.94±0.26; $F=0.07$, $p=0.79$), and remained absent in ventilation rates (CS-, 3.56±0.36; CS+, 3.19±0.22; $F=0.80$, $p=0.37$).

There were no changes from the first to the last extinction trial in animals in the control group. For animals that received CS- training, the change in TER scores from the first to the tenth extinction presentation was not significant (trial 1, 0.32±0.11; trial 10, 0.74±0.15; $F=5.12$, $p=0.07$), nor was the change in ventilation rate (trial 1, 3.54±0.20; trial 10, 3.56±0.36; $F=0.01$, $p=0.96$). In conditioned animals, the change in TER from the first to the final extinction trial was not significant (trial 1, 1.03±0.21; trial 10, 0.94±0.26; $F=1.91$, $p=0.77$), but there was a marginal change in the ventilation rates across the course of extinction for conditioned animals (trial 1, 3.87±0.27; trial 10, 3.19±0.22; $F=7.53$, $p=0.04$).

Recovery at 6 hrs post-extinction (Presentations 21-30)

The behavioural responses of conditioned animals in the six-hour recovery period show the clearest evidence of spontaneous recovery. Overall both tentacle extension and ventilation rates appear to be elevated across the course of the ten presentations of the light pulse (Figs 4.12a, b and 4.13a, b).

In the first presentation at six hours post-extinction, conditioned animals showed mean tentacle extension responses of 1.50±0.21, which was significantly higher than TER in animals that had received control (CS-) training, (mean TER for controls, 0.26± 0.16, F=25.34, p=0.0007). However there was no change in TER scores from the final extinction trial to the first recovery trial in conditioned animals, indicating that recovery was not expressed in the first presentation at six hours (F=1.01, p=0.33). There was also no change in TER in animals that had received CS- training between the final extinction trial and the first recovery trial (F=1.79, p=0.10).

The pattern for ventilation rates in the first recovery trial at six hours was similar to the pattern for TER. Animals given CS+ training ventilated on average 4.20±0.40 times per five-second bin, a significant increase when compared with animals given CS- training (mean for CS-, 3.06±0.13; F=8.50, p=0.017). In comparisons between the first recovery trial and the final extinction trial, again there were no significant differences in either conditioned or control animals (CS+, F=5.25, p=0.05; CS-, F=1.15, p=0.29).

By the tenth presentation in the six-hour recovery period the difference between behaviour in animals given CS+ and CS- training had disappeared, although this is due mainly to increases in the behavioural measures among animals in the CS- treatment, whereas the behaviours of animals that received CS+ training remained relatively

constant. In the CS- treatment, animals showed mean TER scores of 0.80±0.34 in the final presentation, compared with 1.60±0.31 for conditioned animals (F=1.40, *p*=0.32). There was also no difference in ventilation rate between the two conditions in the final presentation at six hours. Animals that received CS- training ventilated on average 3.53± 0.24 times every five seconds, compared with 3.97±0.45 in conditioned animals (F=0.73, *p*=0.42).

Recovery at 12 hours post-extinction (presentations 31-40)

Behaviour across the later recovery periods was highly variable, and there were no robust differences between conditioned and control animals. In the first presentation at the twelve-hour recovery interval, animals in the control group showed low tentacle extension responses, as did conditioned animals (mean TER for CS-, 0.17±0.17; Mean TER for CS+, 0.40±0.24; F=2.67, *p*=0.17; Fig. 4.12b). There were also no differences in ventilation rates between the two groups (mean ventilation in CS- animals, 3.43±0.41; mean ventilation rate in CS+ animals, 3.13±0.19, F=0.39, *p*=0.55, Fig. 4.13b).

In the tenth presentation of the light pulse at twelve hours post-training, TER scores for both CS- and CS+ conditions had increased from the first presentation, but only the change in CS- was significant (CS-, F=7.20, *p*=0.014; CS+, F=0.98, *p*=0.33). Mean TER in the final presentation was 1.44±0.41 for animals that had received CS- training, compared with 1.00±0.45 in conditioned (CS+) animals (F=0.27, *p*=0.63; Fig. 4.12b). There was also no difference in ventilation rate when compared between CS+ and CS- conditions in the tenth presentation. Animals that had received CS- training ventilated on average 3.94±0.67 times per five-second bin, compared with 3.50±0.53 in

conditioned animals (F=0.26, *p*=0.62). Comparisons of ventilation rates between the first and final presentation at twelve hours also yielded no significant changes in either control (CS-) or conditioned (CS+) animals (CS-: F=0.90, *p*=0.39; CS+: F=0.40. *p*=0.58; Fig. 4.13b).

Recovery at 24 hours post-extinction (presentations 41-50).

Behaviour across the last recovery period was also highly variable, and again there were no robust differences between conditioned and control animals. In the first presentation at the 24-hour recovery interval, animals in the control group showed mean TER scores of 1.14±0.58. Animals that had received CS+ training showed mean TER scores of 0.83±0.48 (F=0.30, *p*=0.61; Fig. 4.12b). There was also no difference in ventilation rates between the two groups (mean ventilation in CS- animals, 4.17±0.62; mean ventilation rate in CS+ animals, 3.94±0.43, F=0.09, *p*=0.77, Fig. 4.13b).

In the final presentation of the light pulse at 24 hours post training, TER scores for both CS- and CS+ conditions were almost identical to TER scores from the first presentation. Mean TER in the final presentation was 1.19±0.16 for animals that had received CS- training, compared with 1.36±0.35 in conditioned (CS+) animals (F=0.12, *p*=0.72, Fig. 4.12b). There was also no difference in ventilation rate compared between CS+ and CS- conditions in the final presentation. Animals that had received CS- training ventilated on average 3.47±0.21 times per five-second bin, compared with 3.64±0.30 in conditioned animals (F=0.21, *p*=0.65).

Effect size estimates.

The computations of estimated standardised effect sizes (EES) for selected comparisons within Experiment 2 showed very similar trends to the results of the ANOVA results listed above. Effect-sizes were estimated for conditioned animals for training by comparing TER (Fig.4.14) and ventilation rates (Fig. 4.15) between the first and final (tenth) training presentation. The effect of extinction was estimated by comparing the first and final extinction presentations (presentations 11 and 20), then recovery effects at each interval were estimated by comparing the final extinction presentation to the first recovery presentation at each of the three recovery intervals (presentations 20 to 21 for 6 hrs, 20 to 31 for 12 hrs, and 20 to 41 for 24 hrs). None of the estimates showed a genuine effect.

Over the course of the ten training trials, the estimated effect on TER was 0.62, with upper and lower 95% CLs of 1.78 and -0.54 respectively. The estimated effect size of training on ventilation rate was -0.21, with upper and lower CLs of 0.93 and -1.34 respectively.

The effect of extinction presentations on TER was 0.14, with upper and lower CLs of 1.28 and -0.99. The effect on ventilation rate was -0.98, with upper and lower CLs at 0.22 and -2.18 respectively.

At 6 hours after extinction, the effect on TER scores was 0.38, with upper and lower CLs of 1.52 and -0.76, and the EES for ventilation was 1.05, with upper and lower limits of 2.25 and -0.16 respectively. At 12 hours after extinction, the effect of recovery on tentacle extension was 0.83, with CLs of 0.35 and -2.00, and the EES of 12-hr recovery on ventilation was -0.08, with CLs of 1.05 and -1.21. The final recovery period at 24 hours after extinction also produced no genuine effects of recovery on either

tentacle extension or ventilation rate. The EES for tentacle extension was -0.27, with upper and lower 95% confidence limits of 0.86 and -1.41 respectively. For ventilation rate the EES was 0.72, with CLs of 1.89 and -0.45.

4.5 Discussion

4.5.1 Memory Retention

Chambered Nautilus exhibits temporally separated short- and long-term memory stores. While the short-term memory curve in nautilus is comparable to other cephalopods, long-term memory is considerably shorter. The results show that the memory profile of *N. pompilius* is biphasic. There is a clear conditioned response elicited soon after training is complete, then a lag period where no memory expression is apparent before re-emergence of the conditioned response between one and six hours after training. The response of the animals is remarkably similar to the response curve elicited in both juvenile and adult cuttlefish (Messenger, 1971; Agin et al., 1998, 2003, 2006). The characterisation in this chapter of 'short-term' and 'long-term' memory 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 studies.

In adult cuttlefish (*Sepia officinalis*), Messenger (1971) found a response recovery period at 22 minutes post training, indicating that memory of the aversive 'prawn-in-the-tube' procedure had decayed by that time. In *N. pompilius* these results

show that there is some memory apparent at least 30 minutes post-training and probably slightly beyond, but minimal accessible memory at one hour post-training. The apex of the response curve is also similar – in cuttlefish STM is expressed strongly between two and eight minutes post training in adults (Agin et al., 1998) and declines to baseline levels at around 20 minutes post-training (Messenger, 1971). In nautilus there is a very strong behavioural response in CS+ trials at three minutes post-training, suggesting rapid memory formation consistent with STM. The memory apparent at 30 minutes post-training may be the last expression of the original STM trace, in which case STM persistence is only slightly longer that that expressed by adult cuttlefish. Alternatively, it may indicate the presence of intermediate-term memory (ITM), which may arise later and persist longer than the STM that was almost certainly responsible for the spike at 3 minutes. This possibility warrants further investigation – ITM has been proposed as the underlying factor in the re-appearance of memory between 20 and 60 minutes in adult cuttlefish (Agin et al., 2006). ITM and LTM are physiologically distinct processes and both occur in the gastropod mollusc *Lymnaea* (Sangha et al., 2003). Further examination of these two distinct processes would certainly aid our understanding of the neurophysiology underlying memory in nautilus, and represents an intriguing future direction for these studies. STM lasting minutes followed by ITM lasting an hour or more has also been reported in *Aplysia* (Ghirardi et al., 1995) along with *Lymnaea* (Lukowiak et al., 2003) as well several other vertebrate and invertebrate taxa (e.g., rats, Rosenweig et al., 1993; crabs, Pedriera et al., 1998).

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 hours post-training. In

measurements taken during the development stage of the procedure there was no memory expressed at either 36, 48 or 72 hours 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, occurring several hours after training, but the durations observed in both octopuses and cuttlefish are considerably longer – certainly beyond 24 hours and possibly lasting weeks in *Octopus* (Boal et al., 2000; Young, 1961). If LTM genuinely does not persist beyond 24 hours in nautilus, the mechanisms underlying such a short retention period are worthy of consideration. There are several reasons why this short memory trace may have occurred. The first and most obvious explanation is simply that the conditioning procedure was not optimal to produce and sustain LTM. It is well known that reinforcement schedule and training procedures can have a dramatic effect on performance in invertebrates. In the crab *Chasmagnathus,* massed and spaced conditioning produced differences in the behavioural response (Pereyra et al., 2000) and in the pond snail *Lymnaea* different forms of memory can be promoted by using different training procedures (Lukowiak et al., 1998). It is probable that longer-term memory could be elicited through simple alterations to the ITI, ISI or combination of stimuli used.

I was also unable to train to a performance criterion using this paradigm. The very brief (500ms) light pulse that served as the CS and the short ISI (~2 s) did not permit clear determination of which animals demonstrated acquisition of the task during the ten training trials they received. Although it seemed likely that ten training trials

were ample for animals to acquire the association, changes to this approach may provide a clearer picture of when acquisition has occurred.

In octopus, Young (1960a) found no substantial differences in performance of normal octopuses (*O. vulgaris*) trained in an operant procedure with either a five minute or one hour ITI, but found a considerable difference in performance in animals which had their vertical lobes removed. This finding is particularly interesting given the absence of a vertical lobe complex in nautilus. Identifying the site of memory storage in nautilus may provide valuable insights into how memory is encoded and retrieved using different neural architecture.

It is possible that given the absence of analogous 'octopus-like' dedicated learning and memory centres in the nautilus brain, individuals are simply not able to store or retrieve LTM after 24 hours. In coleoids the vertical and subfrontal lobe complexes are implicated in visual and tactile memory respectively (Boycott and Young, 1955; Fiorito and Chichery, 1995; Maddock and Young, 1987; Robertson et al., 1996; Young, 1965c, 1991). Nautilus lacks both these dedicated regions (Young, 1965b) and it is possible that the pleisiomorphic neuroanatomy retained by *N. pompilius* lacks the capabilities of its more derived relatives. This is interesting from both an evolutionary and ecological perspective. There are remarkable if superficial similarities between modern nautiluses and the externally shelled ancestors of the coleoids (Teichert, 1988; Clarke, 1998b). Both lineages of cephalopods remained strikingly similar in appearance until relatively recently, when the coleoid descendents of the belemnite lineage internalised or lost their shells and radiated into predator niches, presumably exerting considerable selective pressure on neuroanatomy and behaviour (Aronson, 1991; Hanlon

and Messenger, 1996; Packard, 1972). The resulting differences in lifestyle may have promoted corresponding changes in the neural architecture of the two lineages. During the Mesozoic and onward, coleoids adopted a fast, visual, predatory lifestyle geared toward avoiding bony-fish predators (Aronson, 1991; Packard, 1972). Nautilids, on the other hand, retained their external shell and their energetically inexpensive locomotory design (Packard, 1972; Ward, 1987) occupying the niche of a solitary, slow growing scavenger living at depths where teleost predators are limited.

If nautilus' inability to retain LTM for long periods has been shaped by its ecology and therefore represents a secondary loss of complexity, we should expect to see some considerable need for LTM at periods longer than one hour but shorter than 24 hours. *N. pompilius* makes daily migrations up and down the reef face in order to avoid predators and locate food. Several studies have tracked animals in the wild through a number of these daily migrations (Carlson et al., 1984; Ward et al., 1984). An animal tracked for six days and nights showed that foraging occurred in shallow water in periods of around 6 hours after the animal made a rapid (2-3 hour) ascent. The subsequent descent was completed around 10-12 hours after the beginning of the ascent (Ward et al., 1984). Perhaps this 12-hour migration cycle, which presumably takes the animal across roughly the same area of reef, has influenced the shape of the LTM curve and promoted the evolution of LTM stores optimised for recovery over relatively short periods. Further detailed study of nautilus both in the wild and in laboratory experiments will be required to reveal the true extent of its behavioural and cognitive abilities and resolve these competing hypotheses.

4.5.2 Extinction and Recovery.

This experiment yielded results that were less informative than the first experiment. Although it appeared that there were trends within the behaviours that suggested extinction and recovery did occur, the variation both within animals and across test periods was very large, rendering the results difficult to interpret. There was little difference between control and conditioned animals in either training or extinction procedures, suggesting that animals given CS+ training habituated rapidly to the conditioning procedure, and that both groups habituated to the experimental context across trials. In Experiment 1, retention testing at one hour post-training revealed that there was little difference in behaviour between trained and untrained animals, reflecting the period between the last expression of STM and the earliest expression of LTM. In the first extinction trial in Experiment 2, somewhat counter-intuitively, there is a pronounced difference in the first presentation in tentacle extension, but not ventilation rates, between control and conditioned animals. This difference appeared to result from the very low TER scores for control animals, not an increase in response by conditioned animals, and probably represents a difference in habituation level between CS+ and CS- conditions. A similar effect is evident at six hours and twelve hours after extinction, where animals in the control group had low behavioural scores in the first trial. In effect, the animals in the control group may be expressing their own conditioned response to the experimental arena, after receiving two essentially identical experiences of un-reinforced light pulses prior.

The evidence for spontaneous recovery is equivocal. There was an increase in the levels of both TER and ventilation rates in conditioned animals at six hours after

extinction, both compared with control animals at the same interval, and compared against the responses of the conditioned animals in the extinction and the training procedures. Although the results show considerable variability, it appeared that the conditioned response was resistant to decay over the ten additional extinction trials at six hours after the first extinction procedure. It is possible that either ten extinction trials were insufficient to induce extinction once LTM was encoded, or that the extinction trials at one hour instead acted to reconsolidate rather than extinguish the memory and thus its expression at 6 hours was particularly strong.

The two final recovery intervals showed no evidence of recovery as both conditioned and control animals expressed highly variable behaviours. At 24 hours post-training in Experiment 1 there was no memory of the conditioning procedure apparent, and it appears that this is reflected in the results of Experiment 2. This variation in response is probably representative of the true decay process of the LTM trace, which in Experiment 1 appeared to be completely inaccessible at 24 hrs post-training. Presumably there is some interaction of the effects of extinction and training in CS+ animals, but there is no clear trend in these later recovery intervals. In control animals there are also very large jumps in behavioural measures between trials, suggesting also perhaps that their memories of the experimental context were also decaying during these two periods. Alternatively it is possible that the intensive handling and experimental procedures prior to these tests had produced a sensitisation effect in animals in both conditions, causing large changes in behaviour between presentations.

Given the ambiguous results of this experiment there is not a great deal of information that can be gleaned from it at this stage, although there are suggestions of

trends that are worthy of closer investigation. It is possible that further refinements to this procedure, combined with a better understanding of the mechanisms underlying the bi-phasic curve in Experiment 1, might provide more robust conclusions about the nature of learning, memory and the memory-decay processes in nautiluses.

Chapter 5. Spatial Learning and Memory

5.1 Introduction.

Both the evolutionary history of nautilus and its ecology make it an interesting subject for studies of spatial learning and memory. Whereas the classical conditioning procedures provided initial baseline data on memory retention and learning ability, spatial memory tasks such as those reported in this chapter can provide more ecologically relevant tests of behavioural plasticity.

There is evidence that memory of the spatial environment is used by at least some species of coleoids in their natural environment (foraging excursions in reef octopuses, e.g., Mather 1988, 1991a,b; Forsythe and Hanlon, 1997). Several laboratory studies also suggest spatial learning in octopus (Boal et al., 2000) and in cuttlefish (Alves et al., 2006, 2007a,b; Karson et al., 2003). Octopuses are typically central-place foragers that return to home dens to shelter from predators (Mather, 1991a), thus memory of their spatial environment is necessary for their day-to-day activities.

Nautilus makes large-scale, daily migrations through its complex reef habitat, encountering potentially the same spatial features over successive trips, especially if it returns to familiar locations during the daily cycle of sheltering during the day and foraging at night (Carlson et al, 1984; Ward et al., 1984). However, there is no concrete evidence that animals maintain location fidelity in the wild and may instead drift passively with the current, making the need for large-scale spatial memory less pressing. However, as nautiluses are relatively weak swimmers and are almost certainly subject to

strong tidal currents in their natural habitat, it is plausible that some form of short-term or small-scale spatial memory may be highly advantageous. A feeding animal might find itself drawn away from its foraging zone by the current, and since odour plumes are patchy and do not maintain a concentration gradient, following an odour plume back to its foraging zone using no other cues may be difficult (Moore and Atema, 1991). In such a situation, an ability to *remember* the location of the food, either based on egocentric cues such as body position and swimming speed, or geocentric cues provided by visual landmarks or other features of the environment, may be of considerable value.

Despite our lack of knowledge about natural behaviour, demonstrating spatial memory in nautilus would allow comparison with the coleoids, and provide an interesting counterpoint to their associative-learning capabilities. Demonstrating spatial learning in nautilus may also illuminate our understanding of the ecology of the ancestors of both nautilids and coleoids.

The aim of this experiment was thus to examine spatial learning and memory on a small spatial scale (within 10-20 body lengths). The experiment design tested specifically whether captive nautiluses could learn and remember visual features in their surroundings, and then use these features to navigate successfully towards a goal. This procedure was similar to the Morris water-maze task (Morris, 1981), a paradigm that was developed to test spatial memory in rodents.

5.2 Methods

5.2.1 Animals.

Wild-caught adult or sub-adult *Nautilus pompilius* (n=10) were obtained from the same commercial supplier used previously (Sea Dwelling Creatures, Inc., California). Animals were maintained in their home-tanks for at least two weeks before being used in any experimental procedure. As this spatial learning study required that animals swim downward through the water column, those that either floated or sank persistently after the two-week acclimation period were excluded.

Animals were fed every four days with a 2cm cube of frozen Tilapia (*Oreochromis niloticus*) head, except if this coincided with a testing day. On testing days, all animals were fed upon conclusion of the experimental procedure once they had been returned to their holding tank.

4.2.2 Apparatus

The spatial memory tasks were conducted in one of the two home tanks (Fig. 5.1a,b), which had been fitted with a circular PVC open-field maze. All animals were housed temporarily in the remaining tank while the experiment was conducted in the tank fitted with the maze. The maze was a circular PVC platform made from a modified home-tank lid (Aquatic Ecosystems, Inc.), resized to fit tightly within the cylindrical home-tank. The maze was reinforced and held level by fixing a grid of plastic mesh to its underside. The upper surface of the maze platform was smooth and black, and identical in appearance and texture to the inner walls of the home tank. A single hole (diameter

20cm), cut into the platform close to one edge served as the goal location for all memory trials (Fig. 5.1b). This goal was the only point of exit into preferred, deeper water (under the maze) within the home-tank. A visual and tactile beacon surrounded the goal. The beacon was constructed from a donut-shaped piece of bubble-wrap, scored across the bubbles to release the air, and with strips of white tape underneath. This combined a rough surface with an obvious striped pattern that contrasted strongly with the smooth black surface of the platform, acting as both a proximal (tactile) and distant (visual) navigation aid to the animal.

The test arena (Fig 5.1) was lit by four fluorescent tubes, located directly above the tank, providing a non-directional light source that was constant across trials. Within the tank, there was a gentle, anticlockwise flow of water (~1cm/second) from the inlet pipe below the maze platform to the outlet pipe above it. This flow pattern was identical to that found in the home tank under normal housing circumstances.

All trials were videotaped using a digital camera (Hitachi CCD, model KP-M2U) suspended above the tank, and a Sony DV mini-recorder (Sony DV walkman, model GV-D900). The experimenter monitored the animal from behind a blind using the mini-recorder display.

4.2.3 Experimental Procedure.

4.2.3.1 Experimental Trials

Before the experimental trials began, animals (n=10) were kept in a consistently darkened home-tank for a week. As bright light was an important aversive stimulus in this experimental design, pre-exposure to constant darkness increased the motivating effect of bright light during the trials themselves.

Animals were tested in random order. Five minutes before a trial began, one animal was removed from its home tank and placed in an uncovered bucket containing home-tank water. Most animals responded to the sudden exposure to bright light in the experiment room by swimming rapidly about in the bucket. Immediately before the trial commenced, the maze platform was secured within the trial tank and the water level above the platform was set to the same depth as the test subject's shell height, such that the animal was in contact with the platform as it swam. The shallow depth of the water above the platform acted as an additional aversive stimulus, as animals avoid shallow areas in their tank if possible.

Video recording of the experimental arena began 30 seconds before the animal was moved from the bucket and placed onto the maze. At the start of each trial, the animal was placed in a consistent start position, which was located opposite (180° degrees) to the escape hole. Within the tank, the goal location (exit) was marked by the beacon but otherwise the tank was featureless, as all interior surfaces were made from identical black PVC plastic. Flow within the tank provided local hydrodynamic cues. In addition, there were a number of global cues (outside the maze) available to the subjects. There was a light gradient within the room created by an algal-culture stand in one corner, which was lit brightly by broad-spectrum fluorescent lights, and other features of the room such as tanks and signs on the walls may also have been visible to the subjects.

The subject was held in the start position for five seconds, facing into the centre of the tank. In this position the natural backwards-swimming response pushed the animal back against the wall of the tank. After five seconds, the subject was released and allowed ten minutes to locate the goal and swim through the exit hole and into deeper

water. Once the animal reached the escape hole and descended below the level of the platform, it was allowed to remain undisturbed in the deeper water for a further ten minutes, as a positive reinforcement for completing the task. Animals that failed to complete the task within ten minutes were guided gently by hand into the escape hole as soon as the ten-minute trial time expired. This was done either by allowing the animal to attach its tentacles to the experimenter's fingers, who then towed the animal at its normal swimming speed into the hole. If the tentacles were retracted, it was pushed from behind at swimming speed until it contacted the hole and exited. Once inside the hole, subjects descended usually without further prompting, and were then left under the maze for ten minutes as above. After ten minutes the animals were retrieved from the darkened tank with a net, and the procedure was repeated on that animal.

All subjects received a single block of five, ten-minute training trials, with an inter-trial interval of ten to twelve minutes. The variation in ITI occurred as animals sometimes attached tightly to the side of the tank, or were out of sight of the experimenter, and extra care was taken to remove these animals at the end of the trial without causing undue distress. Retention of spatial memory was tested with a single probe trial, identical to the training trials, at either 18h, 24h, 36h, 48h, 72h, 96h, 7 days or 3 weeks after the initial training period. Not all animals were tested at every retention interval (Table 5.1).

4.2.3.2 Baseline Activity Recordings.

Video recordings were also taken of animals swimming freely in the cylindrical home-tanks. These recordings served to establish the general activity level of the subjects before the experiment commenced, as well as the use of vertical and horizontal

spaces within the home-tank when the maze platform was not in place. Animals (n=6) were tested individually. One hour before the recordings began, all the animals were transferred to one of the two home-tanks, leaving the other (the same tank as used in the experimental trials) empty. Five minutes before the recording period began, one animal was removed from the holding tank and placed in an uncovered bucket, in a procedure identical to the preparation for the spatial memory trials above. The animal was transferred to the recording tank and held for five seconds in the 'start' position that was used in the experimental trials, except that the animals was held suspended at the surface of the open tank, rather than resting on a maze platform. The subject was released then allowed to swim uninterrupted in the open tank for ten minutes. Overhead video recording (equipment and placement the same as above) of behaviour commenced as soon as the animal was released and continued for the ten minute 'trial'. The time animals took to settle either on the bottom or side of the tanks was recorded, and three-dimensional route maps were plotted for each animal from the video recordings, but detailed statistical analysis was not carried out on these data.

