

**Food-associated conditioning in the smoothhound shark *Mustelus
mustelus* (Linnaeus, 1758)**

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Smoothhound sharks *Mustelus mustelus* (Chondrichthyes: Triakidae) were conditioned to associate a compound auditory and electrical conditioned stimulus (CS) using the presentation of food as the unconditioned stimulus (US). This was done to investigate the general conditioning process of sharks, specifically the use of conditioned stimuli, the time frame required for conditioning, the retention of conditioned responses and the individual learning capabilities of sharks in classical conditioning experiments. Conditioning was done in two CS experiment replicates, in which the CS and the US were paired to elicit a conditioned response (CR: entering of a feeding area). Shark behaviour in these replicates was compared with those of sharks in a CS control. In the CS control, the CS and US were not paired; consequently no CS-US association was formed. In contrast, half of the sharks in the 1st and 2nd CS replicates formed the conditioned CS-US association after five and six days of conditioning training, respectively. This conditioned association was also retained over a two-month rest period. However the rate of conditioning of sharks varied, which was thought to be due to differences in motivational drives, particularly hunger. These experiments demonstrated that classical conditioning could provide a mechanism whereby smoothhound sharks can adapt to utilise new food sources.

However these experiments could not demonstrate whether auditory or electrical cues are more biological relevant signallers of the presence of food for smoothhound sharks. According to the “belongingness” phenomenon, for conditioning to be successful, the CS should be biologically relevant to the US. Therefore by comparisons of the conditioning success with different conditioned stimuli, the biological relevance of

each CS can be determined. To determine the biological relevance of each component of the compound CS, an auditory or electrical CS was paired with the presentation of food (US), in separate experiments. The conditioned CS-US association formed in the majority of the sharks after 60 and 57 conditioning trials in the auditory CS and electrical CS experiment, respectively. This suggests that both auditory and electrical cues are biologically relevant signallers of food, facilitating the opportunistic feeding behaviour of smoothhound sharks. However, after five days of conditioning, conditioning with the electrical CS had proceeded further and the electrical CS-US association was stronger than the auditory CS-US association. This indicates that electrical cues are more biologically relevant due to past feeding experiences. Thus, classical conditioning during past feeding experiences can influence the feeding behaviour of smoothhound sharks.

Opsomming

Gladde-hondhaaie *Mustelus mustelus* (Chondrichthyes: Triakidae) is gekonditioneer om 'n gekombineerde klank en elektriese kondisionerings stimulus (CS) met voedsel, die ongekonditioneerde stimulus (US) te assosieer. Dit is gedoen om die kondisionerings proses, spesifiek die gebruik van kondisionerings stimuli, tyd benodig vir kondisionering, behoud van die gekonditioneerde gedrag en individuele leer-vermoëns van haaie in klassieke kondisionerings eksperimente te ondersoek. Kondisionering is gedoen in twee CS eksperiment replikate, waarin die CS en die US saam aangebied is, om 'n kondisionerings reaksie (CR: om in 'n voedings area in te beweeg) teweeg te bring. Die gedrag van haaie in die CS replikate is vergelyk met die van haaie in 'n CS kontrole. In die CS kontrole is die CS en die US nie saam aangebied nie en gevolglik is geen gekonditioneerde CS-US assosiasie gevorm nie. Daarenteen is die CS-US assosiasie by die helfte van die haaie in die 1st en 2^{de} CS replikate gevorm, na vyf en ses dae, onderskeidelik. Hierdie gekonditioneerde CS-US assosiasie het selfs behoue gebly na 'n twee maande rus periode. Die tempo van kondisionering het egter verskil, moontlik a. g. v. motiverings verskille, veral hongerte. Hierdie eksperimente het gedemonstreer dat klassieke kondisionering kan dien as 'n meganisme waarmee gladde-hondhaaie kan aanpas om nuwe voedsel bronne te benut.