5.2.4 Data Analysis and Statistical Procedures.

Escape times were recorded from video footage of each trial. Time to escape was calculated from the time of release at the start of the trial to the time when the animal's shell dropped into the escape hole. The number of times the animal changed direction during each trial was also recorded. 'Change in direction' was defined as a greater than 15 degree change in heading angle. This amount of deviation excluded the natural slight rotational movement of animals as they swam over the surface of the maze. Escape times and number of turns were compared with repeated-measures ANOVAs (proc mixed)

implemented in SAS 9.1. Mean escape times and mean number of turns were compared, 1) Between the first and last training trials, and 2) Between the last training trial and the probe trial at each retention interval.

5.3 Results.

5.3.1 Baseline Activity Recordings

When animals were released and allowed to swim unrestricted in their home-tank, they swam in a downward, spiral motion before coming to rest in the lower third of the tank. In all cases, the animals followed the contour of the inner walls of the tank, swimming backwards and downward until they either came to rest on the bottom of the tank or attached to the wall using their tentacles. Direction of swimming always followed the current (anticlockwise), and the swimming time varied from 34 seconds to a maximum of 5:03 min. Animals that sat on the bottom of the tank without attaching with their tentacles drifted passively with the current for the remainder of the trial period, circling the outer perimeter of the tank bottom. In every case, animals descended below the surface almost immediately, and quickly came to rest in deeper water. These recordings show a generally thigmotactic swimming habit that followed the current within the tank, with a clear preference for deeper water.

5.3.2 Spatial Memory Procedures

Initially, animals placed on the platform appeared to have difficulty adjusting their swimming action to compensate for the additional drag on the base of their shell as

it rested on the platform. In the early trials animals tended to jet sideways to move over the platform surface, but this resulted mostly in the subjects spinning in place. A noticeable change in swimming method occurred soon after the training trials began, sometimes within the first trial. The jetting angle changed to a strong straight-down siphon jet, which propelled the animals forward in short bursts. This swimming method was adopted by all animals within a short period and persisted throughout the retention trials.

Means and standard errors for escape times and number of turns, with p-values of pair-wise comparisons of times and turns are summarised in Table 5.2 and shown in figures 5.2 and 5.3.

5.3.2.1 Training trials (Trials 1-5).

In the first trial, animals took an average of 447.6±78.8s to escape (Fig. 5.2). Seven of the ten animals failed to escape within the time allotted and were moved to exit hole at the conclusion of the trial. An escape time of 600s was recorded for these individuals. Animals changed direction an average of 9.0±2.1 times during the first trial (Fig. 5.3).

In the second trial only three animals failed to complete the task and required prompting at the end of the trial. The mean escape decreased significantly to 259±90.1s (repeated-measures ANOVA, p=0.0019). The mean number of turns made by animals was 10.1±3.2, a non-significant increase.

In trial 3, all animals escaped successfully within the ten-minute trial. Mean escape time was 66.2±17.0s, a significant decrease from trial 2 (p=0.0015) and mean number of turns was 8.9±1.7s, a non-significant change.

Trials 4 and 5 had mean escape times of 54.7±15.3s and 39.5±13.7s respectively (Fig. 5.2), which were not significantly different from trial 3 (trial 4, p=0.85; trial 5: p=0.69). Animals made fewer turns in trial 4 and trial 5 when compared with naïve animals in trial 1 (Fig. 5.3). Mean number of turns for trial 4 was 4.0±0.72 (p=0.015), and 3.8±1.7 in trial 5 (p=0.030).

5.3.2.2 Test Trials (Trials 6-13, Retention intervals 18hrs to 3 weeks).

In all the retention intervals tested, the pattern of escape times and changes in direction was very similar. Escape times remained significantly lower across retention intervals than in the first training trial, and were not significantly higher than the final training trial. The pattern for changes in direction was less pronounced, with number of turns remaining lower across most retention intervals than the first training trial, and not higher than number of turns made in the final training trials. Table 5.2 summarises means and standard errors of means for test trials at each retention interval and lists p-values of pair-wise comparisons (detailed below).

At 18 hours after training, animals took the same amount of time to locate the exit as in the fifth training trial. The mean escape time (48.8±17.0s, Fig. 5.2) and the mean number of turns (3.2±1.0, Fig. 5.3) were not higher than those of the fifth training trial (Table 5.2, time: p=0.92, turns, p=0.82). This trend continued at 24 hours after training. Neither mean time to escape (47.4±13.3s) nor mean number of turns (4.1±0.6)

had increased significantly (time: p=0.91, turns, p=0.91, Table 5.2) from the final training trial. At 36 hours after the final training trial, the mean time to escape was 52.9±20.6s (Fig. 5.2) and mean number of turns was 2.7±0.4 (Fig. 5.3), another non-significant change from the fifth training trial (time: p=0.86, turns, p=0.65). At 48, 72 and 96 hours after training was complete, the mean escape times and the mean number of turns made remained unchanged from the final training trial. (48hrs: time=42.2±9.9s, p=0.97, turns=3.8±0.6, p=0.97; 72hrs: time=41.0±16.5s, p=0.99, turns=4.6±0.6, p=0.78; 96hrs: time=30.0±5.4s, p=0.91, turns=3.4±0.2, p=0.87). This trend weakened but continued at the longest retention intervals, one week and three weeks post-training. At one week post-training, the mean time to escape was 56.2±13.2s (p=0.81) and the mean number of turns was 4.8±0.6 (p=0.68): slightly higher than the shorter retention intervals, but significantly lower than values for the first training trial (time, p<0.0001, turns, p=0.042). At three weeks post-training, although animals took longer to find the escape hole and made more changes in direction when compared with the fifth training trial, neither of these increases was significant (time=111.8±49.7, p=0.30, turns=4.8±0.7, p=0.69). The mean time to escape at three weeks was significantly lower than in naïve animals in their first training trial (p<0.0001), and the number of turns showed a marginally significant decrease (p=0.046).

5.3.3 Effect size estimates

Estimates of standardised effect sizes were computed for escape time (Fig. 5.4) and number of changes of direction (Fig 5.5), by comparing results from each trial to the

results of the first training trial. For both behavioural variables, the results of this analysis are very similar to the results from the ANOVAs described above.

Escape times

An effect of training on escape time appears in the third training trial. The estimated effect size (EES) when comparing escape time of the first and third trials was -2.04 with upper and lower 95% confidence limits of -0.96 and -3.12 respectively (Fig. 5.4). As these confidence limits do not overlap zero, this is a genuine effect. This effect persists in all subsequent trials, mirroring the results of the ANOVA. In the fourth training trial, the EES was -2.11 with upper and lower CLs of -1.02 and -3.20. In the fifth training trial, the effect size was slightly larger, -2.21 with upper and lower CLs of -1.10 and -3.33. In the retention test at 18hrs, the EES was -1.82, with upper and lower confidence limits of -0.63 and -3.01. At 24 hours, the EES was -1.97 and upper and lower CLs were -0.75 and -3.19 respectively. In the retention test at 36 hours after training, the EES was -1.80 and upper and lower CLs were -0.61 and -2.99 respectively. At 48 hours, the EES was -1.95 and upper and lower CLs were -0.73 and -3.16 respectively. In the retention test at 72 hours after training, the EES was -2.05 and upper and lower CLs were -0.82 and -3.29 respectively. At 96 hours post-training, the EES was -1.82, with CLs of -0.62 and -3.01. At the longest retention intervals of one week and three weeks after training, the effect on escape time persisted, with an EES of -1.85 and CLs of -0.65 and -3.05 at one week, and an EES of -1.36 at three weeks, with CLs of -0.39 and -2.34.

Changes of Direction

An effect of training on the number of turns animals made appeared in the fourth training trial, in agreement with the results of the ANOVA. The estimated effect size (EES) when comparing escape time of the first and fourth trials was -1.43 with upper and lower 95% confidence limits of -0.95 and -2.41 respectively (Fig. 5.5). This effect persisted in all subsequent trials. In the fifth training trial, the EES was -1.38 with CLs of -0.41 and -2.36. In the retention test at 18hrs, the EES was -1.35, with upper and lower confidence limits of -0.24 and -2.47 respectively. At 24 hours, the EES was -1.29 and upper and lower CLs were -0.18 and -2.40 respectively. In the retention test at 36 hours after training, the EES was -1.55 and upper and lower CLs were -0.40 and -2.69 respectively. At 48 hours, the EES was -1.35 and upper and lower CLs were -0.23 and -2.46 respectively. In the retention test at 72 hours after training, the EES was -1.37 and upper and lower CLs were -0.26 and -2.49 respectively. At 96 hours post-training, the EES was -1.08, with CLs of 0.00 and -2.16. The effect on changes of direction persisted at the longer retention interval of one week and three weeks. At one week after training the EES was -1.17 with upper and lower CLs of -0.16 and -2.17 respectively. At three weeks post-training the EES was -1.35 at three weeks, with CLs of -0.38 and -2.32.

5.4 Discussion.

Nautilus can remember the location of a goal exit signified by a beacon for at least three weeks. This is a considerably longer interval than the maximum 24hr retention period observed during classical conditioning. In this experiment there was

no biphasic memory curve apparent, although the longer retention intervals tested here most likely failed to capture the period between STM decay and LTM consolidation if indeed a similar period occurred. Instead these retention tests captured a long-term memory trace that arose before the first retention test and persisted beyond the last.

Although this spatial memory task differed substantially from the classical conditioning experiments, it contained an associative component that was similar to the CS-US relationship of blue light and food odour: the beacon provided a strong associative relationship between cue and goal. Although this may not demonstrate 'true' spatial learning (i.e., use of path integration vectors or formation of a cognitive map), the presence of an associative component within the task does not necessarily preclude it.

This experiment was designed to examine considerably longer retention times than the classical conditioning experiments described in Chapters 3 and 4. Longer memory retention was predicted based on the more 'active' nature of the task, the substantially longer inter-trial interval (ITI), and the longer running time of each complete training block. There is a considerable body of evidence that longer ITIs promote longer and more stable memory traces (eg, Lukowiak et al., 2003), and it seems plausible as well that a more ecologically relevant task may do the same.

Nautiluses acquired this task quickly, after only two or three training trials, finding the goal escape point within one minute or less within the first three trials. Their paths became straighter as well, with animals making fewer corrections (direction changes) over the first three training trials. These results are comparable to those observed in spatial memory experiments with cuttlefish (Karson et al., 2003). In

a simple alley maze, cuttlefish showed a marked drop in escape time by the fourth trial when trials were given in blocks of five. In the nocturnal reef octopus *O. bimaculoides*, Boal et al. (2000) showed that animals placed in a circular open-field maze similar to the setup in this experiment learned the location of a burrow (an escape point) within the first three trials, and retained this memory for at least a week.

Similar to the coleoids above, nautiluses not only learned the location of a goal in just a few trials, but also remembered the goal location for at least three weeks after training. This is longer than the memory observed by Boal et al. (2000), however the authors do not report results from beyond one week. It is likely that in octopuses memory persists for some considerable time beyond the one week reported in this study, given the obvious retention of the task by the animals at the conclusion of testing. In octopuses the vertical lobe complex is known to be the site of learning and memory in the brain (see Hochner et al., 2006, for review). The absence of a vertical lobe complex in nautilus makes this long retention period particularly surprising, and suggests that although a dedicated learning and memory centre in the nautilus brain has not been identified, such a region may not be necessary for 'coleoid-level' memory retention.

The three-week memory test showed more variability in escape times than the retention tests at one week and earlier, suggesting that three weeks may have been approaching the limit of retention in at least some of the animals tested. Despite this, a three-week retention period in even some of the animals is remarkable, given the relatively short training period and the lack of memory retention beyond 24 hours in classical conditioning experiments. These long retention times in this experiment

suggest that some aspect of this task made it more 'memorable' than the associative task. It was certainly a more naturalistic setup than that of the classical conditioning experiments. Animals were unrestrained and were able to explore their environment actively as they gathered the information necessary to complete the task. The motivation and reinforcement in this procedure may also have been more salient, as nautiluses showed strong aversion to both bright light and shallow water in preliminary tests. Alternatively, spatial memory may be more ecologically relevant than purely associative memory, and as such may be more efficiently encoded and retrieved in *N. pompilius*.

The speed with which the escape route was learned and the duration of memory are somewhat suggestive of a role for this type of learning in the natural environment. Nautiluses live in excess of ten years in the wild (Saunders and Ward, 1987) and may cover large areas of reef in their daily foraging expeditions. Retaining memories of features of their environment over a considerable period of time might be useful, perhaps to identify areas where animals have foraged recently, or to identify the limits of a suitable foraging zone or territory. Although there is no evidence currently that animals employ such decision-making processes in their natural environment, further studies of both captive and wild nautiluses may shed more light on the possible roles for spatial learning and memory in the life of this ancient species.

Chapter 6: Navigational Strategy in Spatial Learning and Memory

6.1 Introduction

6.1.1 Background

'Navigation' is the process by which an animal orients itself in space and determines its course toward some goal point (Franz and Mallott, 2000). To be successful, navigational strategies must provide means by which the individual can identify both its current position and its relationship to the desired endpoint of its journey: a 'map' of its surroundings, and a 'compass' to allow the animal to identify its position relative to a goal. This implies an ability to collect and use either environmental (geocentric) cues such as those provided by landmarks, or internal (egocentric) cues, such as a record of the path taken thus far, or a measure of distance travelled.

Navigational strategies used by animals tend to fall into one of several broad categories, with the contribution of egocentric and geocentric cues varying between each. Path integration (PI) is an egocentric strategy whereby an animal relies on an internal odometer and a directionality vector that is updated constantly. Through a combination of these two estimators it keeps track of the absolute direction of movement and the distance travelled between two points. Predominately egocentric strategies such as path integration may be used by animals that inhabit environments where visual cue availability is low (ants; Muller and Wehner, 1998; Wehner et al., 2006; Kohler and Wehner, 2005; crabs; Layne et al., 2003; meerkats, Manser and Bell,

2004; dogs; Cattett and Etienne, 2004; other mammals; Etienne et al., 1996). However, as egocentric strategies such as PI accumulate errors over distance and time, animals may use another geocentric strategy in parallel, switching between the two as one or the other becomes less reliable (e.g., Harkness and Maroudas, 1985; Knaden and Wehner, 2005).

Geocentric strategies can be divided broadly into map-based navigation and beacon-directed navigation. Beacon-based navigation (Gallistel, 1993) utilizes an association between a cue that signals the exact position of the goal, and the goal itself (e.g., the landmark used in Experiment 3, a striped, bubble-wrap circle in direct contact with the exit hole). In beacon homing, an animal must be able to maintain visual contact with the beacon to find the exit, and should orient to the beacon irrespective of starting position or travel direction. In contrast, map-based navigation, or 'piloting' (Cheng and Spetch, 1998; Gallistel, 1993) posits that the animal is capable of forming an internal representation of multiple local and global cues, as well as their relation to each other. Piloting animals can take a novel route to their goal from a new starting location. Complex cue encoding is used by numerous species (e.g., pigeons, Cheng et al., 2006; corvids, Kamil and Jones, 1997; Kamil and Cheng, 2001; rats, O'Keefe and Nadel, 1978; squirrels, Vlasak, 2005; fish, Lopez et al., 1999; ants, Collett et al, 1998; insects, Wehner et al., 1996), but a true 'cognitive map' is difficult to demonstrate with certainty.

A number of studies have examined spatial learning in cephalopods, (Boal, 2000; Hartwick et al., 1978, 1984; Hvorecny et al., 2007; Karson et al., 2003; Mather, 1991b; Wells, 1964) but only a few have systematically investigated the cue types and

navigational strategies underlying the ability to form spatial memories. In detour experiments with octopuses, Wells (1964) found that visual cues (geocentered) rather than body position (egocentered) were used to navigate a detour. In laboratory experiments with *O. rubescens*, animals trained to track a beacon signalling a food reward oriented to the beacon even when it was moved around the arena, and were thus relying on a single cue to locate their desired position (Mather, 1991b). In field experiments, Mather (1991a) manipulated landmarks around the octopus' dens, to provide a conflict between local and distant visual cues. Animals oriented using the distant cues that remained unchanged and ignored the local landmarks that were moved. It was also possible that octopuses were using path integration to return to their dens, however this was not tested explicitly. Cuttlefish (*S. officinalis*) are capable of using either a route memory or a goal-directed cue to orient to an escape point in a T-maze (Aves et al., 2007b), indicating that cuttlefish can use multiple strategies to locate a goal depending on the types of visual cues that were available during training.

6.1.2. Purpose.

The relative importance of egocentric cues vs. geocentric cues (and global (distant) and local (proximate) cues within the geocentric realm) can be separated by removing one set of cues or creating conflicting information between the cue types. The navigational decisions an animal makes when cues have been manipulated or cue reliability has been altered can reveal which strategy an animal is using.

This experiment tested competing hypotheses about the navigational strategy employed by *N. pompilius* in a small-scale spatial memory test*:* 1. Nautilus uses

beacon-based homing to locate the platform exit hole. A beacon was defined as a single, proximate cue located close to or directly over the goal. 2. Nautilus uses path integration to locate the exit. Path integration involves animals using internal information such as body position and movement direction to locate a goal, independent of external information. 3. Nautilus uses a cognitive map to locate the exit. A cognitive map was defined as an internal representation of a known environment based on numerous local and global reference points, their relation to each other and to the goal.

6.2 Methods.

6.2.1 Animals

This final experiment used the same 10 individuals used previously in the spatial memory experiment (Experiment 3). A period of approximately two months elapsed between experiments 3 and 4, during which time animals were maintained in darkened home tanks. All husbandry and handling procedures were as described previously.

6.2.2 Apparatus

The experiment used the same general tank and maze setup as Experiment 3, the spatial memory experiment (see Fig. 5.1). The circular open-field maze and the tank surroundings were modified slightly, depending on the hypothesis being tested. The maze was divided for the purpose of video analysis into four quadrants, based on the position of the exit hole (Table 6.1). No marks were made on the maze itself, quadrants

were measured from video footage only. The quadrant containing the exit hole in training trial defined as quadrant one, and the remaining quadrants were numbered clockwise from two to four. There were six different maze configurations used in this experiment, which are described in detail below in section 6.2.3. All video recording equipment and blinds were identical in make and position to those described in chapter 5.

6.2.3 Maze Configurations

There were six maze configurations used in this experiment. Training and probe-trial configurations are described below and shown in Table 6.1.

1. *Beacon*: The hypothesis that animals were using beacon-based homing (BBH) was tested by training with a maze configuration identical to that of Experiment 3. During training, the beacon (striped, bubble-wrap circle) surrounded the escape hole. The start position and escape hole were kept constant in training and probe trials, but beacon position differed. During the probe trial the beacon was moved anticlockwise into the adjacent quadrant (from Q1 to Q4) where there was no escape hole, while the escape hole itself was unmarked and remained in the training position (Q1). If animals oriented to the beacon in probe trials, even when it was not located in the original training position, this suggests that the animals were using beacon-based homing as a means to locate the exit. If they did not orient to the beacon and moved instead to the unmarked exit, then the beacon was not acting as the primary navigational cue and other information (global or local) was guiding the animals.

2. *Path Integration:* To test whether animals were using path integration (PI) to locate the exit hole, animals were trained as above with the beacon around the hole.

During the probe trial, the start and exit points were kept constant, but the beacon was removed, and all external landmarks were obscured by a blind around the tank. If animals swam directly toward the exit in the absence of visual cues (either the beacon or extra-maze cues) to guide them, then this supports the notion that the animal is using internal (PI) information to solve the spatial problem. If animals did not locate the exit during probe trials, this suggests that PI is not used in this situation and that external cues are necessary for successful completion of this task.

3. *Path Integration*: In a second test of path integration (PI AS), training was identical to that described above. However, in the probe-test trials the start position was moved 90 degrees clockwise, to the quadrant adjacent to the training start position, and was identical for all animals. Animals were also deprived of global cues as above. These probe trials were designed to show whether animals moved to where they would expect the exit-hole to be if the start position had not been moved (i.e., they took a straight path across the maze in the same relative direction as during training). If animals took a straight path to the opposite quadrant, this is suggestive of a route-memory, one component of path integration. If animals oriented to the exit, this would suggest some non-visual, external cue such as water current was used to navigate, and if animals failed to swim either directly to the actual or expected maze position, this would eliminate both of these possibilities.

4. *Cognitive Map*: To test whether animals were forming a cognitive map (CM) of their environment, animals were trained with a different configuration from above. Here in training there was no beacon marking the exit-hole. Instead, there were three landmarks (a white airstone, a white PVC x-junction, and a white piece of plastic egg

crate). The three landmarks were positioned around the edge of the arena, one in each of the three quadrants not containing the exit. Directly adjacent (clockwise, bridging Q3 and Q4) to the start point there was a 20cm x 15cm piece of white plastic egg-crate. The PVC fitting was located to the left of the start position in Q4, and the air-stone was on the right, in Q2. During probe trials the landmarks remained in a constant position relative to the exit and to the experiment room. The start position was shifted 90° to the adjacent quadrant (Q4), alongside the PVC fitting. If animals were not able to locate the exit from this novel starting position, it would be unlikely that map-based navigation was being used.

5. *Proximate vs. Distant Intra-Maze Cues*: To test whether animals were relying on proximal (close to the exit) or distant (around the edge of the arena) intra-maze navigational cues, the training trials were repeated with both the beacon (proximal cue) and the landmarks (distant cues) present. Thus this experimental setup included the bubble-wrap beacon, air-stone, egg-crate and cross-junction. During training the beacon was positioned at the exit-hole, and the start position and the position of all landmarks was as above (start position in Q3, eggcrate left of start position, PVC fitting in Q4, air-stone in Q2). In probe trials the beacon was moved to the adjacent quadrant to the exit (Q4, the same location used for the probe trial for beacon-based-homing), but the landmarks and the start position remained constant. If animals oriented to the beacon and ignored the distant intra-maze landmarks, this would suggest they were relying on information from the proximate beacon to locate the exit. If animals swam directly to the exit in probe trials, this would suggest that the three distant intra-maze landmarks were acting as the primary navigational cues.

6. *Global vs. Local Cues*: As it was possible animals were navigating using extra-maze (global) cues, this hypothesis was tested separately. Animals were trained in the maze with the three distant landmarks (local, intra-maze cues) present, positioned as described above. In probe trials, the maze and the intra-maze cues array were rotated 180 degrees relative to their training position and to the experiment room. Animals started their probe trials in the 'correct' start position relative to the intra-maze cues, not global extra-maze cues. If animals swam directly to the exit hole then they were relying upon intra-maze cues to navigate, as these remained the same relative to the exit and start positions. If animals did not swim to the exit hole from the start position in the probe trial, and instead swam to where the exit-hole would be coded by extra-maze cues, this suggests that global cues were being used rather than local intra-maze cues.

6.2.4 Experimental Procedure

Six subjects were selected randomly for each different maze-configuration that was tested (the ten animals were used multiple times but not necessarily in every configuration, see Table 6.2). Configurations 1 (BBH), 2 (PI) and 4 (CM) were tested first. Configurations 3 (PI AS), 5 (ALL) and 6 (L-G) were tested two to three weeks later in a follow-up stage, once initial results were analysed. Animals were randomly assigned to a test order within each set of tests (i.e., animal 1 received maze configurations in the order 1,2,4; animal 2 received 4,1,2; animal 3 received 2,4,1, etc.), to control for the effects of repeated training trials. The maze configurations for each training block and its corresponding probe trial are shown in Table 6.1.

The running procedures for each trial were effectively identical to the spatial memory experiment described in chapter 5. The training period consisted of a single

block of a maximum of four, ten-minute trials with an inter-trial interval of ten to twelve minutes, or until a criterion was reached of 2 successful escapes in less than two minutes. The single ten-minute probe trial occurred two hours after the completion of training. Four training trials were not always required to reach criterion, as repeated tests on the subjects resulted in very fast acquisition in later trials.

6.2.5 Data Analysis and Statistical Procedures.

Escape times and routes taken were recorded from video footage of each trial. Time to escape was calculated as the time from release of the animal at the start of the trial to the time when the animals' shell dropped into the escape hole. If an animal failed to locate the escape hole within the ten-minute trial, an escape time of 600 seconds was recorded for that subject. The number of changes of direction made during each trial was also recorded, but similar to Experiment 3, this measure was largely uninformative. Instead, route maps of each path were recorded by hand from real-time video footage of each trial, with animal position plotted every two seconds. Escape times were compared with repeated-measures ANOVAs implemented in SAS 9.1. I compared mean escape time and mean number of turns between the final training trial and the probe trial of each configuration. I computed standardised effect sizes for escape times, compared between the final training trials and the probe trials for each maze configuration.

6.3 Results

6.3.1 Beacon –based homing (BBH)

Animals (*n*=6) received four training trials in this configuration. On average, animals took 153.0±92.4s to escape in the first trial (Fig. 6.1a). By the fourth training trial they escaped more quickly (22.0±7.9s). In the probe trial, when the beacon was relocated from around the exit to quadrant four (Q4, see Table 6.1), animals took longer to escape (341.7±116.9s, F=6.69, *p*=0.0271).

Animals made an average of 6.50±1.36 changes of direction in their first training trial. This decreased to 2.50±0.65 in the fourth training trial (Fig. 6.2a). When the beacon was moved to Q4 in the probe trial, animals made significantly more direction changes when searching for the exit (probe trial: 7.0±1.51 turns; F=4.63, *p*=0.041).