Hierdie eksperimente kon egter nie demonstreer óf klank stimuli óf elektriese stimuli meer biologiese relevante seine van die teenwoordigheid van voedsel is vir gladde-hondhaaie nie. Volgens die "belongingness" verskynsel, moet die CS biologies relevant to die US wees, vir kondisionering om suksesvol te wees. Dus kan die biologiese

relevansie van verskillende kondisionerings stimuli bepaal word deur die kondisionerings sukses van elke CS te vergelyk. Om die meer biologiese relevante komponent van die gekombineerde CS te bepaal, is 'n klank of elektriese CS saam met voedsel aangebied in aparte eksperimente. Die gekonditioneerde CS-US assosiasie is gevorm by die meeste van die haaie na 60 en 57 kondisionerings proeflopieë in die klank en elektriese CS eksperimente, onderskeidelik. Hierdie resultate dui daarop dat beide klank en elektriese stimuli biologies relevante siene van voedsel is, wat die oppurtunistiese voedings wyse van gladde-hondhaaie fasiliteer. Kondisionering met die elektriese CS het egter verder gevorder en die elektriese CS-US assosiasie was sterker as die klank CS-US assosiasie na vyf dae van kondisionering. Dus, mag elektriese stimuli meer biologies relevant wees weens vorige voedings ervarings. Dit illustreer dat klassieke kondisionering tydens vorige voedings ervarings die voedings gedrag van gladde-hondhaaie kan beïnvloed.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Classical conditioning experiments, with the presentation of food as the unconditioned stimulus (US), have been performed on numerous animal species. Some of the first were undertaken on dogs by Pavlov (1927), and subsequently similar experiments have been performed successfully on several animal groups, including teleost fish. However, few studies of this nature have been undertaken on any elasmobranch fish. Most studies of this group have focused on determining the visual (Aronson *et al.* 1967; Clark 1959; Graeber 1978; Graeber & Ebbesson 1972; Tester & Kato 1966; Wright & Jackson 1964) and auditory capabilities (Aronson *et al.* 1967; Clark 1959; Davies *et al.* 1963; Wisby *et al.* 1964; Wright & Jackson 1964) of sharks. These studies demonstrated that the conditioning rate of sharks was comparable to those of mammals, birds and teleost fish, but did not illustrate the role of conditioning in shark behaviour.

Classical conditioning experiments can be invaluable in studying the behaviour of sharks in the laboratory, which would normally be difficult to study in the wild. This is due to the adaptive approach to the conditioned response (CR) and the “belongingness” phenomenon. According to the adaptive approach, the CR has adaptive value by preparing the animal to optimise interaction with the US (Domjan 1992; Hollis 1982). If

the US is food, the CR should increase feeding efficiency, which in turn would translate into a reproductive advantage, thereby contributing to fitness (McCleery 1978).

Several studies with teleost fish have provided support for the adaptive approach. Even though the conditioned responses were energetically costly, a conditioned stimulus (CS) preceding a rival fish (US) elicited attacks from territorial male Siamese fighting fish *Betta splendens* (Thompson & Sturn 1965) and conditioned stimuli preceding food (US) elicited approaches from several fish species (Brandon *et al.* 1982; Squier 1969; Woodard & Bitterman 1974). Clearly this anticipatory CR must confer some advantage on these individuals, possibly in preparing them for encounters with the US. Subsequent studies have shown this to be the case. Territorial male blue gourami fish *Trichogaster trichopterus* could defend their territory more successfully when a CS signalled an intruder than when unsignalled (Hollis 1984). This competitive advantage had both short and long term effects. Most likely due to neuro-endocrinological factors, males for whom attacks were signalled, were more likely to win the encounter as well as subsequent encounters, than those for whom attacks were not signalled (Hollis *et al.* 1995). Similarly mating success of blue gourami males to which the presence of a female was signalled, was higher than those not signalled (Hollis *et al.* 1989; Hollis *et al.* 1997). These studies demonstrated that classical conditioning plays an important role in several animal behavioural systems.

The role of different senses in a particular behavioural system can be further investigated in view of the “belongingness” phenomenon. This approach predicts conditioning will be successful if the conditioned stimulus (CS) and US “belong” together or are biologically relevant (Domjan 1992; Hollis 1997). This was described by Garcia & Koelling (1966) in food-aversion conditioning in which rats were presented with two