Route maps showed that all the animals oriented first to the beacon in probe trials, and three failed to locate the escape hole entirely and instead settled on or near the beacon after a short search period. Three located the exit after swimming first over the beacon then recommencing searching (Fig 6.3a,b)

6.3.2 Path Integration – removal of visual cues (PI)

This training configuration was identical to BBH. In the first trial animals took 173.2±97.6s to escape (Fig. 6.1b) which declined to 91.5±42.1s by the fourth training trial. When all visual cues were removed in the probe trial, animals took longer to search for the exit, with a mean escape time of 459.0±86.6s (F=10.15, *p*=0.0097, Fig. 6.1b).

Animals made an average of 4.50±0.34 changes of direction in their first training trial. This increased slightly to 6.50±1.19 in the fourth training trial (Fig. 6.2b). When

visual cues were removed in the probe trial, animals did not make significantly more direction changes when searching for the exit (mean number of turns in the probe trial: 4.83±1.33; F=2.37, p=0.148).

Four of the six animals failed to locate to exit in the ten-minute probe trial (Fig. 6.3c,d), and the route maps for each animal show a search pattern that appeared to be random.

6.3.3 Path Integration – removal of visual cues and altered start position (PI AS)

This configuration was tested in the second phase of experiments, approximately three weeks after the first. Animals (n=6) showed very fast acquisition of the task in these later trails, and all animals required only two training trials to reach criterion in this configuration. In the first trial animals took 26.6±5.88s to escape, and 54.8±9.37s in the second (Fig. 6.1c). The probe trial included an altered start position and the removal of visual cues, and animals took longer to locate the exit as a result (mean escape time of 383.0±109.17s, F=9.00, p=0.017).

Animals in this configuration made an average of 3.00±1.26 changes of direction in their first training trial. This increased slightly to 3.20±0.73 in the second training trial (Fig. 6.2c). When the beacon was moved to Q4 in the probe trial, animals made an average of 4.80±1.66 changes of direction, an increase that was non-significant (F=3.24, p=0.071).

Two of the six animals failed to locate the exit, and the route maps (Fig. 6.3e,f) show a similar search pattern to the first test of path integration, which appeared to be largely random.

6.3.4 Cognitive map-based navigation – altered start position (CM)

Animals (*n*=6) received four training trials. In the first training trial animals took 322.8±110.0s to locate the exit hole. By the fourth trial this had declined to 142.5±46.2s (Fig. 6.1d). In the probe trial animals started the trial from a novel position within the maze. Animals took somewhat longer to escape during probe trials as the mean time increased to 302.3±117.2s, however this was a non-significant change (F=1.61, *p*=0.23).

Animals in this configuration made an average of 7.17±1.58 changes of direction in their first training trial. This decreased slightly to 7.00±1.81 in the final training trial (Fig. 6.2d). When the animals' start position was moved to Q4 in the probe trial, animals made an average of 6.67±0.42 changes of direction, an increase that was non-significant (F=0.03, *p*=0.86).

The route maps (Fig. 6.3g,h) suggest a random search strategy in the early stages of the trial, and a direct escape route after a short period. Only one animal failed to locate the exit in this test configuration.

6.3.5 Proximate vs. distant intra-maze cues (ALL)

This configuration was tested in the second phase of experiments, and again animals showed rapid acquisition of the task. In the first training trial animals took 148.4±113.4s to escape, and by the fourth trial escape time had declined to 94.0±39.9s (Fig. 6.1e). In the probe trial, the subjects located the exit as quickly as in trial 4. The mean escape time was 83.8±43.1s, a non-significant change (F=0.03, *p*=0.86).

In their first training trial, animals made an average of 2.2±0.73 changes of direction. This increased slightly to 3.20±0.58 in the fourth training trial (Fig. 6.2e).

When the beacon was moved to Q4 in the probe trial, animals made an average of 3.00±0.63 changes of direction, a non-significant difference (F=0.05, p=0.82).

The route maps of this configuration show most animals took a direct path to the exit, with only a slight deviation toward the beacon, although animals did not search extensively over the beacon (Fig. 6.3i,j).

6.3.6 Intra-maze (local) vs. extra-maze (global) cues (L-G)

This configuration was also tested in the second phase of experiments. The animals showed rapid acquisition, and mean time to escape remained low across the four training trials (trial 1, 79±31.1s; trial 4, 66.3±23.5s, Fig. 6.1f). In the probe trial, where the maze was shifted 180° with respect to the experiment room, escape time increased significantly to 275±78.0s (F=6.58, p=0.0281).

Animals in this configuration made an average of 7.17±3.40 changes of direction in their first training trial. This decreased to 5.83±3.63 in the fourth training trial (Fig. 6.2f). When the maze was rotated 180° in the probe trial, animals made an average of 10.33±1.89 changes of direction, a significant increase (F=6.58, p=0.028).

The route maps of this configuration showed that animals concentrated their search over the previous exit location, before moving toward the new goal location (Fig. 6.3k,l).

6.3.7 Effect size estimates

For each configuration, the effect of changing the maze configuration in each probe trial was estimated by comparing the escape time of the final training trial to the

escape time of the probe trial. The effect on number of changes of direction was compared among the same trials for each configuration.

In the BBH trials, the estimated effect size (EES) of cue manipulation on escape time was 3.56, with upper and lower 95% confidence limits (CL) of 5.58 and 1.74 respectively (Fig. 6.4). As these limits do not encompass zero, this is a genuine effect, and reflect the result of the ANOVA. The estimated effect size for number or turns was 0.85, with upper and lower 95% CLs of 2.04 and -0.33 respectively. Thus there was no effect on changes in direction, as these limits overlap zero (Fig. 6.5).

In the PI configuration, the EES on escape time was 4.98 with upper and lower CLs of 7.27 and 2.69. In the second configuration testing path integration, PI AS, the estimated effect on escape time was 3.91, with upper and lower CLs of 1.98 and 5.84. The estimated effect size for number or turns in the first test of PI was -0.27, with upper and lower 95% CLs of 0.86 and -1.41 respectively. In the second configuration, PI AS, the EES was 0.52, with upper and lower CLs of 1.67 and -0.63 respectively. There was no effect on changes in direction in either case, as these limits overlap zero (Fig. 6.5).

The effect on escape time in the trials of cognitive map use showed the only disagreement with the results of the ANOVA. The estimated effect size was 1.66, with upper and lower 95% CLs of 2.97 and 0.34. As the CLs do not include zero, this is a genuine effect, whereas the results of the ANOVA showed no significant increase in escape time. In contrast, there was no effect on number of turns, as the EES was -0.09 with upper and lower CLs of 1.04 and -1.23. This follows the result of the ANOVA, which also showed no significant change in directional changes for this configuration.

In trials of the configuration testing all intra-maze cues (ALL), the estimated effect size (EES) of cue manipulation on escape time was -0.23, with upper and lower 95% confidence limits (CL) of 0.91 and -1.36 respectively (Fig. 6.4). As these limits encompass zero there is no effect, and this reflects the result of the ANOVA. The estimated effect size for number or turns was -0.14, with upper and lower 95% CLs of 1.00 and -1.27 respectively. Thus there was no effect on changes in direction, as these limits overlap zero (Fig. 6.5).

In the configuration that tested the animals' reliance on local (intra-maze) vs. global (extra-maze) cues, the estimated effect size (EES) of maze rotation on escape time was 3.34, with upper and lower 95% confidence limits (CL) of 5.10 and 1.59 respectively (Fig. 6.4), a genuine effect. This configuration also showed the only genuine effect of the probe trial on number of changes in direction. The effect size for number or turns was 1.35, and upper and lower 95% confidence limits were 2.61 and 0.10 respectively (Fig. 6.5).

6.4 Discussion

Nautiluses are able to use various geocentric strategies, but could not use egocentric cues to locate a goal. Cue salience depended both on the context and on the other cues that were available. Interestingly, nautiluses were quite adept at using visual cues to navigate, despite the limited acuity of their eyes (Muntz, 1984, 1986, 1987b).

Egocentric cues appeared to be insufficient for successful localisation. In both tests of path integration, animals took far longer to find the escape hole when egocentric cues were the only ones available. When there were no visual landmarks available several animals in each test failed to locate the exit hole entirely, and the search strategy appeared largely random, with no concentrated area of search apparent from the route maps of each animal. Path integration relies on the ability to constantly update knowledge of position relative to a start point or to a goal. This depends heavily on the animal being able to compute some conversion between their own 'effort', for example, a measure of steps taken or distance swum, or of energy expended, into a directional and distance vector. For terrestrial animals, whose movement through their environment is mediated almost exclusively by their own effort (i.e., they are not subject usually to wind-shear or water currents), this computation can be sufficiently accurate to allow precise navigation. Additionally, for path integration to be useful, there must be some mechanism of error correction to re-calibrate the path integrator as it accumulates errors (Collett et al., 1992), such as visual feedback from the environment. Nautilus lives in an environment that is almost completely dark and subject to strong tidal currents, yet it has limited visual acuity and is a relatively weak swimmer. It is likely that for nautiluses, the constant error accumulation that would arise as a result of passive movement by local water currents would be sufficient to render this mechanism of navigation unviable. Additionally, the lack of consistently available visual cues suggests that visual means of error correction would also not be reliable. Thus it is unsurprising that nautilus were not able to use path integration to locate the escape point, even over the very small scale that was tested here. In octopuses there is equivocal evidence that animals may relocate their

home dens using path integration, as return routes tended to be shorter, and animals jetted along more direct paths than outbound, mostly substrate-bound, crawling foraging trips (Forsythe and Hanlon, 1997). In other experiments, octopuses were able to locate their dens even when local visual cues signalled an incorrect location (Mather, 1991b). In both cases, the species tested were shallow-water reef octopuses, animals that live in a complex visual environment and rely primarily on vision to hunt and locate shelters.

In maze experiments with cuttlefish, Alves and colleagues (2007b) showed than animals used a response strategy (i.e., memory of a left or right turn toward the exit) when distant landmarks were present, but not when proximal visual cues were provided. Thus it is possible also that cuttlefishes may rely on a route memory only when local cues are not available.

The tests that involved geocentric cues yielded some surprising results. Overall, it appears that under these conditions, nautiluses are quite adept at using a range of proximate, distant and global visual cues to navigate. In coleoid cephalopods, the use of visual cues in a various learning tasks is well documented (reviewed in Mather, 2007), and unsurprising given the complex structure of their eye and reliance on visual input in hunting, communication and defence. However, nautiluses have simple eyes that are probably not adept at forming images (Muntz 1987), and an optic lobe that is small compared with its relatives (Young, 1965a,b, 1976, 1988). Therefore it is somewhat surprising that they relied on visual input to orient, and appeared not to use egocentric cues at all. Perhaps the very small spatial scale of these experiments, coupled with the high-contrast visual cues that were provided, combined to make a visually guided navigational strategy the most effective. It is possible that animals may have been able to

use egocentric cues if they had been trained as well as tested in an environment where visual cues were not available, such that visual cues would not have overshadowed information that would have allowed formation of a route-based memory. The results from these experiments demonstrate that egocentric cues do not seem to be used as a parallel or alternative strategy to visually mediated navigation, although it is impossible to rule out its occurrence under different conditions.

In the test of beacon-based homing, when the beacon was moved away from the exit, all of the subjects oriented initially toward the beacon, and only 50% of the animals ever left the new beacon position to locate the exit. This suggests that the proximate visual cue was highly salient for navigation in this context, and is a result consistent with that obtained by Mather (1991b) in a similar experiment using octopuses.

In this configuration, the rest of the intra-maze environment was largely featureless, although extra-maze (global) cues were available. Three of the six animals in probe tests eventually located the exit after they had oriented initially to the beacon. The trajectories of the escaping animals appear to show a more direct path to the exit than those in the PI tests, suggesting that the search strategy was not random in this case. It is possible that once the subjects had discounted the value of beacon in signalling the goal, they switched to a secondary navigational strategy based on the unchanged extra-maze cues.

Animals that failed to complete the probe trial all settled on the beacon itself after a short search period. This may have been indicative of an autoshaping response (Brown and Jenkins, 1968), where animals learn to respond to the cue in a way that is similar to the behaviour toward the reward, to the extent that the reward may be ignored

while the cue is present (Hearst and Jenkins, 1974). Autoshaping toward a CS has also been observed in cuttlefish (Cole and Adamo, 2005). They found that animals trained to associate a plastic sphere with delivery of a feeder fish showed increasing tendency to strike at the sphere over the course of the training. On some occasions this resulted in a delay of their attack on the fish while their attention was directed at the ball. In contrast, Purdy and colleagues (1999) found that only the early stages of an attack sequence were directed to the conditioning stimulus, in this case a flashing light, and did not attack the light with tentacle strikes. They considered this evidence of sign-tracking (i.e., associative learning) rather than autoshaping.

In the spatial memory experiment (Experiment 3, chapter 5), I observed that animals that had located the escape hole occasionally remained at the surface of the water and explored the bubble-wrap beacon with their tentacles, sometimes remaining there for the full ten-minute inter-trial interval. Thus it is possible that the bubble-wrap may have come to represent the 'reward' in these trials, and the animals that located it were content to remain in its proximity until the trial was over. Alternatively, the failure of three animals to alter search strategy once the beacon failed to signal the exit may have reflected a drop in motivation to escape over the course of the training.

The result of the test containing all intra-maze cues (ALL) demonstrated that animals were able to switch strategies, from navigating using the beacon to navigating using distant landmarks, with relative ease. When animals were given the proximate cue (beacon) together with the distant intra-maze landmarks (air-stone, egg-crate and pipe fitting), they ignored the repositioning of the proximate cue and navigated successfully using the multiple, distant cues, which included both distant intra-maze cues and global

cues. Although several animals turned toward the beacon in the test trial, they moved quickly toward the exit and did not search repeatedly over the beacon, as they did in the test of beacon-based homing. All subjects located the exit without an increase in escape time, despite the beacon being in the same position as in the test trial for BBH. This is interesting for several reasons. Firstly, it reduces the likelihood that animals oriented to the beacon in the BBH test simply because it was closer and more visible from the start position. Secondly, it suggests that the salience of a given cue and the choice to include it in decision-making was dependent on the array of other cues that were present during the acquisition phase. Similarly, Alves and colleagues (2007b) showed that cuttlefish (*S. officinalis*) were capable of making spontaneous choices between response- and place-based strategies based on the type of cues available and the perception of their reliability.

A similar strategy was apparent in the test of local intra-maze cues vs. global extra-maze cues. In the test of cognitive-map based navigation, animals were able to navigate using the three distant intra-maze landmarks, however when the maze (and all intra-maze cues) was shifted 180° with respect to the experiment room, escape time increased markedly. All of the animals searched repeatedly over the previous location of the hole relative to the experiment room, although only one subject failed to escape within the ten-minute trial. The directed searching over the previous exit position suggests that animals were using navigational cues that were located outside the maze, such as the light gradient within the room, or some fixture in the room that was visible from within the maze. In contrast with the result of the BBH tests, after searching for the exit in its expected location, animals appeared to search randomly for the relocated exit, suggesting they did not use the intra-maze cues as a secondary option once global cues

failed to signal the exit. The greater number of successful escapes in this test compared with the BBH test may have been related to the presence of an obvious 'place-marker' in the BBH test. The presence of the beacon may have encouraged longer search durations at its location, whereas in the test of local vs. global cues, there was no visual cue marking the exact exit location in either training or testing, so animals may have been more motivated to recommence searching.

Despite the primitive structure of the nautilus eye, the test subjects were able to discriminate between several very similar visual cues with some accuracy. For example, in the test setup for cognitive map-based navigation, the animals were positioned such that there were three white, plastic cues in the same spatial relationship to each other and to the animal as in the training trials (one immediately on the animal's left, one approximately 60° to the animal's right, and one almost opposite). If they were unable to discriminate between the three shapes, the route maps would be expected to show straight-line trajectories that brought animals alongside the air-stone, where they would expect the exit to be found if they did not discriminate between the cues. Instead, the search strategy appears largely random, until the point when an animal crossed close to the original start location. At this point subjects oriented toward the exit and assumed a straight-line trajectory toward it. Thus this is not a true 'cognitive map' as animals could not to compute a new, direct route from a novel start position (Bennet, 1996; Healy, 1998; Shettleworth, 1998). Nautiluses had encoded instead the spatial relationships of the landmarks relative to the goal, and were able to recognise when they were in a position where their internal representation matched visual input, similar to the retinotopic 'snapshot' described in other invertebrates (ants: Antonsen and Wehner,

1995; *Drosophila*: Dill et al., 1993). Interestingly, it did not appear that the disoriented animals used the global extra-maze cues in this test, even though their relationship to the exit was consistent.

Overall nautiluses seem to prefer the more distant landmarks of each array. In the absence of visual cues, animals relying on egocentric cues did poorly. When a proximate beacon was the only intra-maze cue available, animals oriented toward it even when it conflicted with any egocentric route-memory, a result also observed in octopuses (Mather, 1991b). When given a choice between proximate and distant intra-maze cues, animals navigated successfully using the distant landmarks, and when intra-maze and extra-maze cues were in conflict, animals chose to navigate using the global cues.

Taken together, these results raise some intriguing insights into navigation in the wild. The use of visual cues was surprising given the presumably limited visual acuity of the eyes (Muntz, 1987b). The contrast of the white cues against the black tank probably meant the shapes were relatively obvious to the animals, particularly given that the experimental arena was lit at a much brighter level than animals would encounter in the wild. The reliance on the most distant cue type available in several experiments suggests that perhaps animals in the wild might orient to their surroundings using a global cue such as the onshore-offshore light gradient, a cue that remains constant and is likely to be visible for at least some part of the daily migratory cycle. The use of local visual cues for small-scale orientation may be useful in the more brightly lit surrounds close to the surface, but is unlikely to be of great use at the depths where nautiluses spend most of their time, even given the light-capturing ability of the eye. Unlike octopuses, nautiluses are not central-place foragers, so needing to locate a home den

after outbound foraging is not a factor in nautilus behaviour. However, given that animals probably scavenge for food, remembering the location of already depleted foraging areas may be of some use. This would only be necessary if nautiluses traversed the same areas of reef over successive foraging trips. If animals instead drift passively over the reef, then they will be brought to new foraging zones by the current and such memory of previous foraging sites would be of little use.

Identifying an ecological role for such complex discriminatory abilities is difficult. At this stage our understanding of nautilus behaviour in the laboratory outstrips our knowledge of its behaviour in the wild by far. This is a situation that will most likely persist for some time, and until detailed and non-invasive studies of wild animals are possible, it is unlikely that a clear ecological role for visual discriminations and multiple navigational strategies will be revealed.

Chapter 7 – General Discussion

These experiments provided surprising and sometimes conflicting results. The classical conditioning experiments indicated that the bi-phasic memory profile in *N. pompilius* was very similar to at least one other coleoid species, *S. officinalis* (Messenger, 1971; Agin et al., 2006), whilst the duration was far shorter. Despite memory being stored in a different region of the brain in nautilus (as there is no vertical lobe or obvious analogue present), the profile was almost identical. As LTM is protein-synthesis dependent, the time to consolidation is dependent on the rate of transcription and translation within the neural tissue, which may not differ necessarily across brain regions, or across closely related species. It is certainly interesting to find such a similar result in nautilus as that reported for *S. officinalis,* a species with a highly developed brain that in many ways is optimised toward learning and memory.

Despite the similarity of the *shape* of the memory curve between nautilus and *Sepia*, certainly the duration of the memory trace was very different. The putative LTM trace appeared around 6 hours after training and persisted for less than 24 hours. Coleoid cephalopods have been reported to retrieve long-term memory over the course of weeks (e.g., Boal et al., 2000), so the brief time to memory decay in a long-lived, slow-moving species such as nautilus was surprising. However, results from the spatial memory experiment (Experiment 3) suggested that the brief LTM trace observed in Experiments 1 and 2 was an artefact of the procedure itself, and did not reflect an innate limit of memory persistence in nautilus. In Experiment 3 memory was stable for at least three

weeks after conclusion of the training phase, demonstrating comparable LTM persistence as octopuses and cuttlefishes (Agin et al., 1998; Cole and Adamo, 2005; Forsythe and Hanlon, 1997; Mather, 1991a,b; Young, 1960). Again, despite the absence of a vertical lobe analogue in nautilus, they were able to perform a simple learning task at a level that was at least comparable and possibly superior to other cephalopods.

Taken together these results raise an interesting question. Novel brain regions such as those found in the coleoid CNS arise in response to sustained directional selection on existing neuroanatomy. Removal or ablation of the vertical lobe in octopuses produces deficits in learning (Agin et al., 2006; Graindorge et al., 2006; Hochner et al., 2003) but behaviour is otherwise quite normal (Young, 1960a). Therefore it is reasonable to infer that the vertical lobe complex functions primarily as a learning and memory centre, similar to the hippocampus of vertebrates. The arrangement of nerve fibres within the vertical lobe show convergent properties (‘en passant’ synapses, short connectives) with vertebrate learning centres, potentially supporting fast processing speed and also flexibility (Hochner et al., 2003, Young, 1998). There is mounting evidence that the brain of modern nautilids is pleisiomorphic (Teichert and Matsumoto, 1987; Shigeno et al., 2007), and not the result of secondary simplification. It is reasonable therefore to postulate that the evolution of the coleoid brain has followed roughly a path from a simple, ‘nautilus-like’ brain, to a complex, multi-lobed structure such as is found currently in octopuses and cuttlefish. Thus at some stage in coleoid evolution, there was sufficient evolutionary advantage conferred on animals with more complex brains that were specialised for learning and remembering. It is an intriguing finding, therefore, that at least in some simple tasks nautilus can perform at similar

levels as its more complex relatives. Of course, the two aspects of learning that were examined in this study (memory profile and memory duration) represent only a small fraction of the incredibly complex matrix of cues, associations and relationships that the coleoid cephalopods may have adapted to learn and remember. Nonetheless it is interesting that some aspects of the performances of nautiluses compared favourably with their more 'advanced' relatives, when faced with tasks that were appropriate to their behaviour and physiology.

The final experiment examining navigational strategy provided insights into the types of cues and information that nautiluses use when making small-scale navigational decisions. Despite their relatively poor visual acuity, animals were able to discriminate between several visual cues that were similar in appearance. This suggested that even with a lensless eye and simple optic lobe (Young, 1960b), animals were able to identify differences in shape and orientation of visual cues, and were able to encode those features into some internal representation of their environment. Overall this experiment suggested that animals rely more heavily on visual cues rather than egocentric cues, and that different cues were preferred under different conditions, with animals sometimes choosing a proximate cue and sometimes a global cue. This is suggestive of redundant cue encoding that permits animals to locate the goal successfully under a range of conditions.

The spatial memory experiments (experiments 3 and 4) were conducted on a spatial scale that is comparably tiny considering the normal daily movement range of wild animals. However, the large-scale vertical migrations of wild animals are most likely mediated by learning-independent cues such as pressure and light gradients, and it

is likely that if memory is used in spatial navigation, it is across the horizontal rather than the vertical plane. Nautiluses spend much of their time in darkness, so visual information is presumably also limited to the immediate surrounds of the individual, although the onshore-offshore light gradient represents at least one large-scale cue that would be available consistently. Thus overall it seems likely that nautiluses may find the greatest need for navigational cues on a relatively restricted spatial scale, as they perhaps move about foraging or locating shelters. Certainly their ability to discriminate between several shapes of similar size and colour suggests that although they may lack the impressive discriminatory abilities displayed by octopuses (Boycott and Young, 1955; Young 1960a; Young 1961), their visual acuity and ability to remember fine details about their surroundings may be greater than hypothesised previously. Detailed study of wild animals may reveal a role for learning and remembering visual features of their environment.

Chapter 8 – Conclusions and Future Directions

This study developed several simple procedures for demonstrating learning and memory in captive nautiluses. Although the procedures had varied success rates, there is potential for each to be developed and refined further. In particular the spatial memory task produced long-term and stable changes in behaviour, suggesting that this type of procedure may be of use in future studies. Its similarity to successful studies on other cephalopods indicates that it may provide important comparative data on spatial learning across a range of cephalopod species.

These experiments raise a number of questions about the nature of neural function and behaviour in nautilus compared with other cephalopods. The procedures were designed to allow easy modification to accommodate different species of cephalopods, such that direct comparative studies would be carried out on a range of species using identical or at least highly similar methods. Behavioural data from animals exposed to identical experimental contexts can provide more concrete evidence of differences between taxa.

There is certainly a need for better understanding of natural behaviour in nautiluses. These experiments have shown that nautiluses may be capable of encoding and recalling detailed features of their environments, and may in fact be more adept at learning and remembering that their relatively primitive brain suggests. Thus at almost every level of inquiry, from neurobiology and behaviour to ecology and evolution, there

is a great deal of information that could be gleaned from studying the behaviour of nautilus.

Identifying the functional analogue of the coleoid vertical lobe complex in nautilids would represent an intriguing direction for both neurobiological and evolutionary studies. At this stage very little is known about the function and organisation of the nautilus brain, making detailed investigations of the sites of memory storage unfeasible until more basic investigations are completed.

Despite their promise as an 'evolutionary snapshot' species, using nautiluses as study organisms poses a number of problems. It is a difficult species to maintain in the aquarium for long periods and is typically unresponsive in experimental situations. Currently 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 as such, field-based behavioural studies are extremely difficult. Conversely, destructive neurobiological techniques required for understanding brain function are equally untenable. Therefore nautilus is unlikely to be a good candidate for extensive laboratory experimentation. Using non-invasive behavioural assays at least permits animals to be used in multiple tests with within-subject design, reducing the requirements for large numbers of experimental subjects. Despite these limitations, these animals offer several highly valuable features to neuroscientists and evolutionary biologists. 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, complementing the rich existing literature on the evolution of learning and memory, as well as adding to our growing understanding of this ancient species.

Table 3.1 An overview of the unsuccessful conditioning paradigms attempted during the development stage of the study.

Conditioning Stimulus	Unconditioned Stimulus	Number animals tested	Method	Result
5 second blue light pulse (480nm) directly at right eye	Tilapia head solution, 1mL	5	• Animal unrestrained • ITI 15 minutes • 10 presentations • Test @ 15 minutes post training	No conditioned response apparent at 15 minutes, animal generally agitated and highly active throughout trial.
5 second blue light pulse (480nm) directly at right eye	Tilapia head solution, 1 mL	6	• Animal restrained in harness • ITI 5 minutes • 10 presentations • Test at 24 hrs and 7 days post training	Strong unconditioned tentacle extension response to the light in conditioned and control animals. Hyponome direction suggested light was highly aversive
5 1-second light flashes behind restrained animal (strobe light effect)	Tilapia head solution, 2mL	4	• Animal restrained in harness • ITI 5 minutes • 10 presentation • Test at 15 minutes post-training	Unconditioned tentacle extension in response to light in some animals only. No evidence of conditioning at 15 minutes
5 second blue light pulse (480nm) directly behind restrained animal	Tilapia head solution, 2mL	4	• Animal restrained in harness • ITI 5 minutes • 5 presentations • Test at 15 minutes post-training	Unconditioned tentacle extension in response to light in some animals only. Equivocal evidence of conditioning at 15 minutes
5 second blue light pulse (480nm) directly at left eye	Tap on hood with blunt glass rod	6	• Animal restrained in harness • ITI 30 seconds • 10 presentations • Test at 30 seconds, 5 minutes and 15 minutes	Significantly lower tentacle extension response to light in conditioned animals at 30 s and 5 mins, no difference at 15 mins. Tentacle extension suppressed throughout, not specifically in response to CS.

Table 4.1 Descriptions and diagrams of each TER response level, based on the proportional extension of the tentacles beyond the hood margin, and in response the conditioning procedure in experiments 1 and 2.

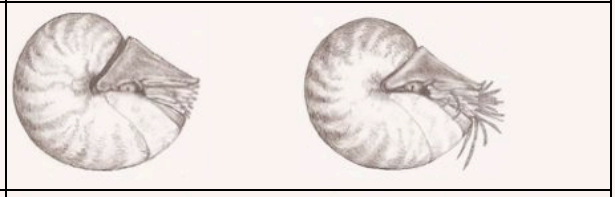
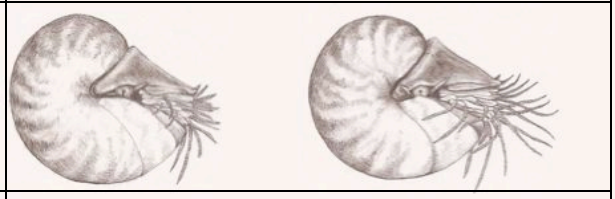
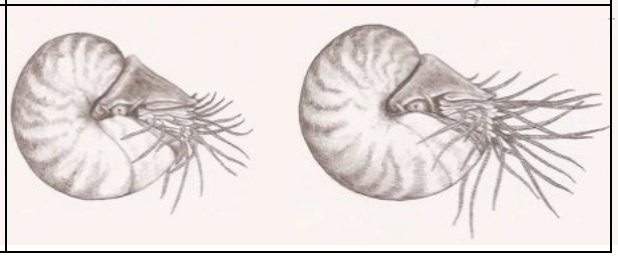
RANK	DEFINING FEATURES	PERCENTAGE RANGE	DIAGRAM
0	Hood closed or open. Tentacles withdrawn into sheaths	More than 50% of visible tentacles extended less than 10% of hood length	
1	Hood open, tentacles extended, digital tentacles dropped, little lateral spread	More than 50% of visible tentacles extended between 10% and 33% of hood length	
2	Hood open, tentacles extended, digital tentacles dropped, moderate lateral spread	More than 50% of visible tentacles extended between 33% and 66% of hood length	
3	Hood open, tentacles maximally extended, digital tentacles fully dropped, strong lateral spread	More than 50% of visible tentacles extended > 67% of hood length	

Table 4.2 Shows details of the within-subjects, counterbalanced experiment design for Experiments 1 and 2. Animals were used multiple times, and were trained in both conditions (CS+ and CS-) at different stages of each experiment. 1 and 2 denotes the order of training within each retention or recovery interval, which was assigned randomly to each animal. Training in different conditions was separated by at least two weeks. Where animals were used to test multiple retention or recovery intervals, training for each was separated by at least six weeks, and the order of CS+ and CS- training was changed.

Experiment 1. Tests of Retention Intervals

Animal	*3 mins*		*30 mins*		*1 hour*		*6 hours*		*12 hours*		*24 hours*	
	CS-	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*
1	-	-	-	-	1	2	1	2	-	-	2	1
2	-	-	-	-	-	-	1	2	1	2	2	1
3	1	2	1	2	-	-	-	-	2	1	-	-
4	-	-	-	-	-	-	2	1	2	1	1	2
5	1	2	2	1	-	-	-	-	1	2	-	-
6	-	-	-	-	-	-	2	1	1	2	1	2
7	-	-	-	-	1	2	2	1	-	-	1	2
8	-	-	-	-	1	2	1	2			2	1
9	2	1	2	1	-	-	-	-	2	1	-	-
10	1	2	2	1	2	1	-	-	-	-	-	-
11	2	1	1	2	2	1	-	-	-	-	-	-
12	2	1	1	2	2	1	-	-	-	-	-	-

Experiment 2. Training, Extinction And Recovery at 6hrs, 12hrs and 24hrs

Animal	*Rec. Interval*	*Training*		*Extinction*		*6 hours*		*12 hours*		*24 hours*	
		CS-	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*	*CS-*	*CS+*
A	6	1	2	1	2	1	2	-	-	-	-
A	12	2	1	2	1	-	-	2	1	-	-
A	24	2	1	2	1	-	-	-	-	2	1
B	6	2	1	2	1	2	1	-	-	-	-
B	12	1	2	1	2	-	-	1	2	-	-
B	24	1	2	1	2	-	-	-	-	1	2
C	6	2	1	2	1	2	1	-	-	-	-
C	12	1	2	1	2	-	-	1	2		
C	24	2	1	2	1	-	-	-	-	2	1
D	6	1	2	1	2	2	1	-	-	-	-
D	12	1	2	1	2	-	-	1	2	-	-
D	24	2	1	2	1	-	-	-	-	2	1
E	6	1	2	1	2	1	2	-	-	-	-
E	12	2	1	2	1	-	-	2	1	-	-
E	24	1	2	1	2	-	-	-	-	1	2
F	6	2	1	2	1	2	1	-	-	-	-
F	12	2	1	2	1	-	-	2	1	-	-
F	24	1	2	1	2	-	-	-	-	1	2

Table 4.3 A list of mean, s.e.m. and *p* values for pair-wise comparisons of TER and ventilation rates between control and conditioned animals in test presentations of each retention interval in Experiment 1. The 60 second test period was comprised of 12, 5s bins. Values for all bins, early bins (the first 30 seconds) and later bins (last 30 seconds) are listed separately. Note the significant differences among the later bins.

Retention interval	*bins included*	*Mean TER*	*Mean TER*	*p value*	*Mean Ventilation*	*Mean Ventilation*	*p value*
		CS-	*CS+*		*CS-*	*CS+*	
3 minutes	all (60s)	0.45±0.24	1.63±0.37	0.06	3.30±0.27	3.79±0.18	0.07
	early (30s)	0.55±0.29	1.55±0.39	0.14	3.33±0.33	3.77±0.20	0.16
	late (30s)	0.36±0.21	1.69±0.40	0.02*	3.27±0.23	3.80±0.17	0.04*
30 minutes	all	0.59±0.21	0.92±0.26	0.34	3.77±0.21	4.44±0.26	0.02*
	early	0.63±0.20	0.63±0.31	1.00	3.83±0.20	4.33±0.33	0.15
	late	0.55±0.25	1.38±0.31	0.06	3.72±0.23	4.55±0.20	0.006*
1 hour	all	0.77±0.26	0.75±0.21	0.93	3.51±0.15	3.76±0.17	0.35
	early	0.77±0.26	1.66±0.38	0.94	3.38±0.14	3.94±0.28	0.15
	late	0.77±0.26	0.69±0.22	0.77	3.63±0.21	3.58±0.15	0.82
6 hours	all	0.98±0.50	2.10±0.38	0.07*	3.15±0.15	3.63±0.32	0.07
	early	1.00±0.51	1.66±0.38	0.22	3.13±0.13	3.50±0.32	0.32
	late	0.97±0.49	2.52±0.40	0.03*	3.00±0.16	4.66±0.28	0.009*
12 hours	all	0.59±0.33	1.15±0.42	0.24	3.33±0.12	3.88±0.19	0.04*
	early	0.69±0.35	1.00±0.43	0.41	3.31±0.12	3.67±0.22	0.14
	late	0.38±0.21	1.58±0.39	0.02*	3.36±0.14	4.11±0.21	0.03*
24 hours	all	0.80±0.43	0.72±0.21	0.81	3.54±0.36	3.47±0.27	0.86
	early	0.86±0.46	0.72±0.22	0.69	3.47±0.40	3.47±0.27	1.00
	late	0.75±0.40	0.72±0.22	0.93	3.61±0.34	3.47±0.29	0.77

Table 4.4 A list of mean, s.e.m. and *p* values for selected pair-wise comparisons of TER and ventilation rates between control and conditioned animals in Experiment 2. Values shown are from the later 30 seconds of each recording period only.

Interval	*Pres*	*Mean TER*	*Mean TER*	*p value*	*Mean Ventilation*	*Mean Ventilation*	*p value*
		CS-	*CS+*		*CS-*	*CS+*	
Training	1	1.02±0.22	1.04±0.24	0.95	3.74±0.28	4.20±0.26	0.23
	2	0.85±0.19	1.60±0.19	0.01 *	3.74±0.26	4.35±0.23	0.08
	3	1.09±0.24	1.64±0.24	0.11	3.79±0.25	4.44±0.24	0.07
	4	1.27±0.27	1.18±0.22	0.79	3.59±0.26	4.01±0.21	0.21
	5	1.35±0.25	1.28±0.22	0.83	4.03±0.25	4.22±0.22	0.57
	6	0.97±0.19	1.23±0.23	0.39	3.80±0.25	4.18±0.25	0.29
	7	1.04±0.20	1.39±0.24	0.28	3.97±0.27	3.92±0.28	0.87
	8	1.26±0.25	1.18±0.23	0.80	3.74±0.22	3.67±0.18	0.83
	9	1.25±0.22	1.28±0.24	0	3.76±0.21	3.97±0.24	0.51
	10	1.09±0.19	1.53±0.27	0.17	3.75±0.19	4.03±0.25	0.38
Extinction - 1hr	1	0.32±0.11	1.03±0.21	0.0062 *	3.54±0.20	3.87±0.27	0.33
	10	0.18±0.15	0.94±0.26	0.79	3.56±0.36	3.19±0.22	0.38
Rec - 6hrs	1	0.26±0.16	1.50±0.21	0.0007 *	3.06±0.13	4.20±0.40	0.017 *
	10	0.8±0.34	1.60±0.31	0.32	3.53±0.24	3.97±0.45	0.42
Rec – 12 hrs	1	0.17±0.17	0.40±0.24	0.17	3.43±0.41	3.13±0.19	0.55
	10	1.44±0.41	1.00±0.45	0.63	3.94±0.67	3.50±0.53	0.62
Rec – 24hrs	1	1.14±0.58	0.83±0.48	0.61	4.17±0.62	3.94±0.43	0.77
	10	1.19±0.16	1.36±0.35	0.72	3.47±0.21	3.64±0.30	0.66

Table 5.1 A list of animals used in the spatial memory experiment. Animals were allocated to test intervals randomly at the outset of the experiment. Each animal was trained twice and tested either four or five times. The two longest retention intervals, 1 week and 3 weeks, were tested in a separate series (red crosses) after the shorter retention intervals (black crosses). All animals were tested at three weeks after training. Each shorter retention interval was tested with six of the ten animals.

Animal	training	18hr	24hr	36hr	48hr	72hr	96hr	1w	3w
A	+ +	+	-	+	-	+	-	+	+
B	+ +	-	+	-	+	-	+	+	+
C	+ +	+	+	+	-	+	-	-	+
D	+ +	+	+	+	+	-	-	+	+
E	+ +	+	-	+	-	+	+	+	+
F	+ +	-	+	-	+	+	+	-	+
4	+ +	-	-	+	+	+	+	-	+
6	+ +	+	+	-	+	-	+	-	+
7	+ +	+	+	-	+	-	-	+	+
8	+ +	-	-	+	-	+	+	+	+

Table 5.2 A list of mean escape times and mean number of turns made by animals per trial in Experiment 3, and associated *p* values for pairwise comparisons of selected trials.

trial no.	N	escape time mean±sem	*p* value, trial 1 to subsequent trials	p value, trial 5 to retention tests	no. turns mean±sem	*p* value, trial 1 to subsequent trials	p value, trial 5 to retention trials
1	10	447.6±72.2	-	-	9.0±2.1	-	-
2	10	259.0±90.1	0.0019*	-	10.1±3.2	0.59 NS	-
3	10	66.2±17.0	<0.0001*	-	8.9±1.7	0.96 NS	-
4	10	54.7±15.3	<0.0001*	-	4.0±0.7	0.015	-
5	10	39.5±13.6	<0.0001*	-	3.8±1.7	0.030	-
6	6	48.8±17.0	<0.0001*	0.92 NS	3.2±1.0	0.022	0.82 NS
7	8	47.4±13.3	<0.0001*	0.91 NS	4.1±0.6	0.026	0.91 NS
8	7	52.9±20.6	<0.0001*	0.86 NS	2.7±0.4	0.006	0.65 NS
9	8	42.1±9.9	<0.0001*	0.97 NS	3.8±0.6	0.016	0.97 NS
10	5	41.0±16.5	<0.0001*	0.99 NS	4.6±0.6	0.081 NS	0.78 NS
11	5	30.0±.54	<0.0001*	0.91 NS	3.4±0.2	0.027	0.87 NS
12	10	56.2±13.2	<0.0001*	0.81 NS	4.8±0.6	0.042	0.68 NS
13	9	111.8±49.7	<0.0001*	0.30 NS	4.8±0.7	0.046	0.69 NS

Table 6.1 Descriptions and diagrams of each maze configuration for training and testing each of the hypotheses of navigational strategy in Experiment 4. S –start position. E – exit hole (goal). Not to scale.

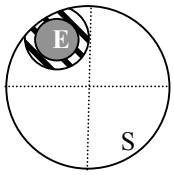
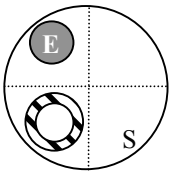
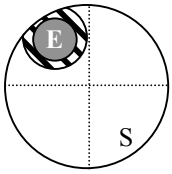
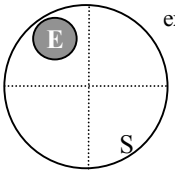
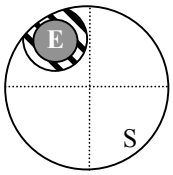
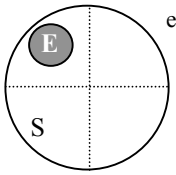
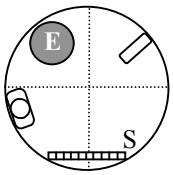
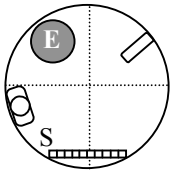
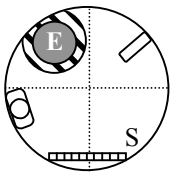
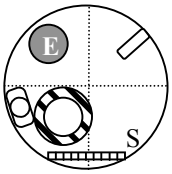
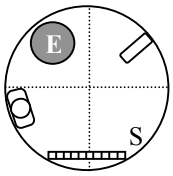
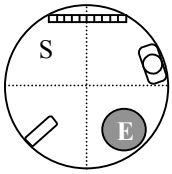
Hypothesis tested	Training configuration	Test configuration
Beacon-based homing (BBH) A single landmark located proximate to the escape point.	 beacon located at exit.	 beacon moved to adjacent quadrant
Path Integration (PI) Navigation tested in the absence of geocentric cues.	 beacon located at exit.	 beacon and extra-maze cues removed
Path Integration (PI AS) Tested in the absence of geocentric cues and with a novel start position	 beacon located at exit.	 beacon and extra-maze cues removed, start position moved 90°
Cognitive Map Use (CM) Tests navigation using known landmark array. Starting from a novel position	 hole unmarked, distant landmarks around maze	 landmarks constant, start position moved 90°
Proximate vs. Distant Cues (ALL) Navigation tested when proximate and distant cues are in conflict	 All intra-maze landmarks present	 proximate and distant landmarks in conflict
Local vs. Global Cues (L-G) Tests the relative contribution of intra- and extra-maze cues to navigation	 hole unmarked distant landmarks around maze	 entire maze shifted 180° with respect to experiment room

Table 6.2 Shows the counter-balanced order of training in each maze configuration for each animal used in Experiment 4. BBH – beacon-based homing. PI – path integration. CM – cognitive map. ALL – all intra-maze cues. PI AS – path integration tested with an altered start position. L-G – local (intra-maze) vs. global (extra-maze) cues.

	Order					
	Series 1			*Series 2*		
Animal	*Week 1*	*Week 2*	*Week 3*	*Week 4*	*Week 5*	*Week 6*
A	BBH	PI	CM	ALL	PI AS	L-G
B	BBH	PI	CM	ALL	PI AS	L-G
C	PI	CM	BBH	PI AS	L-G	ALL
D	PI	CM	BBH	PI AS	L-G	ALL
E	CM	BBH	PI	L-G	ALL	PI AS
F	CM	BBH	PI	L-G	ALL	PI AS

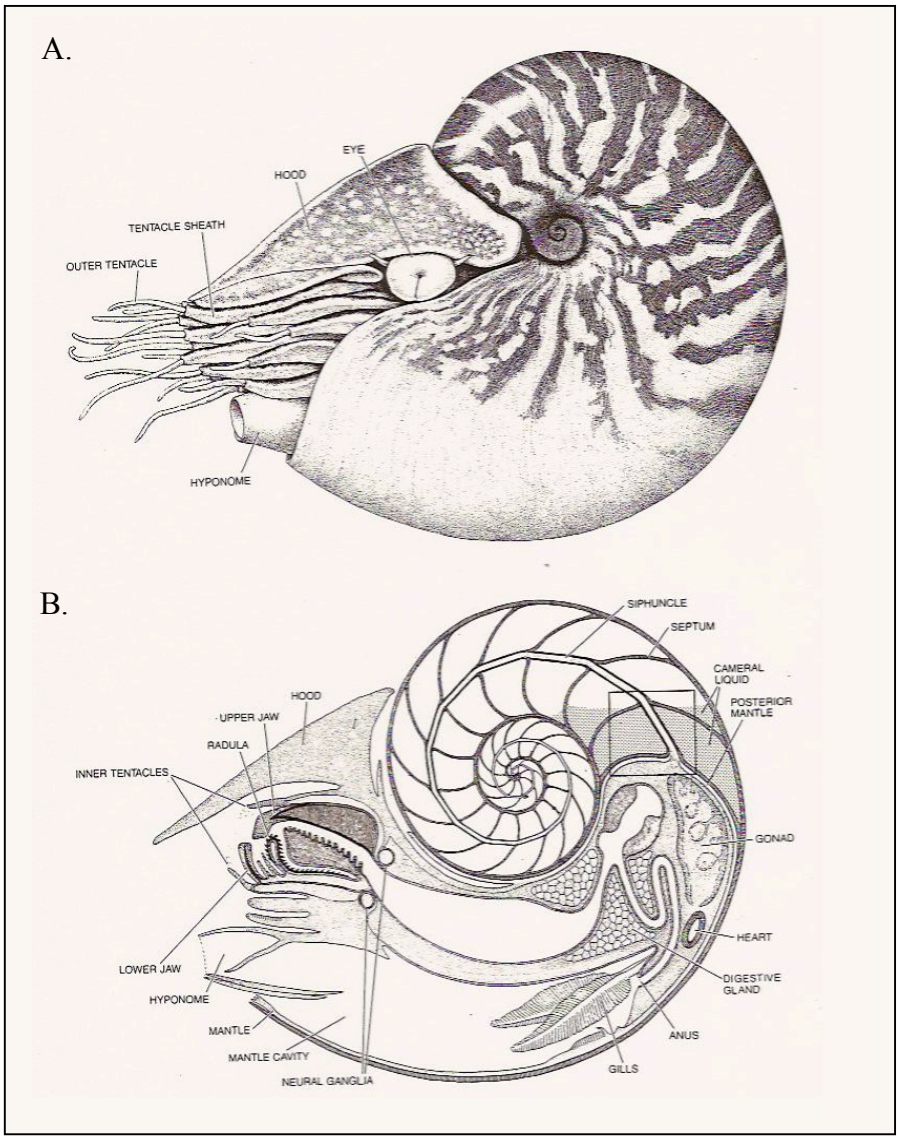


Figure 2.1 A. Lateral view of the gross, external anatomy of *Nautilus macromphalus*. B. Internal anatomy of *N. macromphalus*. Figure reproduced from Ward, 1987.

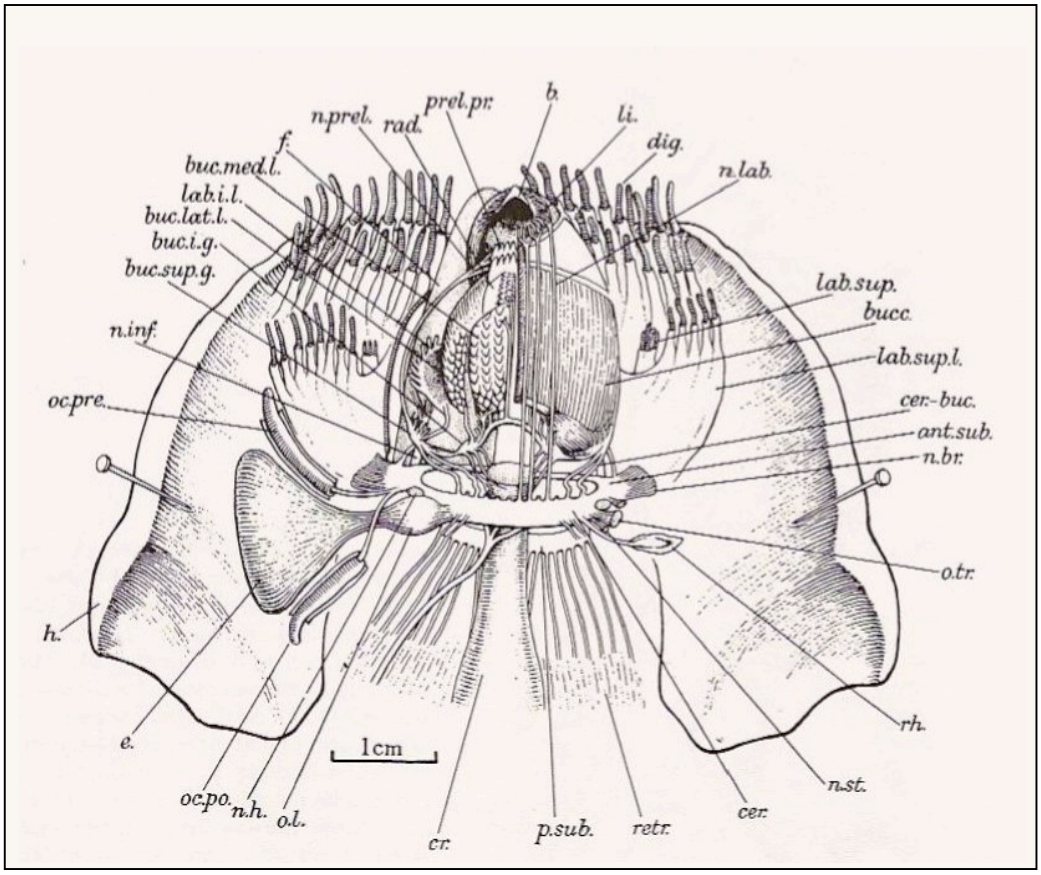


Figure 2.2. Drawing of a dissection of the nervous system and buccal mass. The dissection has been carried deeper on the left. The tentacles are somewhat diagrammatic. Anatomy: (*ant. sub.*) anterior suboesophageal mass: (*b.*) beak; (*bucc*.) buccal mass; (*buc.i.g.*) inferior buccal ganglion; (*buc.lat.l*.) lateral buccal lobe; (*buc.med.l*.) medial buccal lobe; (*buc.sup.g*.) superior buccal ganglion; (*cer*.) cerbral (supraoesophageal) cord; (*cer.-buc*.) cerebrobuccal connective; (*cr.*) crop; (*dig.*) digital tentacles; (*e*.) eye; (*f.*) funnel; (*h*.) hood; (*lab.i.l*.) inferior labial lobe; (*li.*) lips; (*n.br.*) branchial nerves; (*n.h*.) hood nerve; (*n.inf*) infundibular nerve; (*n.lab*.) labial nerve; (*n.prel*.) prelingual nerve; (*n.st*.) static nerve; (*oc.po*.) postocular tentacle; (*o.l*.) optic lobe; (*o. tr.*) optic tract; (*prel.pr*.) prelingual process; (*p.sub*.) posterior suboesopageal mass; (*rad.*) radula; (*retr*.) retractor muscles; (*rh*.) rhinophore. Figure and caption from Young, 1965b.