conditioned stimuli, a taste and audio-visual CS, and two unconditioned stimuli, shock and sickness. Conditioned associations were formed only between the shock US and the audio-visual CS; and between the sickness US and the taste CS (Garcia & Koelling 1966). It was inferred that for rats, with a highly developed sense of taste, taste would be a better cue for food toxicity than an external event, such as shock. However, external events (audio-visual CS) would be reliable signals of physical pain (shock) (Garcia *et al.* 1974). These findings were confirmed by a similar study on rats by Domjan and Wilson (1972). Rozin and Kalat (1971) went further by suggesting that any eating-related cues (cues a species use to identify food) would be effective conditioned stimuli in food – aversion conditioning. This has been confirmed by several studies with birds. Visual cues may be more important for the identification of food for birds than taste cues due to a poorly developed gustatory system, and it was demonstrated that bobwhite quail *Colinus virginianus* (Wilcoxon *et al.* 1971) and chickens *Gallus gallus* (Capretta 1961; Martin *et al.* 1977) could form food-aversions to visual cues. Gillette and colleagues (1980) went on to demonstrate that chickens formed food-aversions more readily with visual than taste cues. Thus, the success of formation and the strength of the conditioned association depend on the biological relevance of the CS to the US (Domjan 1992; Hollis 1997).

This “belongingness” phenomenon was thought to be unique to food-aversion conditioning. However, in operant conditioning studies with pigeons (Foree & LoLordo 1973; LoLordo & Furrow 1976) it was demonstrated that the visual component of a compound CS controlled a CR to obtain food, whereas the auditory component of the compound CS controlled the CR to avoid electrical shock. Subsequently Shapiro *et al.* (1980) demonstrated the “belongingness” phenomenon, once again in food- and shock-associated classical conditioning, with pigeons. As was expected, pigeons formed a

strong conditioned association between food and the visual CS, and not with the auditory CS. Accordingly, the nature of the successful CS would be related to the nature of the US, which would normally activate a particular behavioural system. Consequently, the nature of conditioned stimuli that successfully activates foraging and feeding behaviour, should reflect the sense which detects the unconditioned stimuli which would normally activate this behaviour.

Among the senses that might be of some importance in the feeding behaviour of sharks are the auditory and electrical senses. Both these senses are highly developed. Sharks detect sounds with their inner ear, lateral line and general cutaneous sense and are especially sensitive to low-frequency underwater sounds (less than 1000 Hz) (Bleckmann & Hofmann 1999). Evidence suggests that sharks can use auditory cues to locate prey. Recordings of sounds similar to that made by struggling fish and other shark prey have attracted several carcharhinid and sphyrnid shark species (Nelson & Gruber 1963; Banner 1972).

The electrical sense has also been shown to be important in the feeding behaviour of sharks. Using their ampullae of Lorenzini, sharks can detect electrical fields as small as $2 \mu\text{V/m}$ (Bleckmann & Hofmann 1999). It has been demonstrated in several studies that elasmobranch fish can use electrical cues to locate food. Electrical fields from real prey and electrical fields simulating prey elicited feeding responses from several shark and ray species (Kalmijn 1966, 1971, 1982). The importance of these senses (auditory and electrical senses) in the feeding behaviour of sharks can be investigated further by conditioning studies.

1.2 Review of fish conditioning studies

Conditioning studies with teleost fish and sharks have demonstrated the role of the auditory and electrical senses in the feeding behaviour of these animals. In several conditioning studies, teleost fish have been conditioned to associate auditory cues with food, mainly for aquacultural purposes. Red sea bream *Pagrus major* (Fujiya *et al.* 1980) were conditioned in an aquarium to associate auditory cues with the presentation of food in two weeks. This conditioned association was retained even after a two-month rest period. Several Black Sea fish species, *Symphodus tinca* (Rulena), *S. ocellatus* (Eyespot wrasse), *S. roissali* (Quail wrasse), *S. cinereus* (Gray wrasse) and *Chromis chromis* (Sea swallow) were taught after two weeks in the open sea, to approach a feeding area to obtain food when an auditory cue was presented (Zhuykov & Panyushkin 1991). In an operant conditioning study, common carp *Cyprinus carpio* and thicklipped mullet *Crenimugil labrosus* were trained to make responses for food reward only when an auditory cue was presented (Wright & Eastcott 1982). After 24 hours of training, there were indications that the auditory cue triggered the response for the food reward. Clearly several teleost fish species can be trained to associate auditory cues with the presentation of food.

Conditioning studies also demonstrated that sharks could be trained to associate auditory cues with the presentation of food. In several operant conditioning studies with sharks, auditory cues were paired with an action (pressing of a target) to obtain food (Aronson *et al.* 1967; Clark 1959; Wright & Jackson 1964). In addition, Davies *et al.* (1963) demonstrated several shark species *Carcharhinus obscurus* (Dusky shark), *C. brevipinna* (Spinner shark), *C. leucas* (Bull shark) and *Sphyrna lewini* (Scalloped

hammerhead) could associate auditory cues with the presentation of food in a classical conditioning study. However, the focus of the study was on learning the auditory thresholds of these sharks, and the actual learning processes were largely ignored in this study.