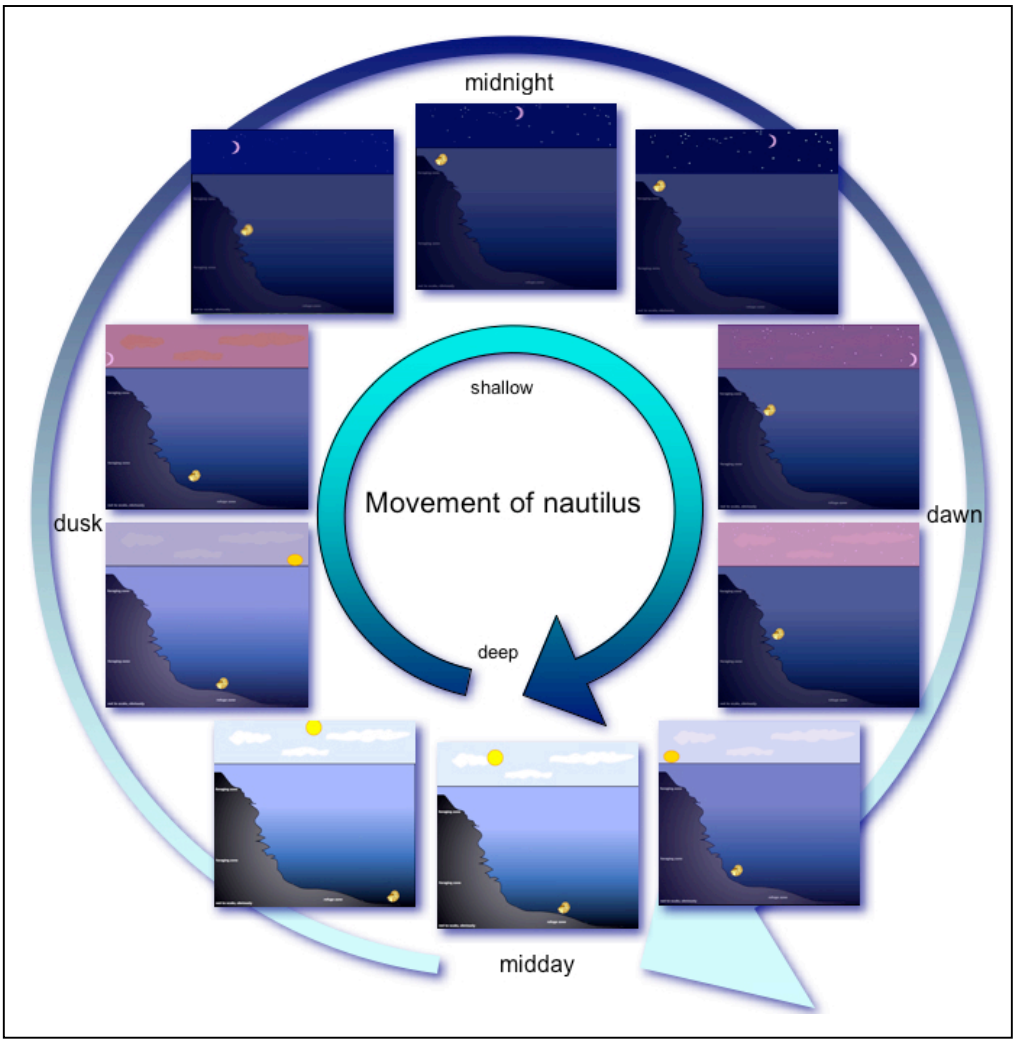


Figure 2.3 Cartoon of the daily movement cycle of nautilus in the wild. Each panel shows a different time in the 24 hr cycle, and the corresponding position of the animal on the reef-face. Diagram not to scale.

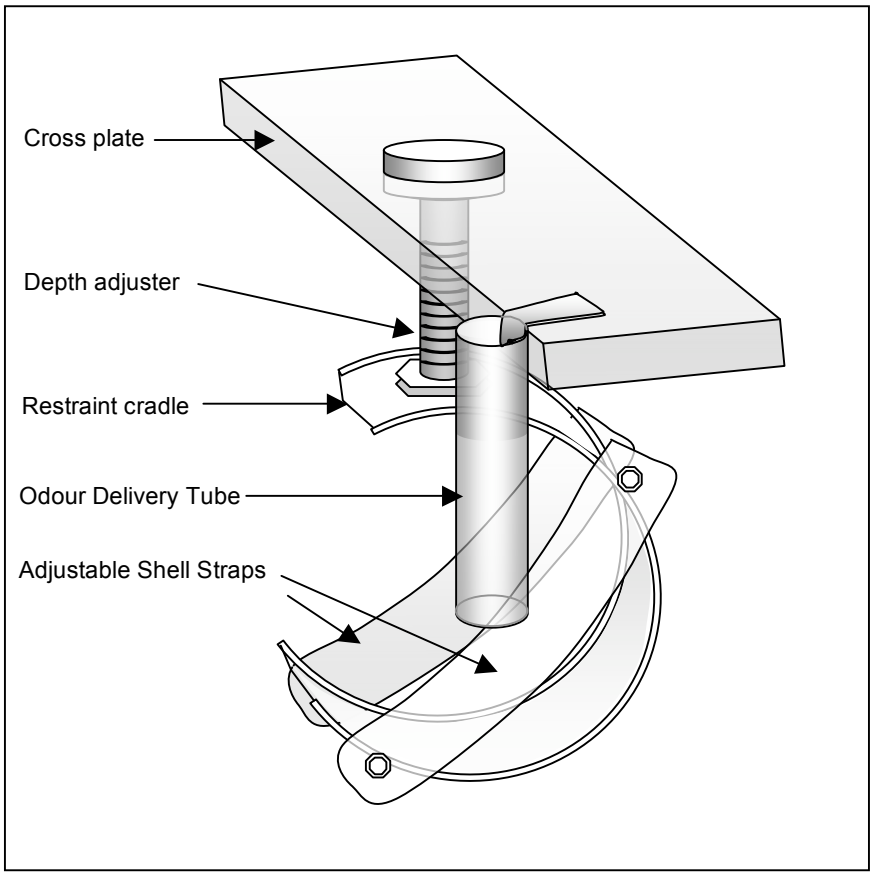


Figure 3.1 Diagram of the restraint harness used in classical conditioning procedures. The harness was constructed from a cross-section of PVC pipe and flexible plastic straps. The animal (not shown) was placed in the harness, facing left in this diagram, and remained immobile during the procedure but had free movement of the hyponome and tentacles. The cross plate rested across the top of the glass aquarium (not shown) that served as the test arena. The depth adjuster allowed the cradle to be lowered under the water to the same depth in every trial. The restraint cradle wrapped around the perimeter of the animals' shell, while the adjustable shell straps held the animal firmly in place. The odour delivery tube allowed the unconditioned stimulus (food odour) to be released in the same position relative to the animal in every presentation. Not to scale.

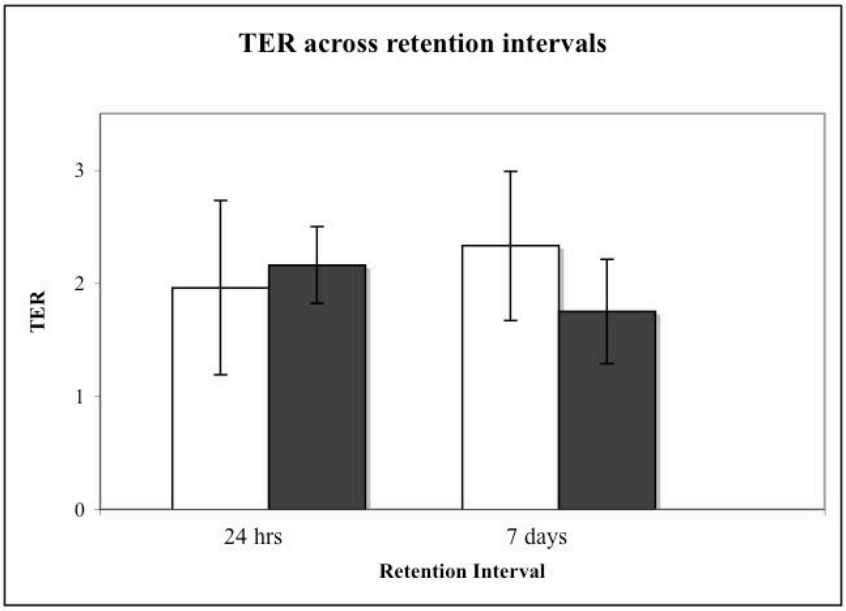


Figure 3.2 Graph of mean (+/- 1 s.e.m.) of tentacle extension responses for control (CS-, white bars) and conditioned (CS+, grey bars) at 24hrs and 7 days post-training. The CS was a 5s light pulse, and the US was food odour. Both CS+ and CS- animals show strong responses to the test presentation of the light pulse.

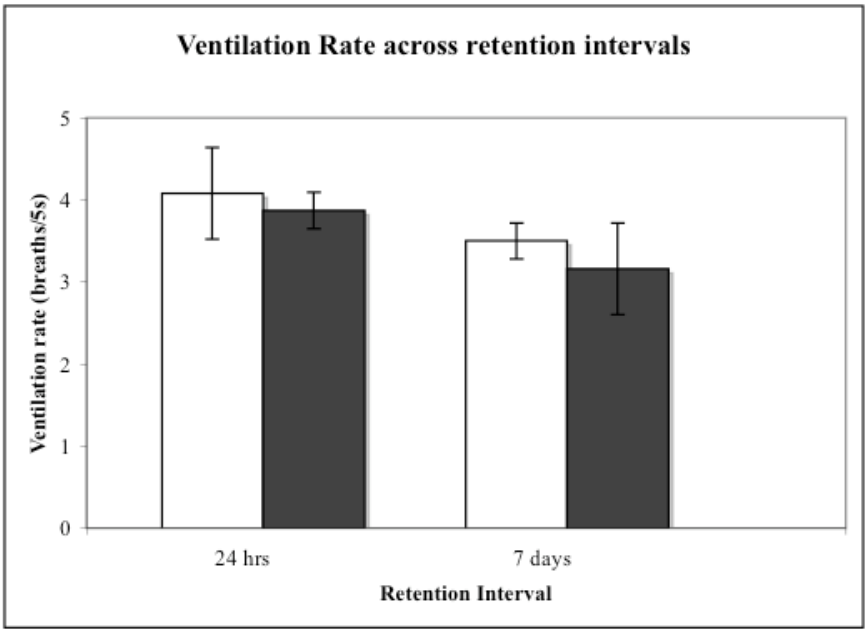


Fig 3.3 Graph shows the mean +/- s.e.m. of ventilation rates for control (CS-, white bars) and conditioned (CS+, grey bars) at 24hrs and 7 days post-training, when the CS was a 5s light pulse, and the US was food odour. Both CS+ and CS- animals show strong responses to the test presentation of the light pulse.

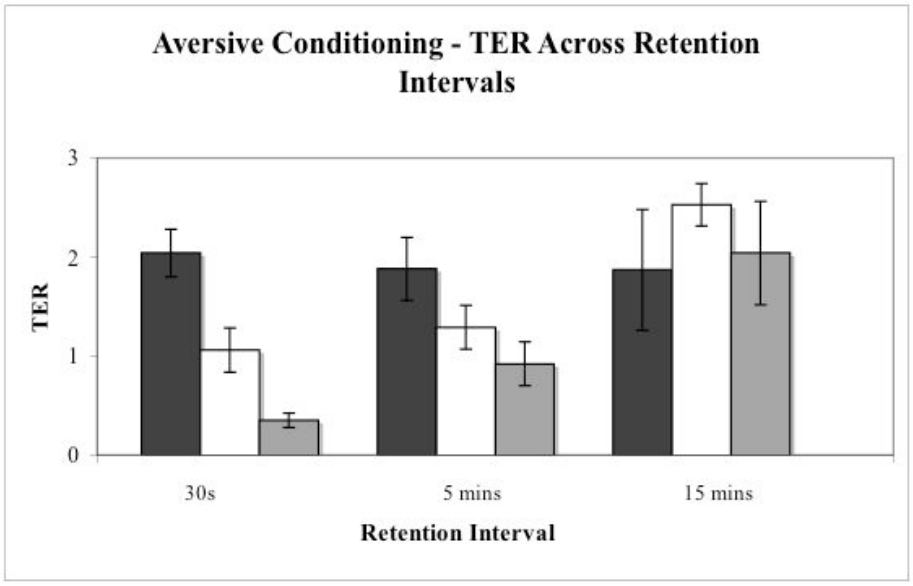


Figure 3.4 Graph shows mean +/- 1 s.e.m. tentacle extension responses among three conditions. Dark grey bars show means of animals trained with the conditioned stimulus only (5s blue light pulse) White bars show means of animals trained with the US only (hood tap) and light grey bars show means of animals trained with paired CS/US. The test presentation consisted of the light pulse only.

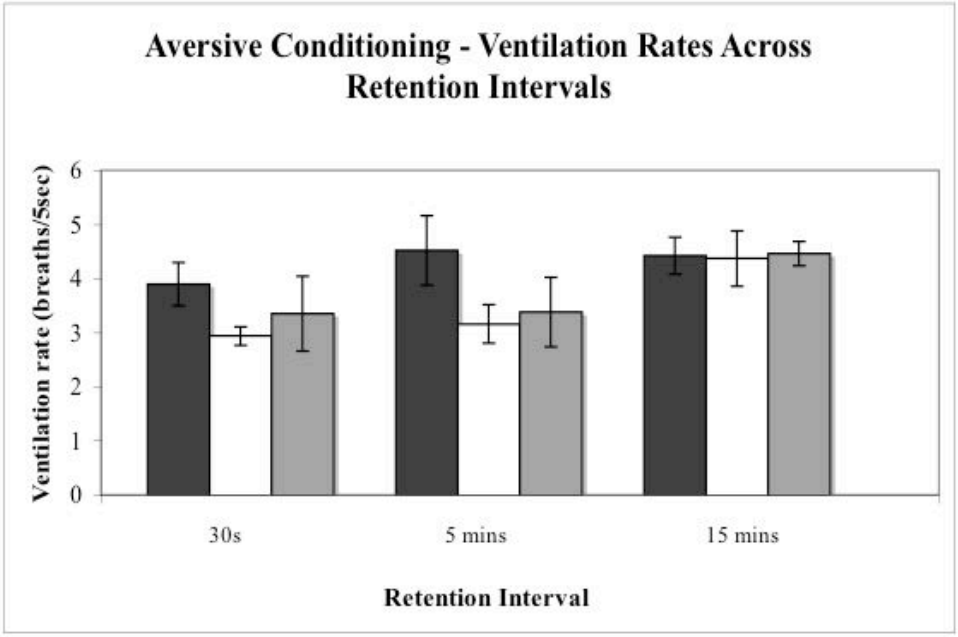


Figure 3.5 Graph shows mean +/- 1 s.e.m. of ventilation rates among three conditions. Dark grey bars show means of animals trained with the conditioned stimulus only (5s blue light pulse) White bars show means of animals trained with the US only (hood tap) and light grey bars show means of animals trained with paired CS/US. The test presentation consisted of the light pulse only.

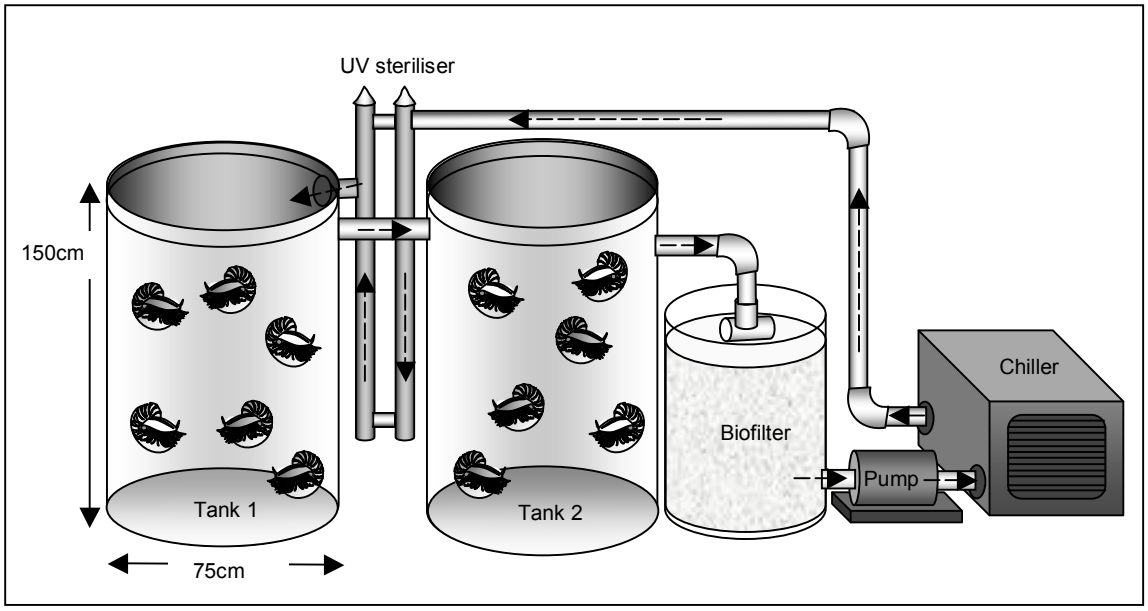


Figure 4.1 Diagram of the housing system and holding tanks for all animals used in this study. Animals were kept in two covered, black polyethylene tanks (150cm x 75cm, covers not shown), connected by PVC tubing to a biofilter. A pump cycled water from the tanks into the chiller, through a UV filter and back into the tanks. Arrows show direction of water flow through the system. Protein skimmers (not shown) were attached to each tank. Diagram not to scale.

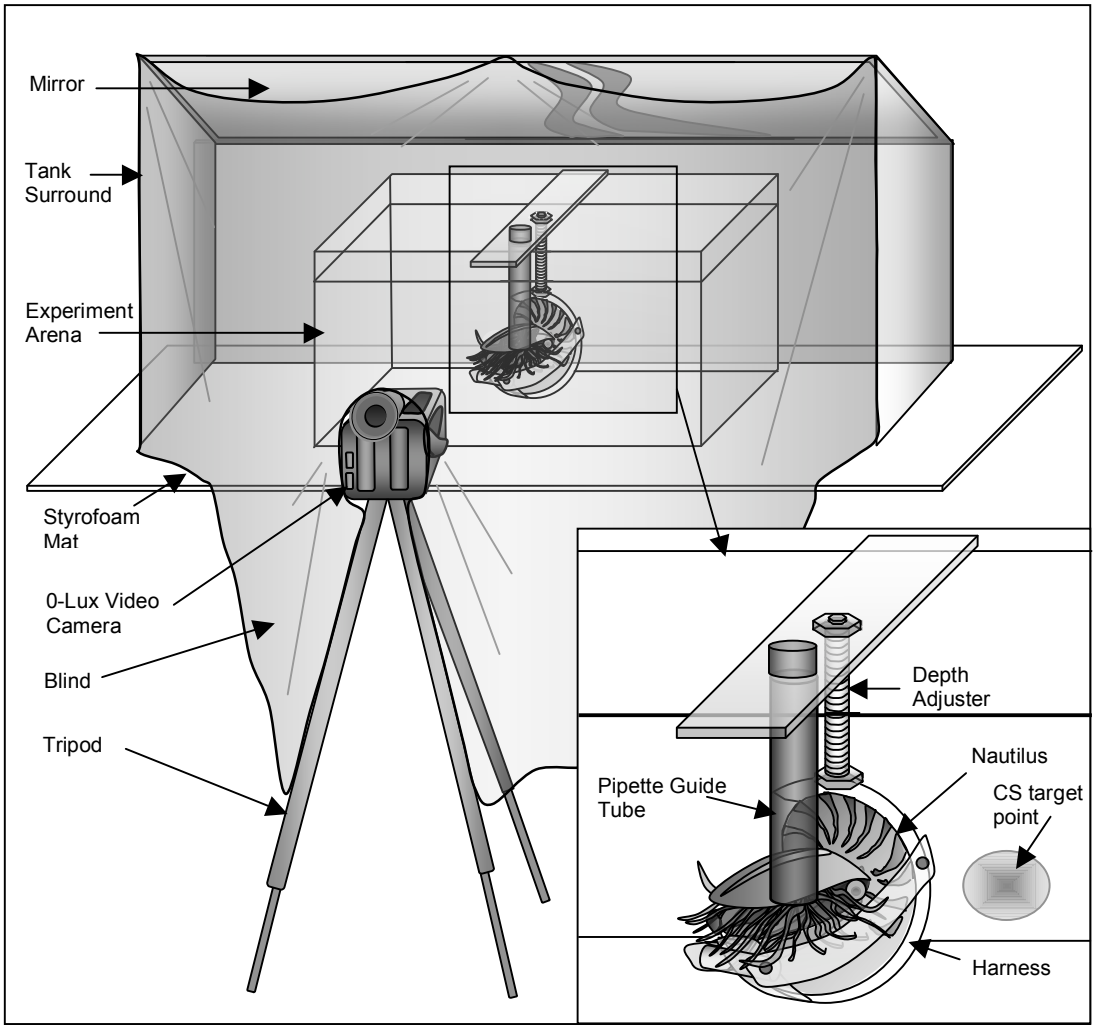


Figure 4.2 Diagram of the apparatus set-up for Experiments 1 and 2. The experimental arena is placed on a Styrofoam mat, and enclosed within a cardboard tank surround. A black plastic blind covers the opening at the front and encloses the video camera and tripod. A mirror angled above the tank allows for simultaneous horizontal and vertical recording. Stimuli are introduced above the tank, through a gap between the mirror and blind. *Inset:* Detail of the restraint. A nautilus is restrained in a PVC harness, which is submerged to a fixed point, controlled by the depth adjuster. Odour is delivered via the pipette guide tube, which controls the position and depth of the pipette (not shown) delivering the US. The CS target point is marked on the tank backing. The light pulse is directed at this point in each trial. Diagram not to scale.

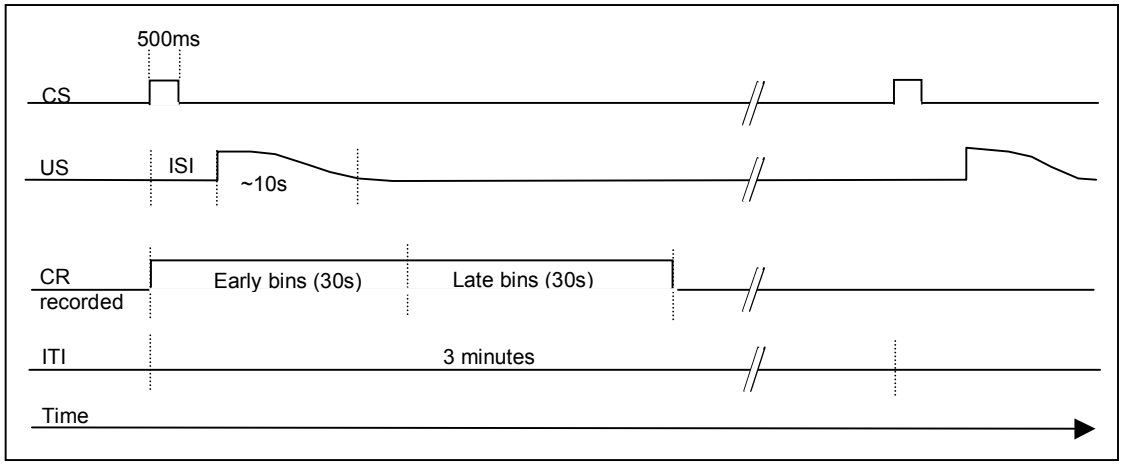


Figure 4.3 Diagrammatic representation of the classical conditioning procedure. CS: conditioning stimulus, a ½ second blue-light pulse. US: unconditioned stimulus, pipetted food-odour solution delivered close to the subjects' tentacles. The intensty of the US probably wanes as the solution diffuses into the tank water. ISI: inter-stimulus interval, the time between CS onset and US onset. As the distance between the pipette tip and animals' tentacles varied with the animals' posture, this interval is appoximate. CR: conditioned response. This was measures for 60 seconds after CS onset. ITI: inter-trial interval, the time between each presentation of the CS. In this experiment the ITI was set at 3 minutes. This procedure was repeated ten times in each training session. The whole session lasted 30 minutes, 45 minutes including acclimation time. Not to scale.

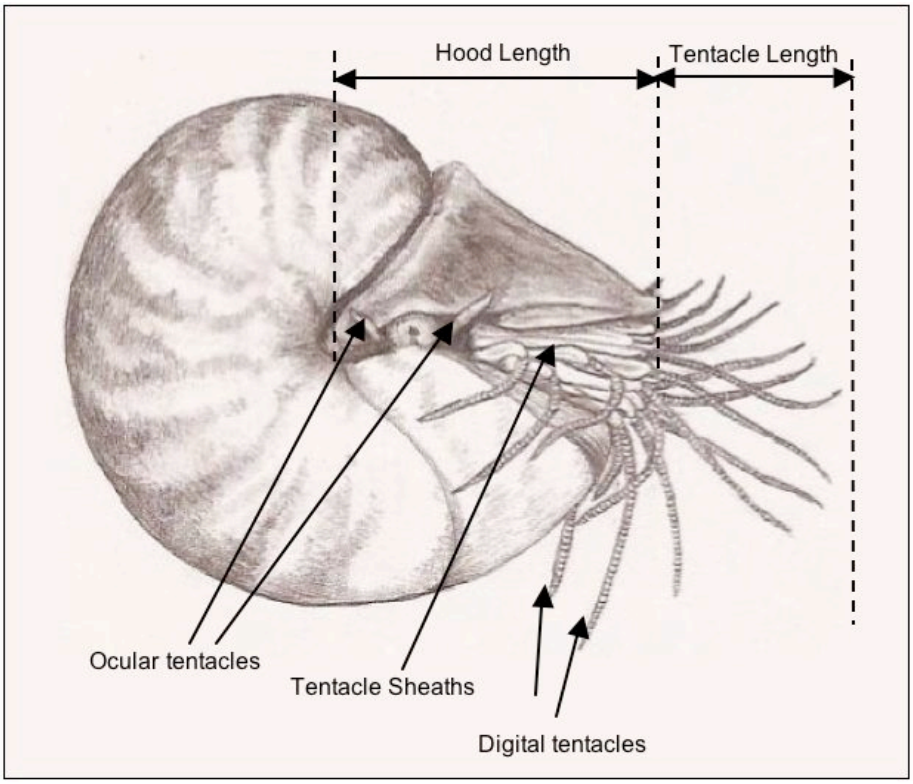


Figure 4.4 Diagram showing measurements and characteristics of ranking system for Tentacle Extension Response (TER) scores. Hood Length is measured from the base of the hood behind the eye to the distal tip of the hood. Tentacle length is measured from the point where tentacles protrude from the hood to a straight-line boundary, defined as a percentage of the hood length. Here the boundary line is drawn at 66% of hood length, thus this posture is ranked as 2.

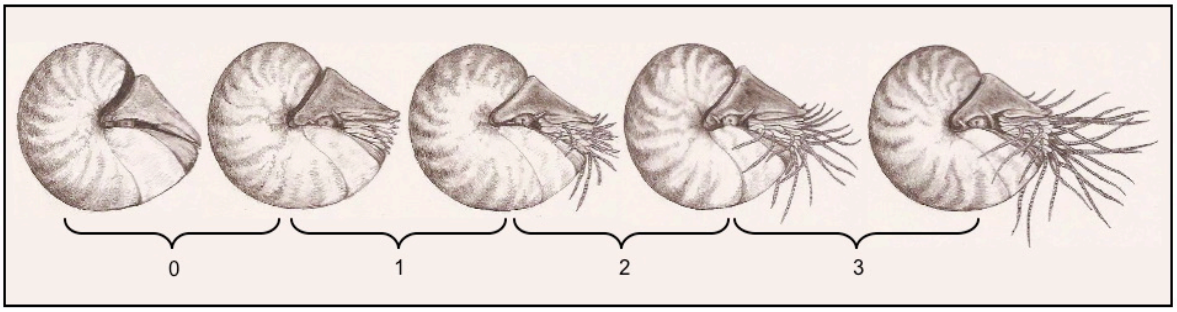


Figure 4.5 Diagram of tentacle extension changes as animals sense novel odours in the water column. Greater tentacle extension reflects higher arousal levels. The length of visible tentacles is ranked from 0 to 3, calculated as a percentage of the length of the hood. A score of 0 is given when the tentacles are withdrawn into the tentacle sheaths (no extension). A score of 1 equates to extension less than 33% of the hood length. Two is given when tentacles are extended between 33 and 66% of hood length. Three is recorded when tentacles extend more than 66% of the hood length.

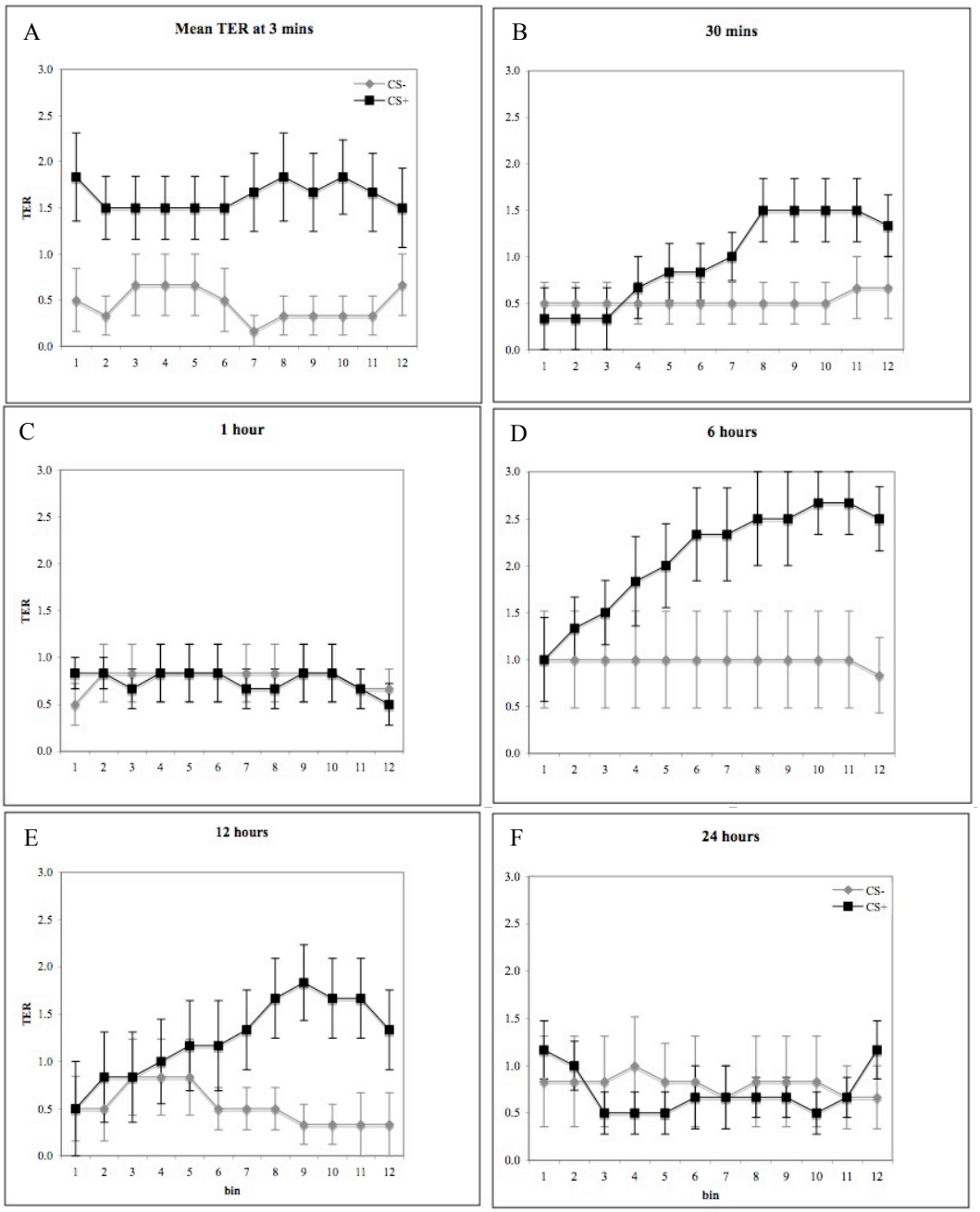


Figure 4.6 Line graphs showing change in tentacle extension across the 60-second test period, taken from each 5-second bin. A. Test period at three minutes. B. Test period at 30 minutes. C. Test period at one hour. D. Test period at six hours. E. Test period at 12 hours. F. Test period at 24 hours.

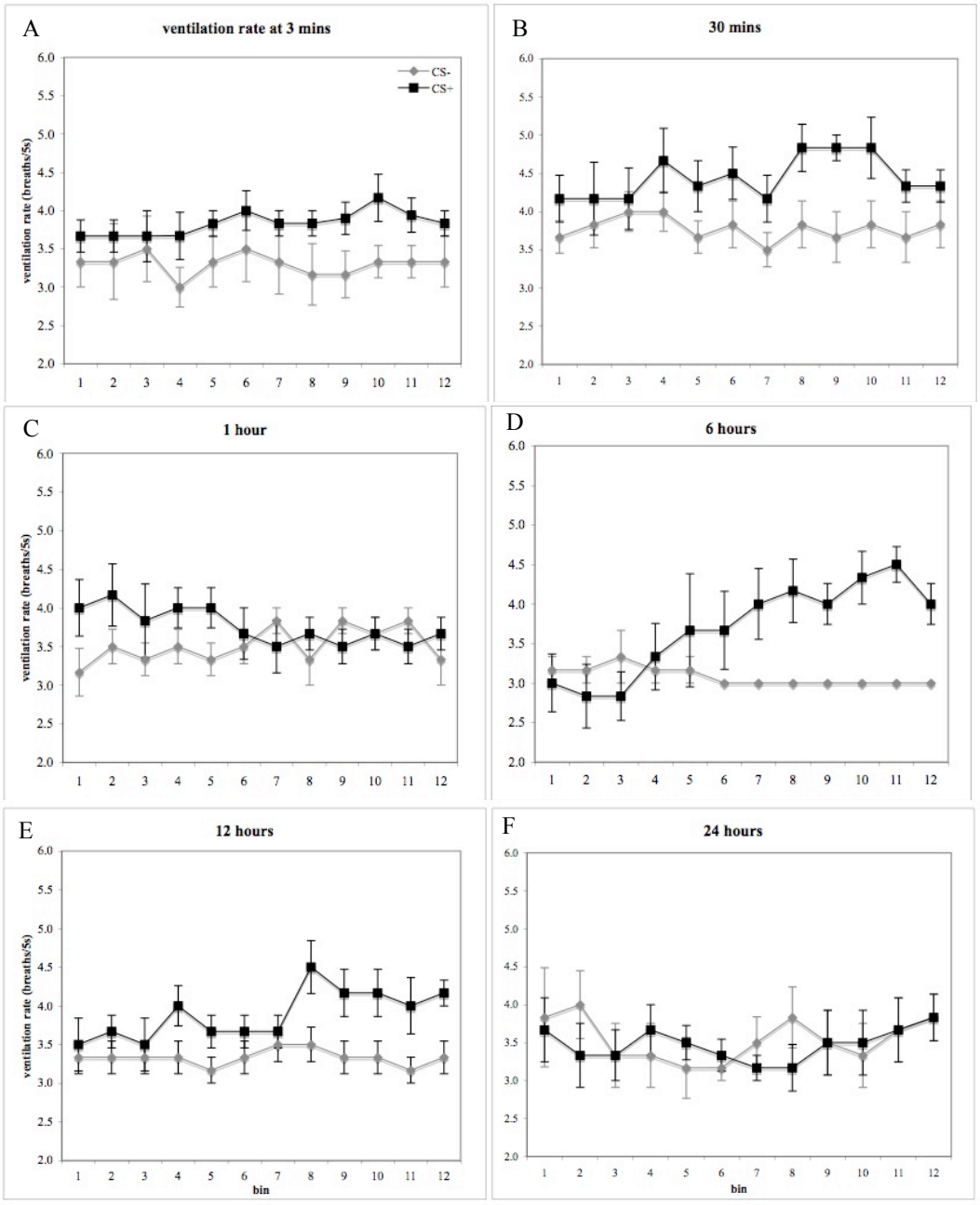


Figure 4.7 Line graphs showing change in ventilation rate across the 60-second test period, taken from each 5-second bin. A. Test period at three minutes. B. Test period at 30 minutes. C. Test period at one hour. D. Test period at six hours. E. Test period at 12 hours. F. Test period at 24 hours.

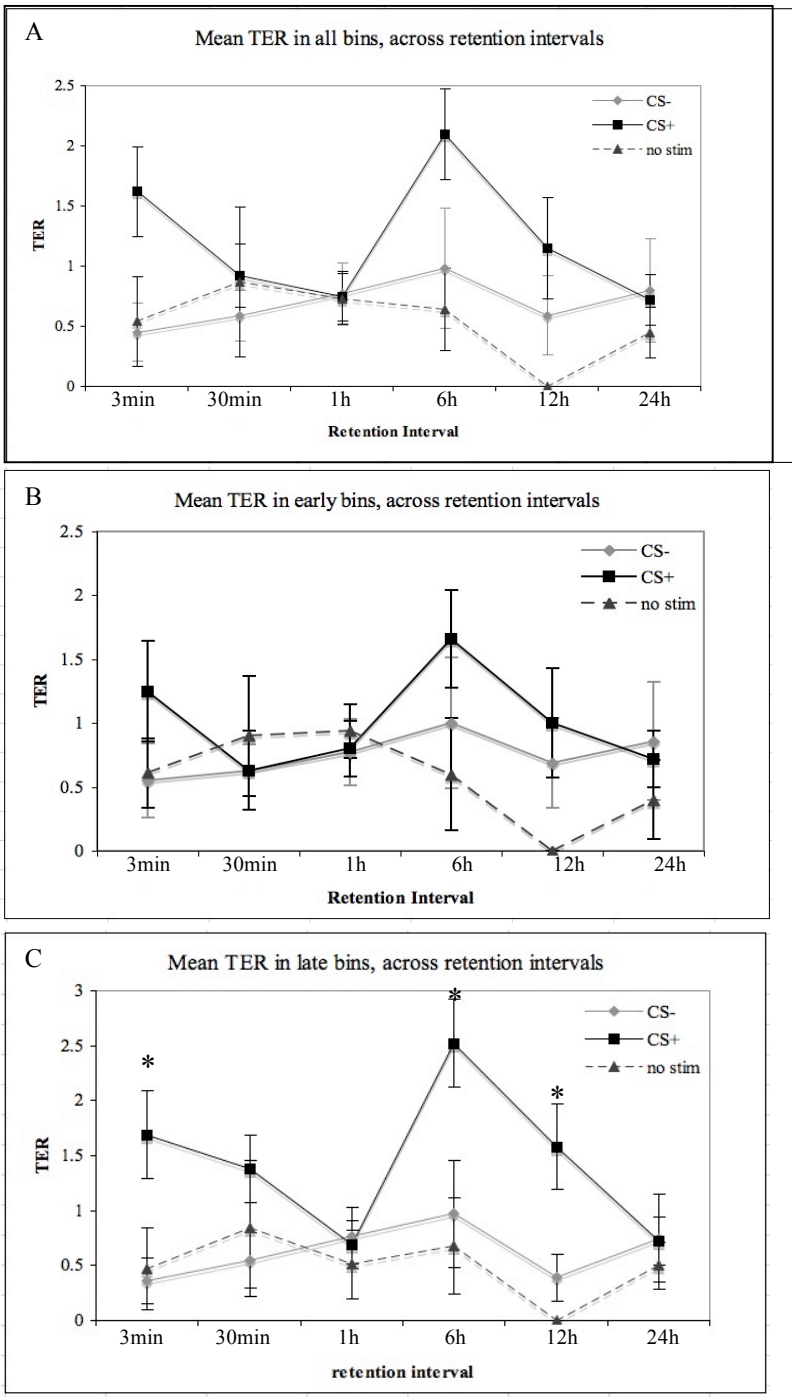


Figure 4.8 Graph of mean (+/- 1 s.e.m.) tentacle extension responses in CS- (grey line), CS+ (black line) and no-stimulus (broken line) conditions for A. the full 60-second test period, across the six retention intervals. B. the first 30 seconds of the test period, and C. the last 30 seconds of the test period. * Denotes a significant difference between CS+ and CS- at each retention interval. Note the strengthening trend in the latter half of the test periods.

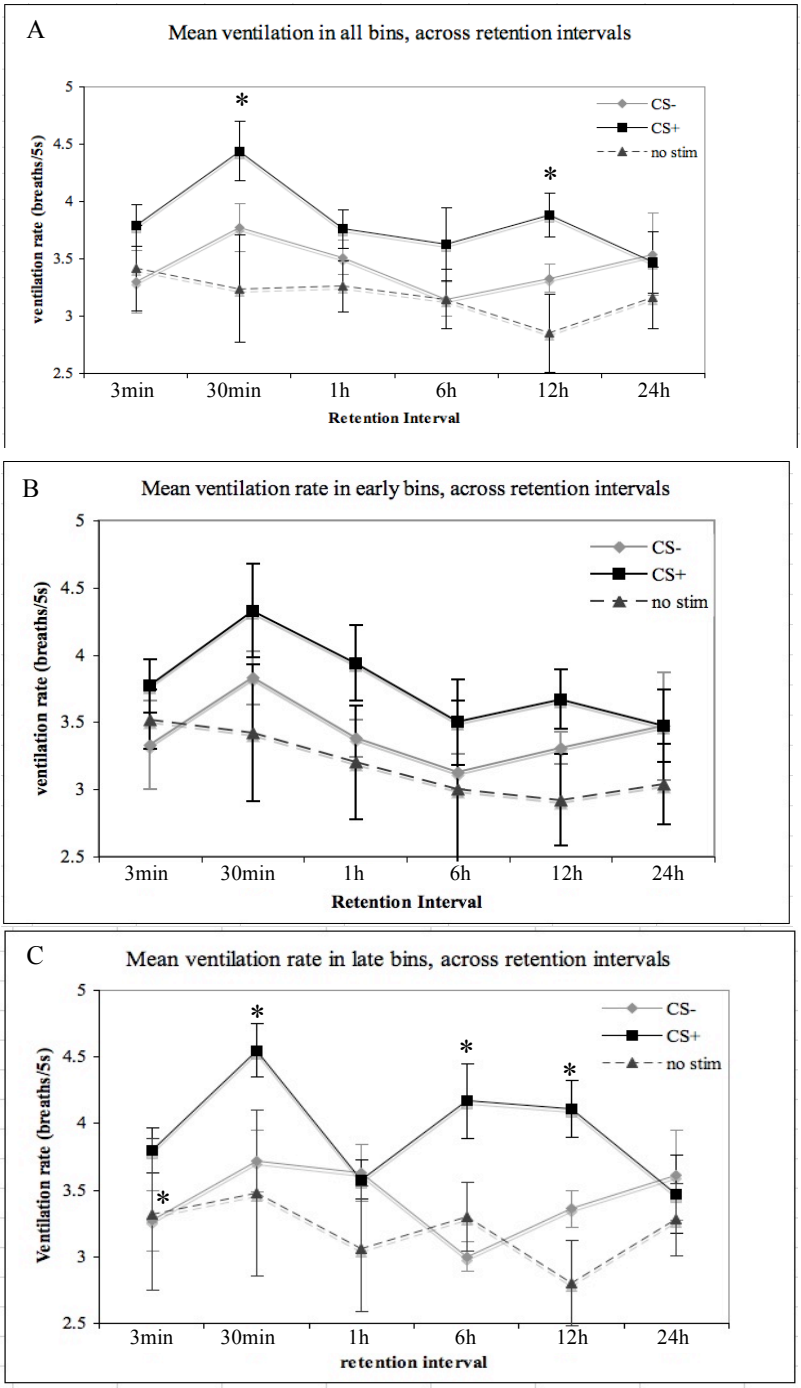


Figure 4.9 Graph of mean (+/- 1 s.e.m.) ventilation rates in CS- (grey line), CS+ (black line) and no-stimulus (broken line) conditions for A. the full 60-second test period, across the six retention intervals. B. the first 30 seconds of the test period, and C. the last 30 seconds of the test period. * Denotes a significant difference between CS+ and CS- at each retention interval. Note the strengthening trend in the latter half of the test periods.

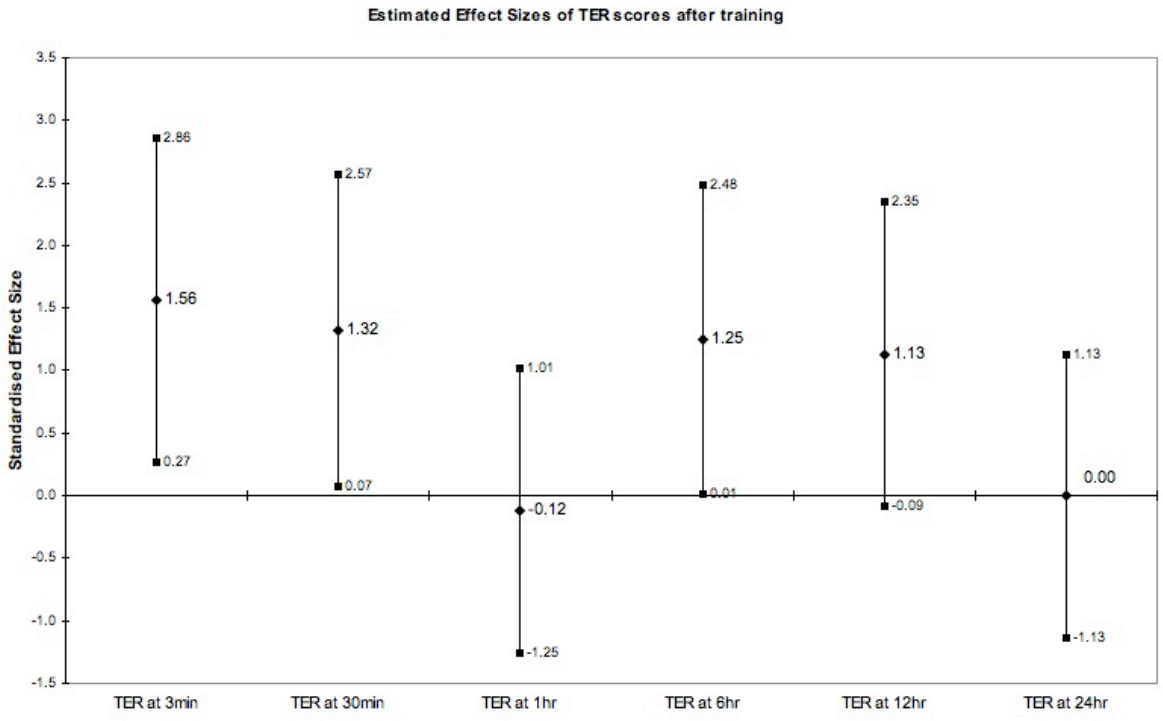


Figure 4.10 Graph shows standardised effect sizes of conditioning on tentacle extension responses in the six retention intervals, taken from the last 30 seconds of each test period. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

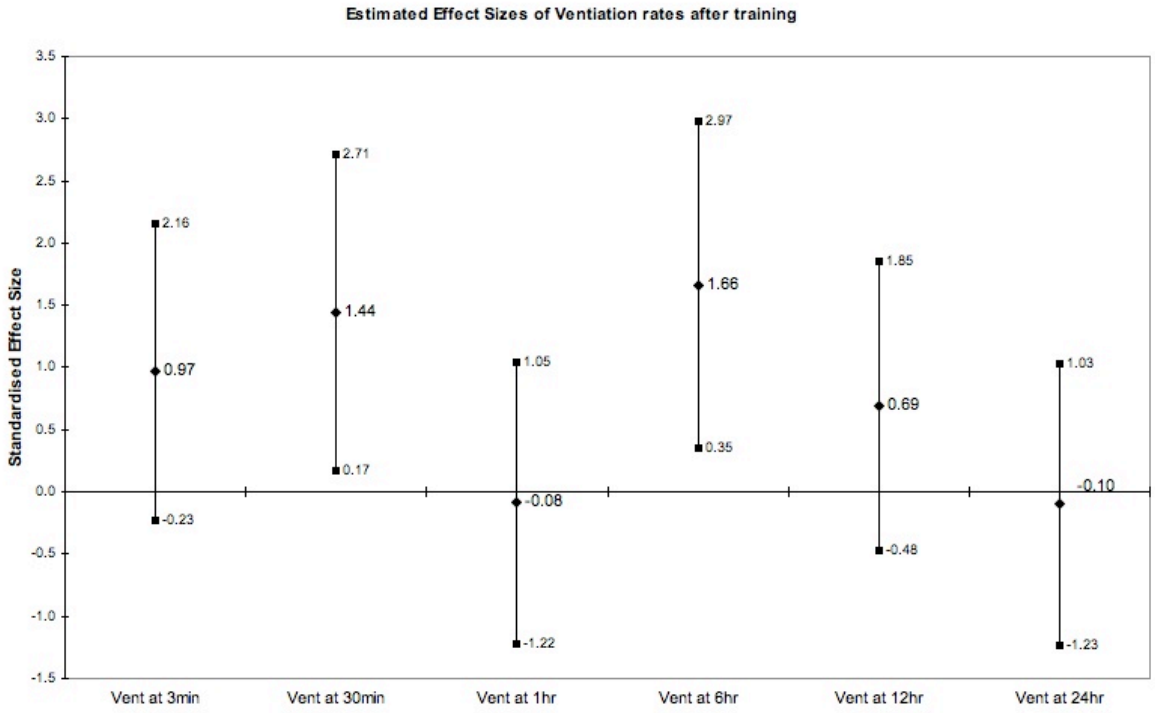


Figure 4.11 Graph shows standardised effect sizes of conditioning on ventilation rates in the six retention intervals, taken from the last 30 seconds of each test period. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

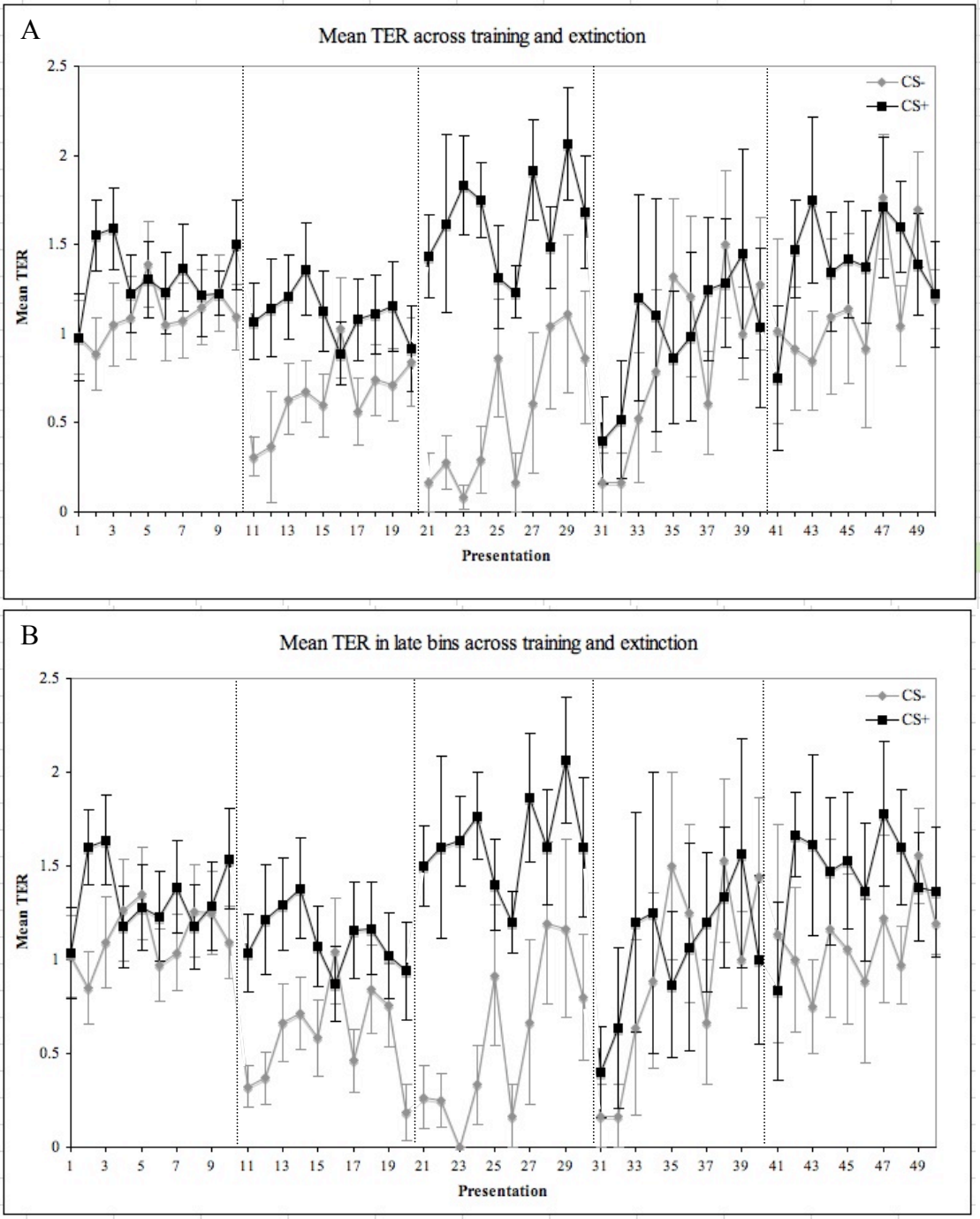


Figure 4.12 Graph of mean (+/- 1 s.e.m.) tentacle extension responses in CS- (grey line), CS+ (black line) conditions for A. the full 60-second recording period, and B. the last 30 seconds of each recording period of each presentation across training (1-10), extinction (11-20) and recovery at 6 hrs (21-30), 12 hrs (31-40) and 24 hrs (41-50).