Few studies with teleost fish have used an electrical cue as a CS. An electrical cue was paired successfully with an aversive US (electrical shock) for goldfish (Breuning & Wolach 1981), but has not been paired with a food US for any teleost fish. Likewise, no study with sharks has paired an electrical CS with the presentation of food, but Kalmijn & Kalmijn (1981) demonstrated that California round stingrays *Urobatis halleri* could associate electrical cues with a food reward in a conditioning study.

Clearly, information about the general conditioning of food-association with auditory or electrical cues in sharks is needed. Neither the role of classical conditioning in the formation of feeding behaviour, nor the relative importance of the auditory and electrical senses in natural feeding behaviour of sharks have been investigated. These are the issues that I attempt to address in this study.

1.3 Study aims

The aims of this study are to: 1. investigate the general conditioning process; 2. infer the role of classical conditioning; and 3. investigate the relative importance of the auditory and electrical senses in the natural feeding behaviour of sharks. This was achieved by attempting to condition immature smoothhound sharks *Mustelus mustelus* (Chondrichthyes: Triakidae) to associate a compound auditory and electrical CS, or

alternatively the separate components of the compound CS, with the presentation of food (US). Smoothhound sharks are strong-swimming epibenthic sharks, feeding mainly on crustaceans (Smale & Compagno 1997). They are ideal for conditioning experiments as they are easily kept in captivity and relatively easy to obtain through trek-net fishing activities in False Bay.

In chapter 2 I describe an experiment in which the compound auditory and electrical CS was paired with the presentation of food to study the conditioning process. More specifically, the types of CS that could be used successfully, the time required for conditioning and retention of the CR (entering of a feeding area), and intraspecific variation in learning capabilities, were investigated. As the CR, according to the adaptive approach, should enable smoothhound sharks to optimise interaction with food sources, the role of classical conditioning in the natural feeding behaviour of this species could be inferred from this study.

In chapter 3 the separate auditory CS and electrical CS was paired with the presentation of food. The conditioning rate and success of conditioning with each of these conditioned stimuli were compared. According to the “belongingness” phenomenon, the CS, which conditions more successfully, should be the more biologically relevant cue to signal the presence of food to smoothhound sharks. Therefore, the relative importance of the auditory and electrical senses, in addition to the role of classical conditioning in the formation of feeding behaviour could be inferred.

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CHAPTER 2

FOOD-ASSOCIATED CONDITIONING IN THE SMOOTHHOUND SHARK *MUSTELUS MUSTELUS* WITH A COMPOUND AUDITORY AND ELECTRICAL CONDITIONED STIMULUS

2.1 Summary

The use of conditioned stimuli, the time frame required for conditioning, the retention of conditioned responses and the individual learning capabilities of sharks in classical conditioning experiments were investigated in the smoothhound shark, *Mustelus mustelus*. In two CS experimental replicates, a compound auditory and electrical conditioned stimulus (CS) was paired with presentation of food as the unconditioned stimulus (US) to elicit the conditioned response (CR: entering of a feeding area). In a CS control the compound conditioned stimulus was not paired with the US, and no association was formed between the CS and US. Half of the sharks in the 1st and 2nd CS replicates were successfully conditioned after five and six days of conditioning training, respectively. This conditioned association was retained over a rest period of two months. Individual variation in the rate of conditioning was observed, which was thought to be due to differences in motivational drives, particularly hunger. As smoothhound sharks are opportunistic feeders, classical conditioning provides a mechanism by which these sharks can adapt to fully utilise new food sources.

2.2 Introduction

Classical conditioning experiments with the presentation of food as the unconditioned stimulus (US) have been performed on numerous animal species. Some of the first were undertaken on dogs by Pavlov (1927), and subsequently similar experiments have been successfully performed on several animal groups, including teleost fish. However, few studies of this nature have been undertaken on elasmobranch fish. Most studies of this group have focused on determining the sensory capabilities of sharks, and involved discrimination learning (Tester & Kato 1966; Wisby *et al.* 1964).

Classical conditioning experiments can be invaluable in studying behaviour of sharks in the laboratory, which would normally be difficult to study in the wild. This is because the conditioned response (CR) has adaptive value (Domjan 1992; Hollis 1982). According to this approach, the CR is essentially preparatory in dealing with the US. Thus, the CR should enable a shark to optimise the interaction with the US and consequently contributes to fitness by increasing feeding efficiency, which in turn would translate into reproductive advantage (McCleery 1978). In addition, it was found that for conditioning to be effective, the conditioned stimulus (CS) and US should “belong” together or be biologically relevant (Domjan 1992; Hollis 1997). Thus, if the US is the presentation of food, the nature of conditioned stimuli that successfully activates the foraging and feeding behavioural system, should be related to the unconditioned stimuli which would normally activate this behavioural system.

The electrical and auditory senses of sharks are highly developed (Bleckmann & Hofmann 1999) and auditory and electrical stimuli may be important in activating foraging and feeding behaviour in sharks. No study conducted on teleost fish could be

found where electrical stimuli (CS) were paired with food presentation (US) and only one study of this nature has been conducted on elasmobranch fish, in which Kalmijn & Kalmijn (1981) conditioned California round stingrays *Urobatis halleri* to associate an induced magnetic pole with a food reward. The stingrays may have detected these magnetic poles by voltage gradients induced when swimming through the magnetic field (Kalmijn 1984). Accordingly, these stingrays used electrical cues to locate the food reward. Auditory stimuli (CS) are more frequently paired with food presentation (US) in classical conditioning studies.

Several classical conditioning studies of this nature have been performed on teleost fish for aquacultural purposes. Red sea bream *Pagrus major* (Fujiya *et al.* 1980) and several Black Sea fishes (Zhuykov & Panyushkin 1991) were trained to feed at a specific place when an auditory stimulus (CS) was presented. In a similar study by Davies *et al.* (1963) several shark species, *Carcharhinus obscurus* (Dusky shark), *C. brevipinna* (Spinner shark), *C. leucas* (Bull shark) and *Sphyrna lewini* (Scalloped hammerhead) were conditioned to associate the presentation of food with auditory conditioned stimuli. However, little was mentioned about the actual learning processes, the time frame needed for conditioning and the individual variation in learning capabilities within shark species.

In this study small smoothhound sharks *Mustelus mustelus* (Chondrichthyes: Triakidae) were used to determine whether elasmobranch fish could be conditioned to associate a compound auditory and electrical CS with the presentation of food (US). Smoothhound sharks are strong-swimming epibenthic sharks (Smale & Compagno 1997) and were chosen as test subjects as they are easily kept in captivity and relatively easy to obtain. The aim of this study was to examine the conditioning process.

Specifically, the type of CS used with success on sharks, the time frame needed for conditioning, the retention of the conditioned response and the individual variation in the learning capabilities of sharks within the species was investigated. In addition the CR, according to the adaptive approach, should enable the smoothhound sharks to utilise food sources better, and the nature of the successfully conditioned CS should reflect a sense normally used by smoothhound sharks to locate food. Accordingly, this study was also intended to investigate what role classical conditioning plays in the feeding behaviour of smoothhound sharks, and the senses involved in locating food.

2.3 Method and Materials

2.3.1 Specimens and holding

Four small smoothhound sharks, one female (B_c) and three males (R_c , RY_c and Y_c) were used in the CS control; four specimens, three females (P_1 , RY_1 and YP_1) and a male (Y_1) were used in the first replicate, and four females (B_2 , RY_2 , RYR_2 and Y_2) during the second replicate of the CS experiment. The letters in the name of the sharks refer to the colour code of their tag, i.e. P = pink, R = red, and the subscript numbers refer to the 1st or 2nd replicate. The specimens were caught in False Bay, Western Cape Province, South Africa (34 ° E; 18 ° S) with trek-nets during April 2000 and December 2000 – January 2001, for the first and second replicate respectively and in March 2001 for the CS control. The total length of all the sharks was between 400 and 700 mm. According to size at maturity data from Smale & Compagno (1997) all these sharks were immature.

All the experiments were conducted at the Sea Fisheries Research Institute (Marine and Coastal Management) research aquarium in Cape Town. The sharks were housed in a circular tank, 3 m in diameter and 7 m deep, in which the experiments were also conducted. Several other teleost fish, including sardine *Sardinops sagax*, white steenbras *Lithognathus lithognathus*, red roman *Chrysoblephus laticeps* and white stumpnose *Rhabdosargus globiceps* were kept with the sharks in this tank. Seawater in the tank was aerated and circulated. The sharks were fed once a day during daylight hours with sardines except during the trial period. A 12:12 hour light: dark cycle was maintained in the aquarium.