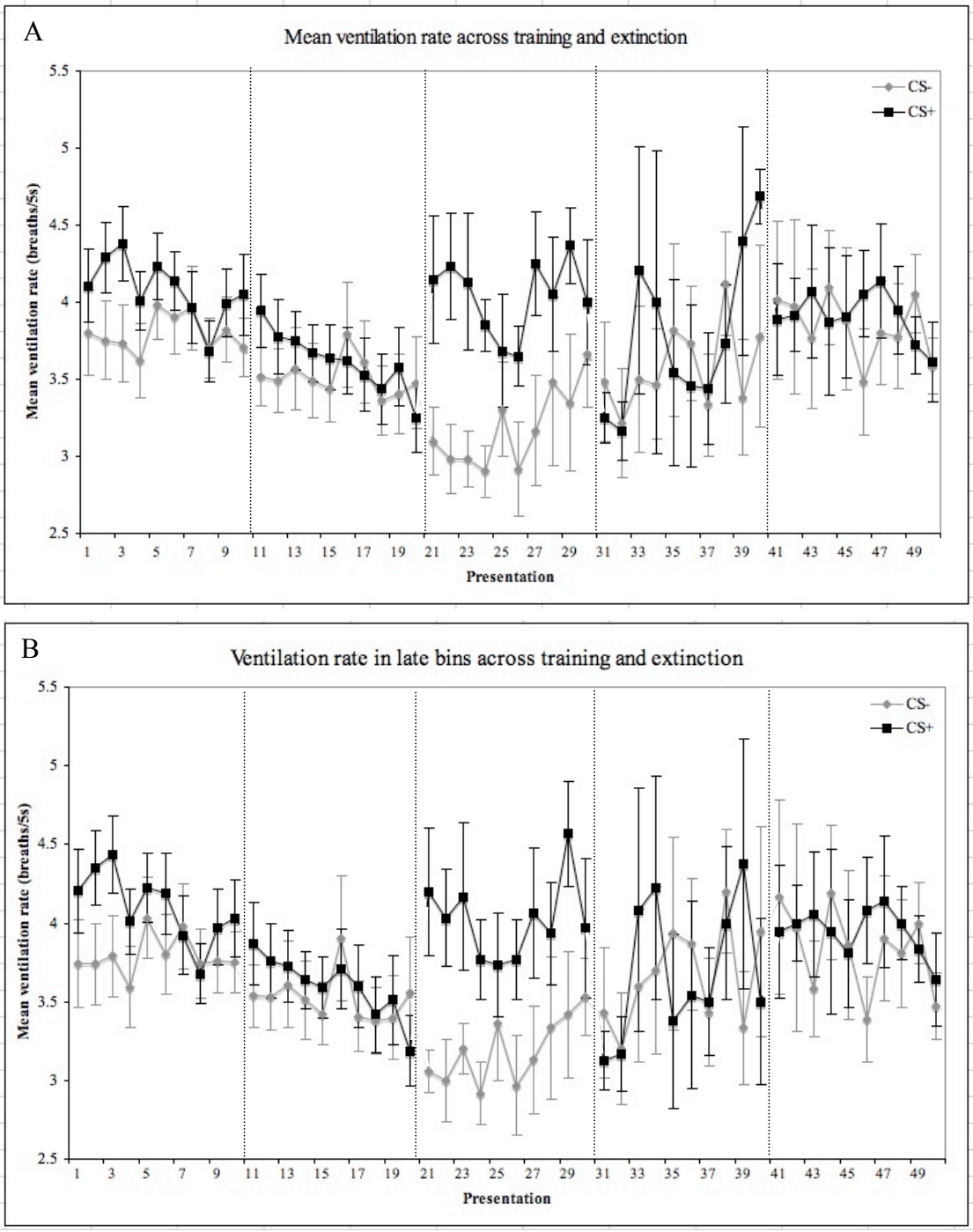


Figure 4.13 Graph of mean (+/- 1 s.e.m.) ventilation rates in CS- (grey line), CS+ (black line) conditions for A. the full 60-second recording period, and B. the last 30 seconds of each recording period of each presentation across training (1-10), extinction (11-20) and recovery at 6 hrs (21-30), 12 hrs (31-40) and 24 hrs (41-50).

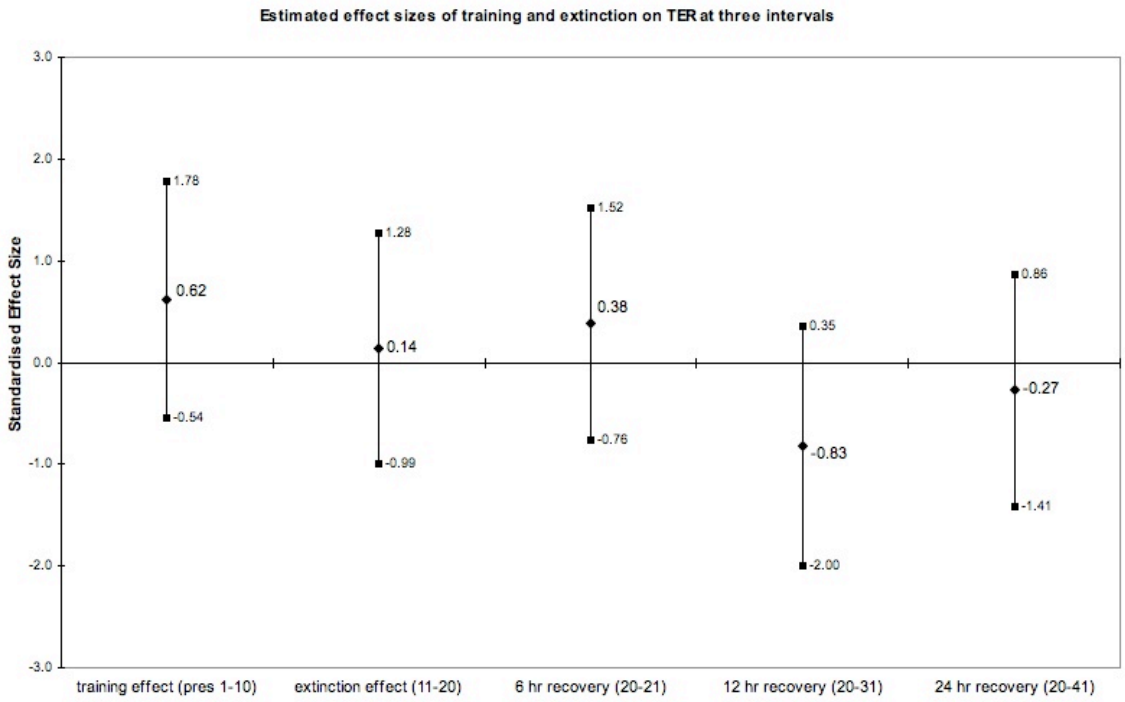


Figure 4.14 Graph shows standardised effect sizes of training, extinction and recovery on tentacle extension response in conditioned (CS+) animals. The effect of training is shown by comparing the first and final training trials, (presentations 1 and 10). The extinction effect size is shown by comparing the first and last extinction trial, (presentations 11 and 20). Recovery at 6 hrs is shown by comparison of the last extinction presentation (20) to the first recovery presentation (21). Recovery at 12 hrs is shown by comparison of the last extinction presentation (20) to the first recovery presentation (31). Recovery at 24 hrs is shown by comparison of the last extinction presentation (20) to the first recovery presentation (41). Data for effect size computations were taken from the last 30 seconds of each test period. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

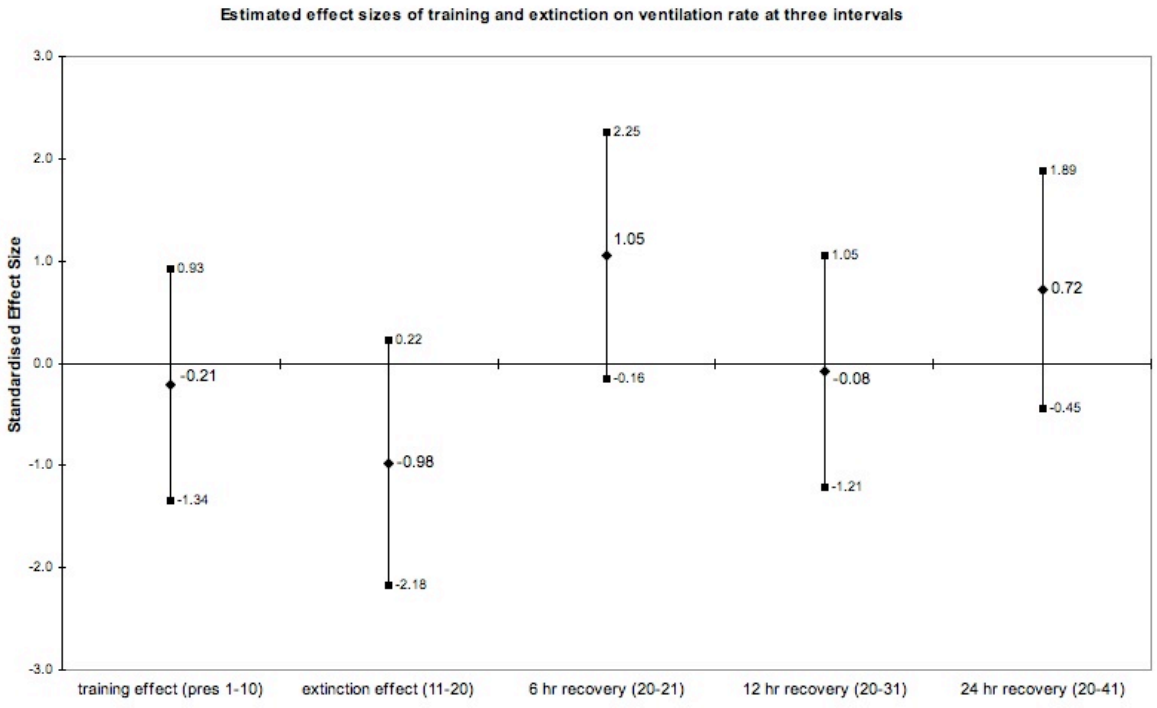


Figure 4.15 Graph shows standardised effect sizes of training, extinction and recovery on ventilation rate in conditioned (CS+) animals. Effect of training is shown by comparing the first and final training trials, (presentations 1 and 10). Extinction effect is shown by comparing the first and last extinction trial, (presentations 11 and 20). Recovery at 6 hrs is shown by comparison of the last extinction presentation (20) to the first recovery presentation (21). Recovery at 12 hrs is shown by comparison of the last extinction presentation (20) to the first recovery presentation (31). Recovery at 24 hrs is shown by comparison of the last extinction presentation (20) to the first recovery presentation (41). Data for effect size computations were taken from the last 30 seconds of each test period. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

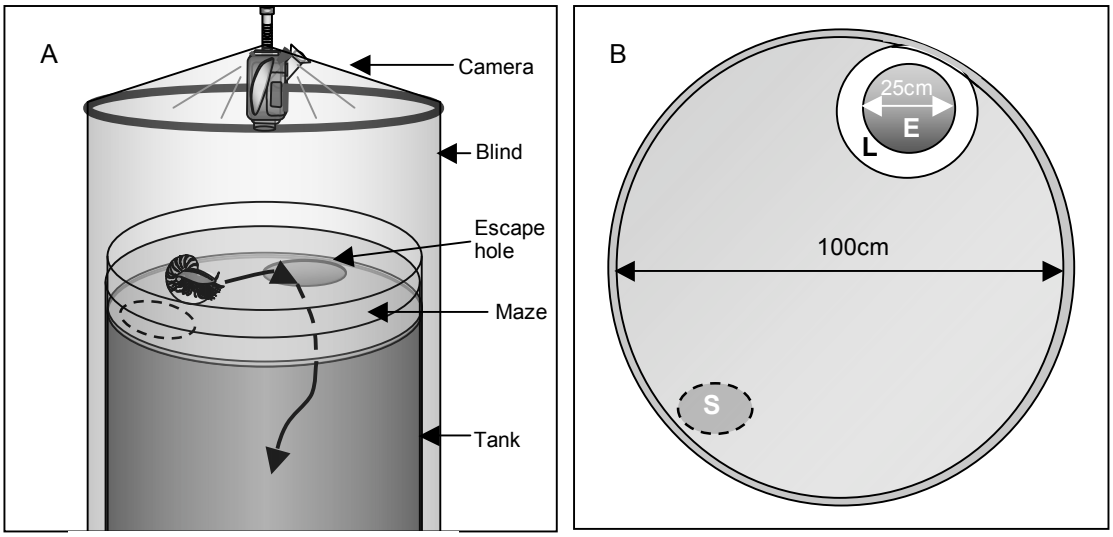


Figure 5.1 Diagram of apparatus setup for spatial learning experiments. A. The maze is immersed in the tank to a depth similar to the height of the focal animal's shell. Camera records the trial from overhead. The entire tank is surrounded by a black plastic blind, which controls the availability of external navigational landmarks. The animal must locate and swim through the escape hole. Arrows show the path the animal takes to escape into the tank below. B. Maze shown from above. S – start position, E – escape hole, L – landmark. Diagram not to scale

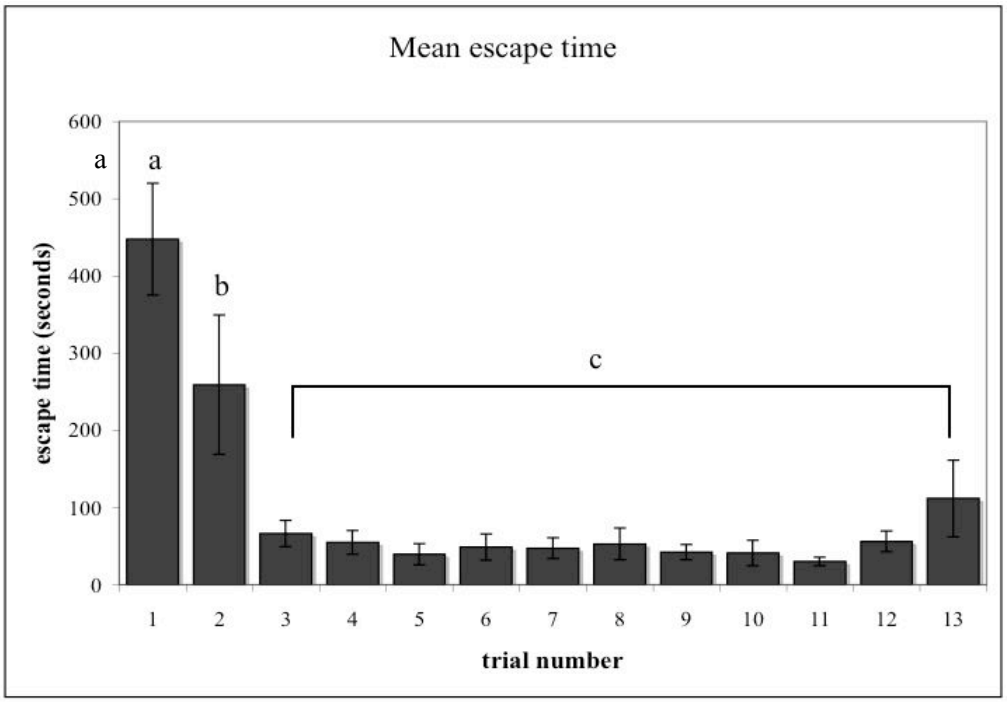


Figure 5.2 Graph shows mean escape times per trial in Experiment 3. Bars with the same letter above are not significantly different. Trials 1-5 are successive training trials. Trials 6-13 are test trials at different intervals. Trial 6: 18h; trial 7: 24h; trial 8: 36h; trial 9: 48h; trial 10, 72h; trial 11: 96h; trial 12: 1 week; trial 13: 3 weeks.

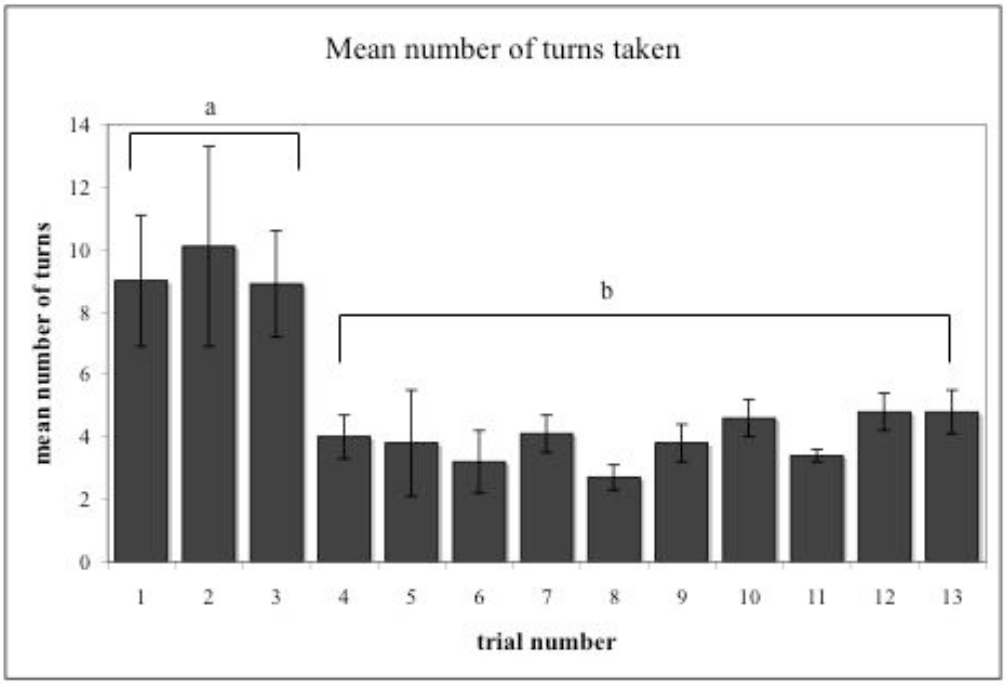


Figure 5.3 Graph shows mean number of turns made by animals (directional change of >15° in heading angle) per trial in Experiment 3. Bars with the same letter above are not significantly different. Trials 1-5 are successive training trials. Trials 6-13 are test trials at different intervals. Trial 6: 18h; trial 7: 24h; trial 8: 36h; trial 9: 48h; trial 10, 72h; trial 11: 96h; trial 12: 1 week; trial 13: 3 weeks.