2.3.2 Apparatus

Customised feeding equipment and the compound auditory and electrical CS were made for the conditioning experiment. For feeding during the experiments, small pieces of sardine were tied onto fishing line hanging from a rod, which was extended approximately 0.600 m under the water surface. This enabled both the manipulation of the feeding area and the amount of feeding.

A recording, made by banging two pieces of wood together, producing several tones between 300 and 1 100 Hz, was used as the auditory component of the compound CS. This was presented via BIGSTON BR-10 cassette tape recorder on an AUTODEK AE-4022 speaker just submerged in the water. The speaker was closed, but not sealed in a plastic container, so water was in contact with the speaker, as a consequence of which the CS had an electrical component. The electrical field produced was 1mV/ cm. The speaker was placed approximately 1.350 m away from where the food was presented. The auditory component could be isolated and presented separately as the auditory CS, by sealing the speaker completely in a watertight container. Like-wise an

electrical CS could be presented alone by removing the tape and turning down the volume.

To record shark behaviour during the experiment, a camera was positioned against a round window, 0.550 m in diameter, on the side of the tank. To prevent distraction of extraneous stimuli, all the windows on the same level were covered with black plastic during the experiment. The position of the conditioned stimuli, feeding rod and camera during training is shown in Fig. 2.1a and 2.1b.

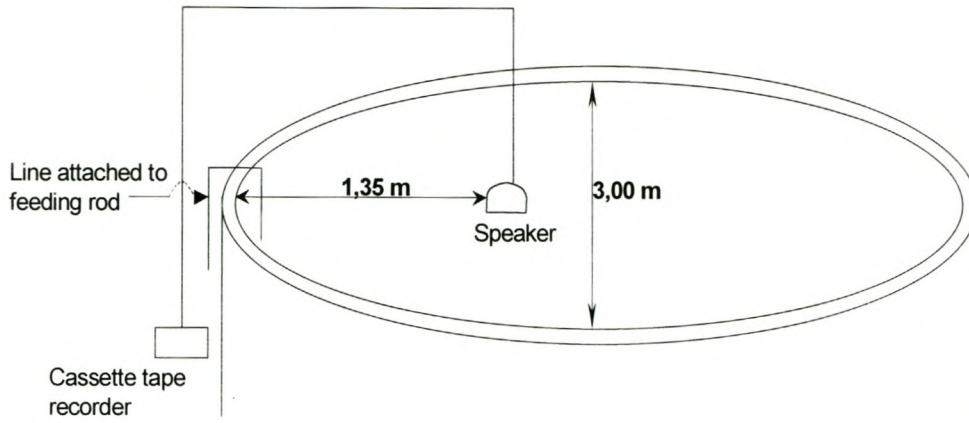


Figure 2.1a) Diagram of side-view of experimental tank with position of conditioned stimulus and feeding rod during training, indicated.

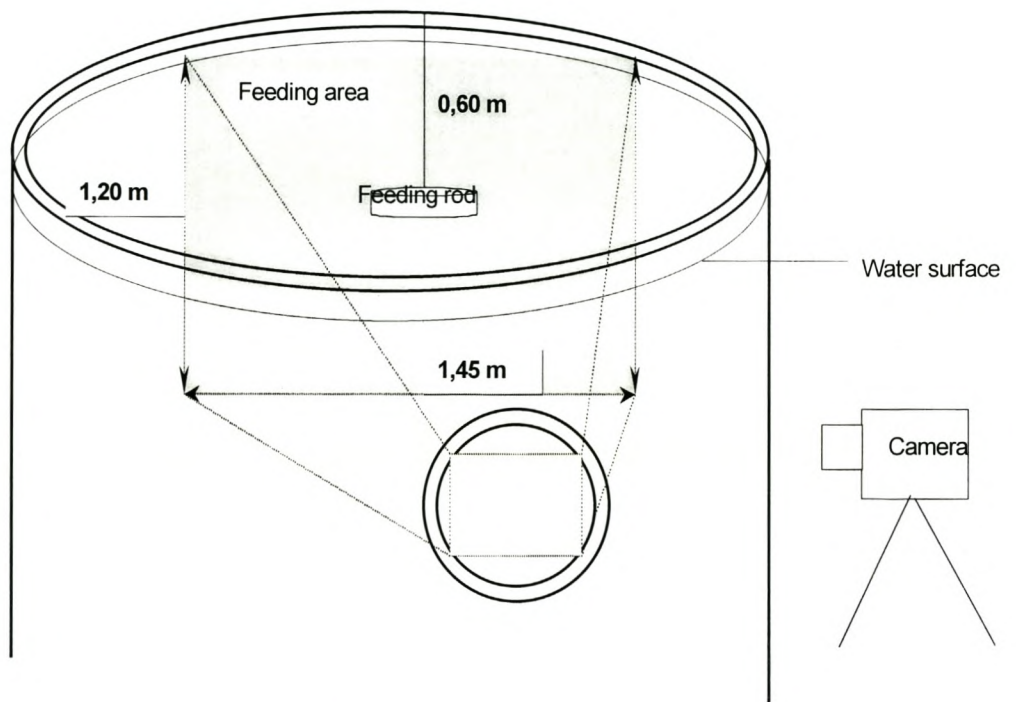


Figure 2.1 b) Diagram of side-view of experimental tank showing position of camera, feeding area and feeding rod.

2.3.3 Experimental procedure

A CS control experiment was done to determine if the responses observed during the conditioning experiments were due to the inherent attractiveness of the CS. The aim of the CS experiment was to condition sharks to associate a compound auditory and electrical CS with the presentation of food (US). In the 1st replicate it was determined how long the CR would be retained, and how long it would take to extinguish the CR.

Training was done in a group-situation in the home-tank. At least one hour prior to the first trial, the other fish in the tank were fed small scraps of sardine. These pieces of food were too small for the sharks to eat. The speaker was also placed in the tank and the windows covered.

During control trials in the CS control, the stimulus used as CS in the CS experiments was presented for 15 s. Shark behaviour was then recorded for 3 min. The inter-trial period was 10 min. These trials were repeated 10 times on a training day.

In the CS experiments, a conditioning trial consisted of the presentation of a CS for 15 s, followed by the presentation of the food (US) on the feeding rod in the feeding area. The feeding area was defined as the area in front of the opposite wall of the tank from the camera, and comprised a square of approximately 1.450 m x 1.200 m around the food.

The CS was then presented for a further 5 s while the food was in the water. The sharks were allowed 2 – 3 min to feed, after which the uneaten food was removed from the water. This was followed by an inter-trial period of approximately 10 min. Trials were

repeated until each shark was reinforced, i.e. received a food-item or a piece of food-item, three times a day.

The CR of the sharks to the CS was their swimming towards the feeding area and circling of the area where food would be given. The latency time was recorded by the camera and was defined as the time elapsed from the initiation of the compound CS until the CR was displayed.

Test and extinction trials in the CS experiments were identical to conditioning trials, except that food was not presented after the presentation of the CS. Shark behaviour was also recorded for 3 min after the presentation of the CS. Ten trials were performed each day. At least an hour after the last test or extinction trial, the sharks were fed by throwing sardine pieces into the water.

2.3.3.1 CS control:

Control trials were conducted from 28/03/01 to 05/04/01 with one female B_c and three males R_c , RY_c and Y_c on every second day until sharks had received five days of training.

2.3.3.2 CS experiments:

a) 1st *replicate* - Trials were conducted as above from 15/05/00 to 25/08/00 with the male Y_1 and three females P_1 , RY_1 and YP_1 on every second day for three days a week. The experiment consisted of an initial training phase and a second training period followed by a test and extinction. Initial training started with a pre-training day (labelled pre-train). On this day the same procedure was followed as with the conditioning trials,

but the CS was not presented, in other words, this day served as a control. This was followed by 5 days of conditioning training. The first day of training is abbreviated as condit_1 , the second day of training as condit_2 , and so forth. Due to poor water visibility, conditioning had to be terminated for eight weeks at that point. Training was then resumed again for another 5 days of conditioning as before (2^{nd} train).