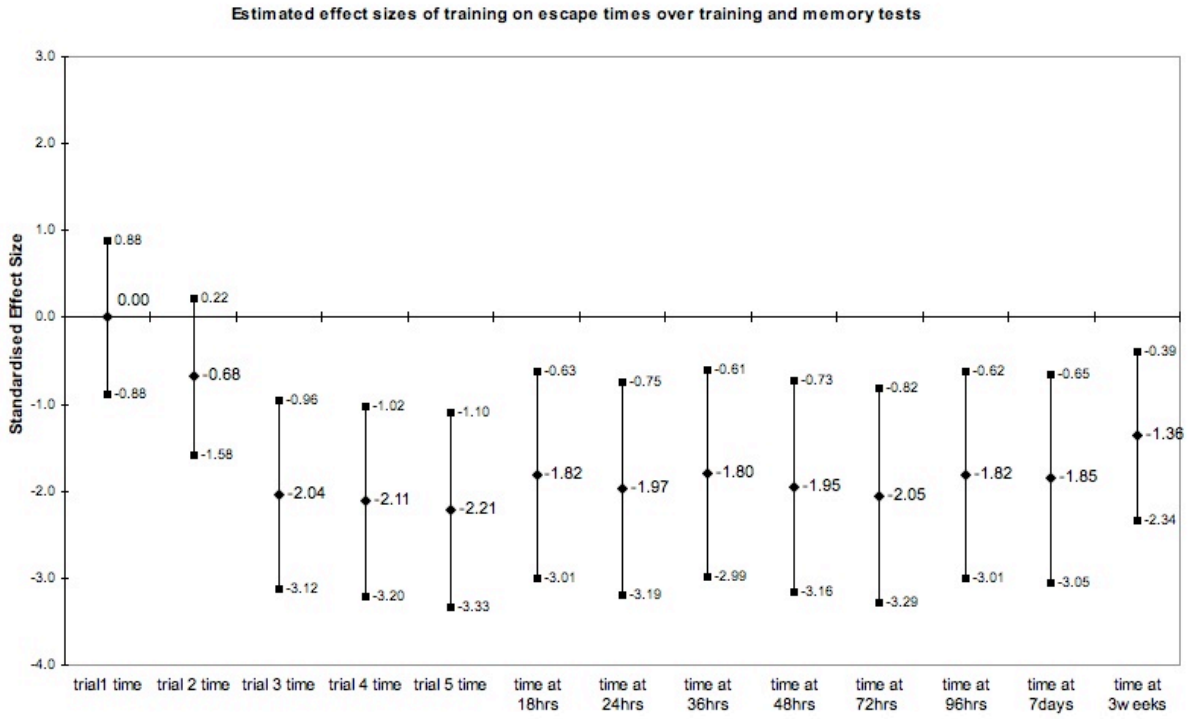


Figure 5.4 Graph shows standardised effect sizes of training on escape times across training and test periods in Experiment 3. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

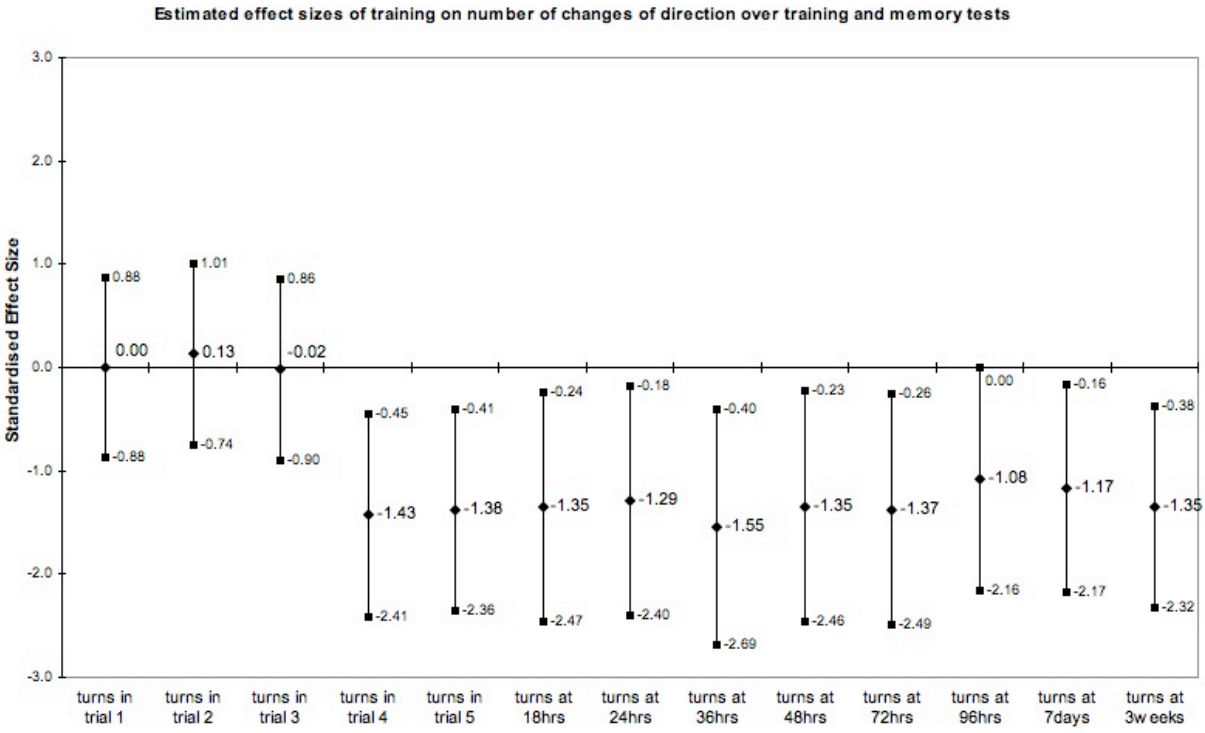


Figure 5.5 Graph shows standardised effect sizes of training on direction changes across training and test periods in Experiment 3. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

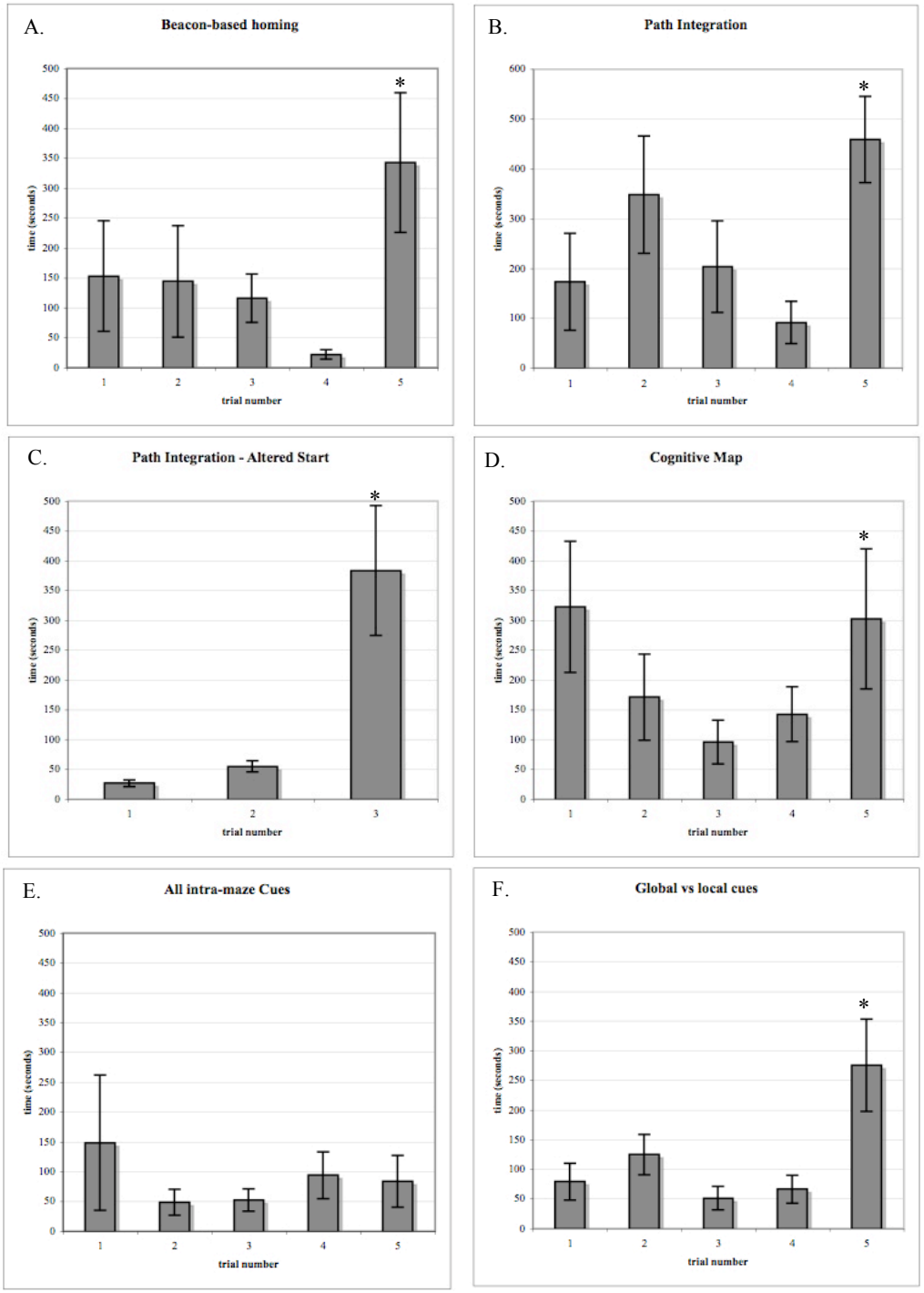


Figure 6.1 Graph shows mean escape times (+/- 1 s.e.m) per trial in Experiment 4. A. Beacon-based homing. B. Path integration. C. Path integration with altered start position. D. Cognitive-map based navigation. E. Proximate vs. distant intra-maze cues. F. Local (intra-maze) vs. global (extra-maze) cues. The probe trial is the last bar in each panel. * denotes a significant increase from the last training trial to the probe trial.

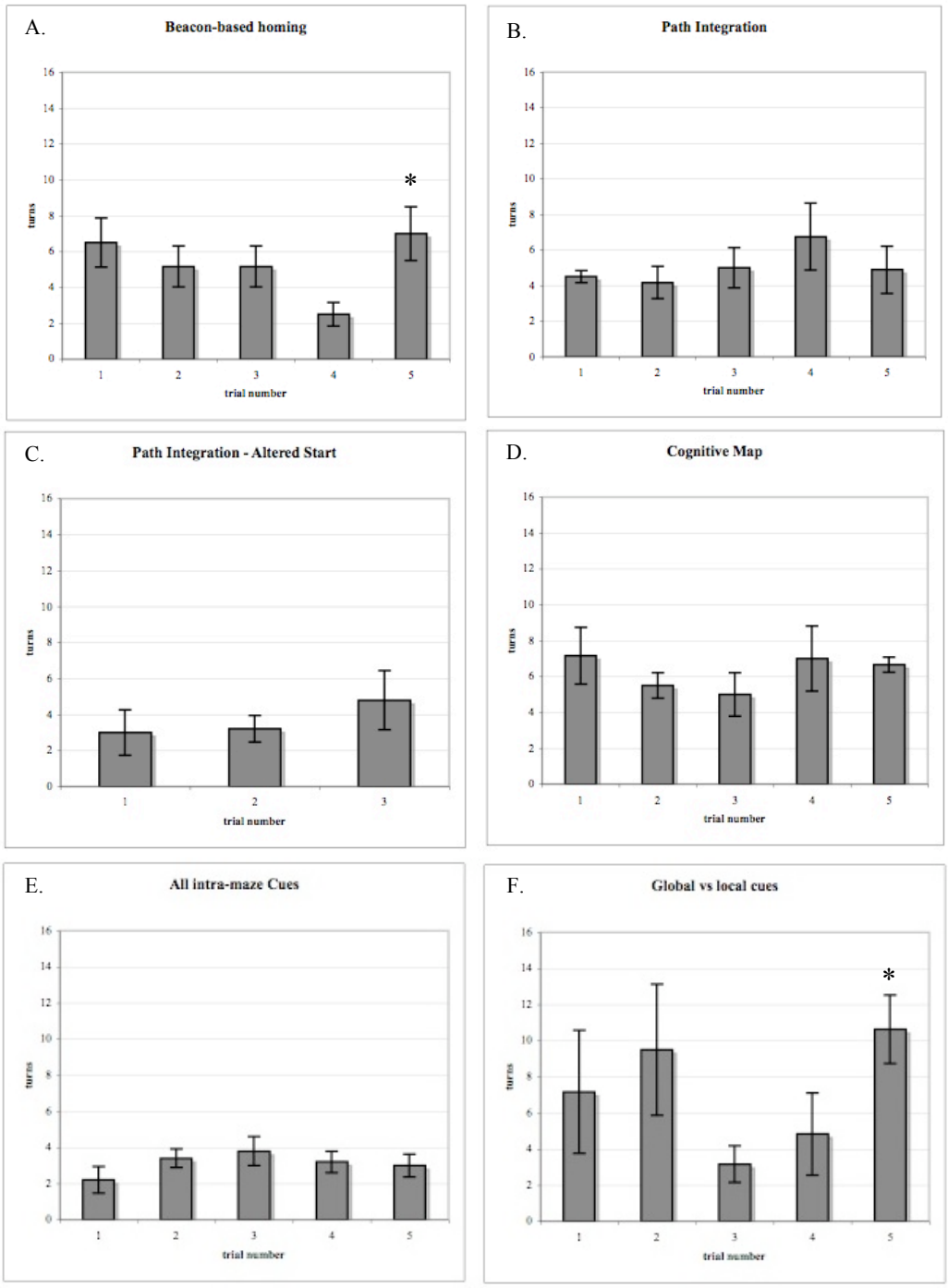


Figure 6.2 Graph shows mean number of direction changes (+/- 1 s.e.m) per trial in Experiment 4. A. Beacon-based homing. B. Path integration. C. Path integration with altered start position. D. Cognitive-map based navigation. E. Proximate vs. distant intra-maze cues. F. Local (intra-maze) vs. global (extra-maze) cues. The probe trial is the last bar in each panel. * denotes a significant increase from the last training trial to the probe trial.

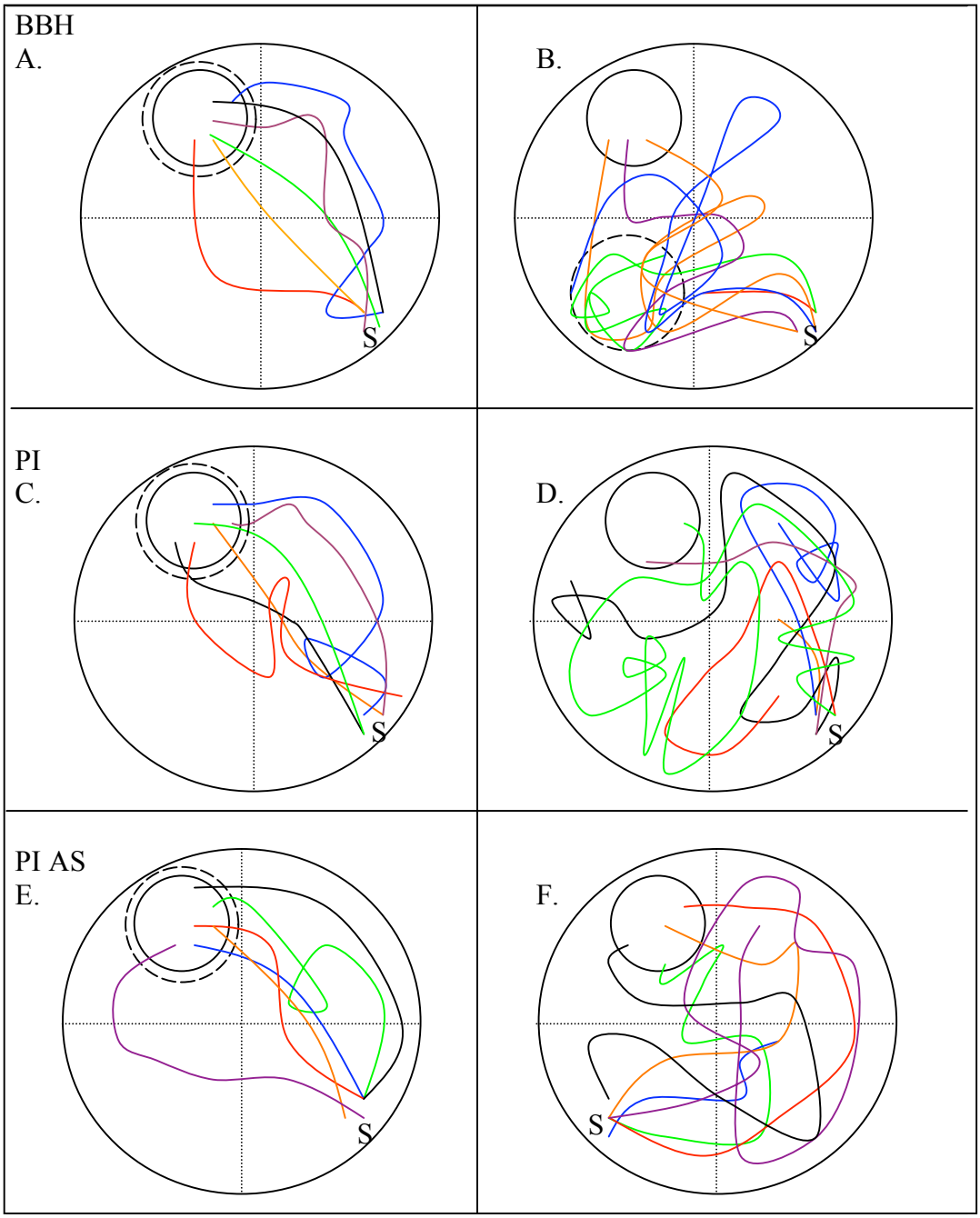


Figure 6.3 Diagrams of route maps for each animal in each maze configuration. A. Last training trial for beacon-based homing. B. Test trial, beacon-based homing. C. Final training trial, path integration. D. Test trial, path integration. E. Final training trial, path integration with altered start position. F. Test trail, path integration with altered start position.

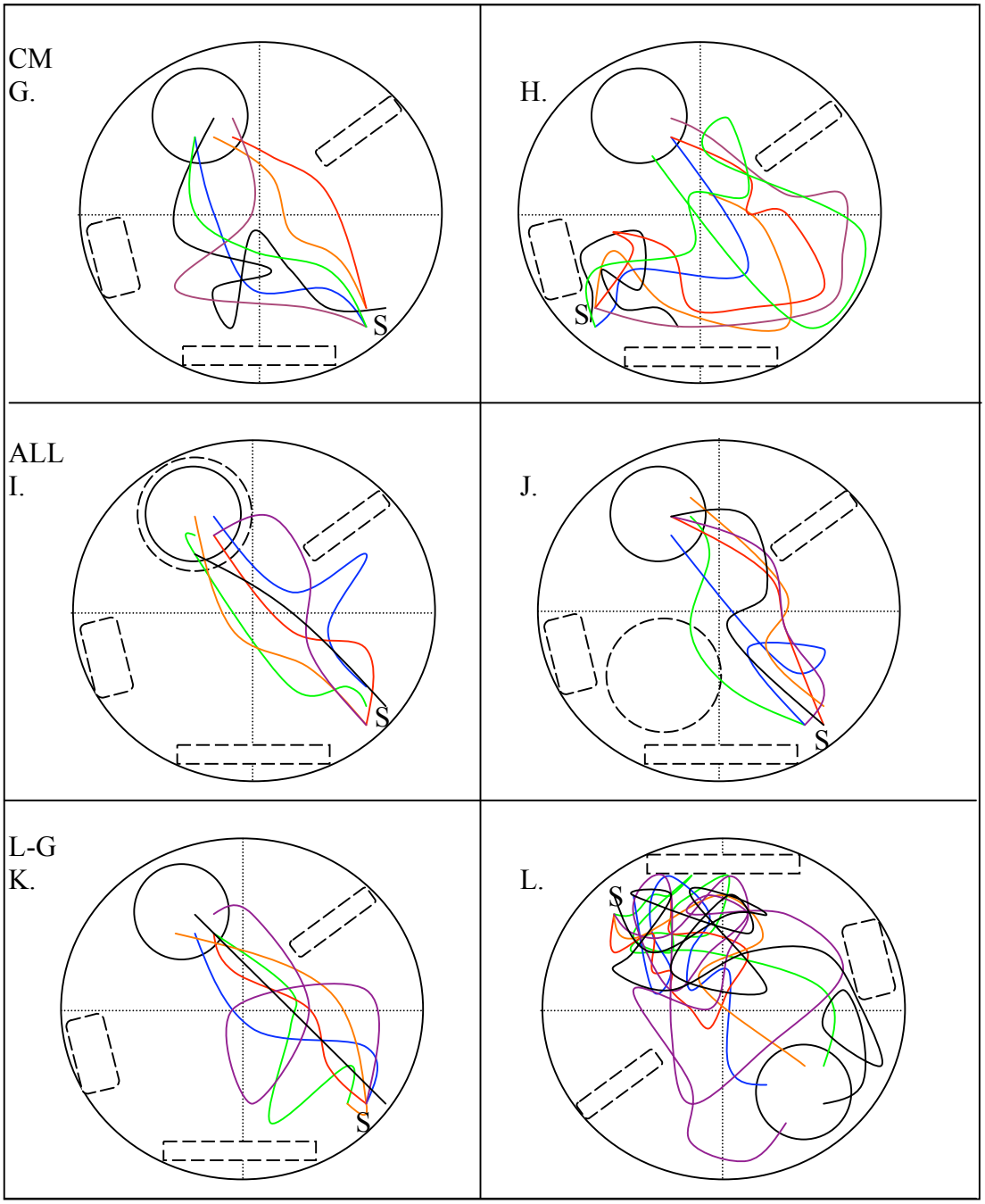


Figure 6.3 continued. G. Final training trial, cognitive map-based navigation. H. Test trial, cognitive map-based navigation. I. Final training trial, proximate vs. distant intra-maze cues. J. Test trial, proximate vs. distant intra-maze cues. K. Final training trial, local (intra-maze) vs. global (extra-maze) cues. L. Test trial, local (intra-maze) vs. global (extra-maze) cues.

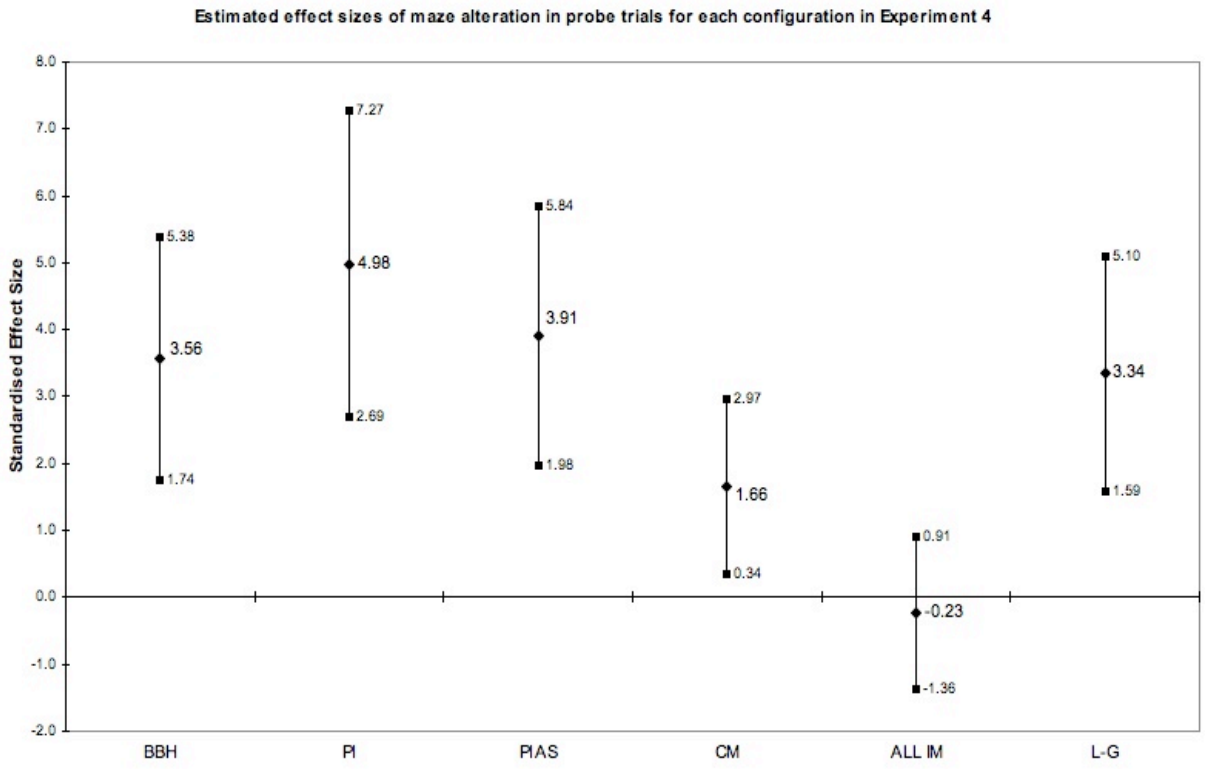


Figure 6.4 Graph shows standardised effect sizes of maze alterations on escape times between the final training trial and the probe trial for each configuration in Experiment 4. BBH - beacon-based homing. PI - path integration. PI AS - path integration with altered start position. CM - cognitive-map based navigation. ALL - proximate vs. distant intra-maze cues. L-G - local (intra-maze) vs. global (extra-maze) cues. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

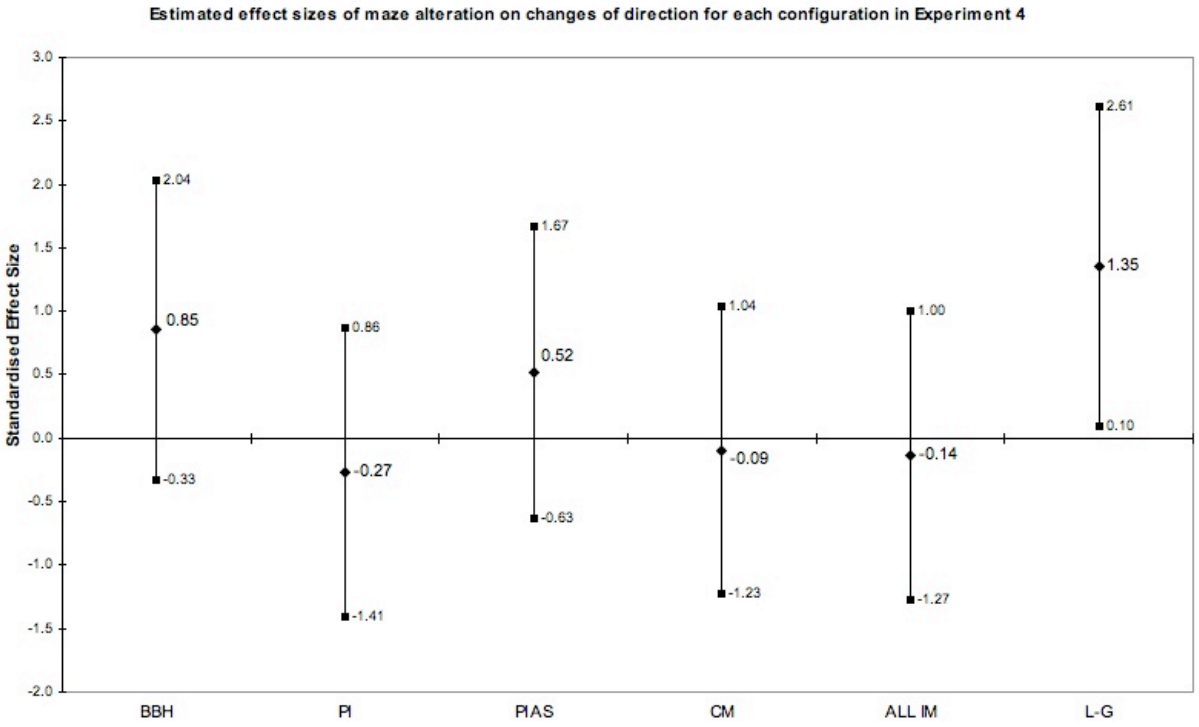


Figure 6.5 Graph shows standardised effect sizes of maze alterations on number of changes of direction made by animals between the final training trial and the probe trial for each configuration in Experiment 4. BBH - beacon-based homing. PI - path integration. PI AS - path integration with altered start position. CM - cognitive-map based navigation. ALL - proximate vs. distant intra-maze cues. L-G - local (intra-maze) vs. global (extra-maze) cues. Bars show estimated effect size and 95% confidence limits. Genuine effects have CLs not overlapping zero.

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