This was followed by the test and extinction of the CR. The test (ext_1) consisted of 10 trials with the compound CS to test if the sharks were fully conditioned. If conditioning occurred, the CR should be maintained during the test. Two days later this was followed by nine days of extinction (ext_2 to ext_{10}), with ten trials on each day. If conditioning did take place, the CR would gradually disappear, but it would not extinguish completely.

b) 2nd replicate - The trials were conducted as in the first replicate from 22/01/01 to 16/02/01 with four female sharks, Y_2 , RY_2 , RYR_2 and B_2 , on every second day. The experiment consisted of an initial training phase and a test phase. Initial training again began with a pre-training day (pre-train), followed by six days (condit_1 to condit_6) of conditioning with the compound CS.

This was followed by the test phase, which began with one day of test trials with the compound CS (ext_1) to test if conditioning occurred. To reinforce the CR, this was followed by one day of conditioning with the compound CS (re- condit_1). Two days later, the sharks were tested with the separate components of the compound auditory and electrical CS. This was followed by one day of conditioning with the compound CS (re- condit_2) to again reinforce the CR. After another two days, the sharks were again tested with the separate components of the compound CS.

2.3.4 Analyses

The first replicate data was analysed separately from the second replicate data. The mean latency time for each day of training (daily mean latency time) was used to plot a learning curve for each shark. The mean of the three best responses on a day was also used to plot a learning curve (best daily mean latency time). The difference in performance of a particular shark over days of training was analysed statistically by Kruskal-Wallis analyses of variance by ranks (Kruskal-Wallis ANOVA's) (Siegel 1956). The source of variance was further analysed with Wilcoxon Mann-Whitney tests, except with the daily mean latency data. For this, Kolmogorov Smirnov two-sample tests were used, as this test is more accurate with small sample sizes than the Wilcoxon Mann-Whitney test (Siegel 1956). For each training day, $n \leq 3$; as a result, probabilities could only be calculated to a significance level of $\alpha \leq 0.100$ (see Table L₁₁ in Siegel & Castellan 1988). Consequently a p -value < 0.1 was taken as significant in this particular case.

To compare the performance of different sharks and under different experimental conditions, the mean latency time of each shark was standardised by the following calculation:

$$c = a / b$$

where c = latency on trial x of training as fraction of daily mean latency on day 1 of training; a = latency time on trial x and b = daily mean latency time on first day of training. The c -values averaged over every 10 trials were used to calculate the differences in performance between sharks in a particular experiment with Friedman one way analyses of variance (Friedman ANOVA's) and Wilcoxon matched pairs tests (Siegel 1956). The c -values for all the sharks in a particular experimental condition was pooled and used to compare performance under different experimental conditions.

These differences were calculated with Kruskal-Wallis ANOVA's and Wilcoxon Mann-Whitney tests. Differences in performance were termed significant at the 95 % confidence limit. All statistical analyses were done on the computer program, STATISTICA.

2. 4 Results

2.4.1 CS control:

All the sharks eventually entered the feeding area after the presentation of the stimulus, but none of the sharks in the control group significantly increased or decreased their response time to the CS (Fig. 2.2 – 2.5). Kruskal-Wallis one-way analyses of variance by ranks (Kruskal-Wallis ANOVA) found no significant differences in the daily mean latencies for any of the sharks over the training period (B_c : $H = 4.123$, $p = 0.390$; R_c : $H = 3.543$, $p = 0.315$; RY_c : $H = 5.353$, $p = 0.148$; Y_c : $H = 4.417$, $p = 0.353$). Thus, these sharks did not learn anything about the CS.

The best daily mean latency data also supports this finding. Statistical analyses of two of the sharks, (B_c and Y_c) demonstrated no significant differences during the control (Kruskal-Wallis ANOVA - B_c : $H = 2.196$, $p = 0.700$; Y_c : $H = 6.974$, $p = 0.137$). A slight upward trend was found for two of the sharks, R_c and RY_c (Fig. 2.3, 2.4) over the five training days (Kruskal-Wallis ANOVA's - R_c : $H = 6.643$, $p = 0.084$; RY_c : $H = 8.436$, $p = 0.038$). For R_c , some of this variance in performance was due to difference in performance between day 2 and day 4 (Kolmogorov-Smirnov: $D = 1$, $p < 0.100$) of training and for RY_c between day 4 and day 2 (Kolmogorov-Smirnov: $D = 1$, $p < 0.100$)

and day 3 (Kolmogorov-Smirnov: $D = 1$, $p < 0.100$). Therefore habituation to the CS may have begun to develop in these two sharks. These two sharks also did not enter the

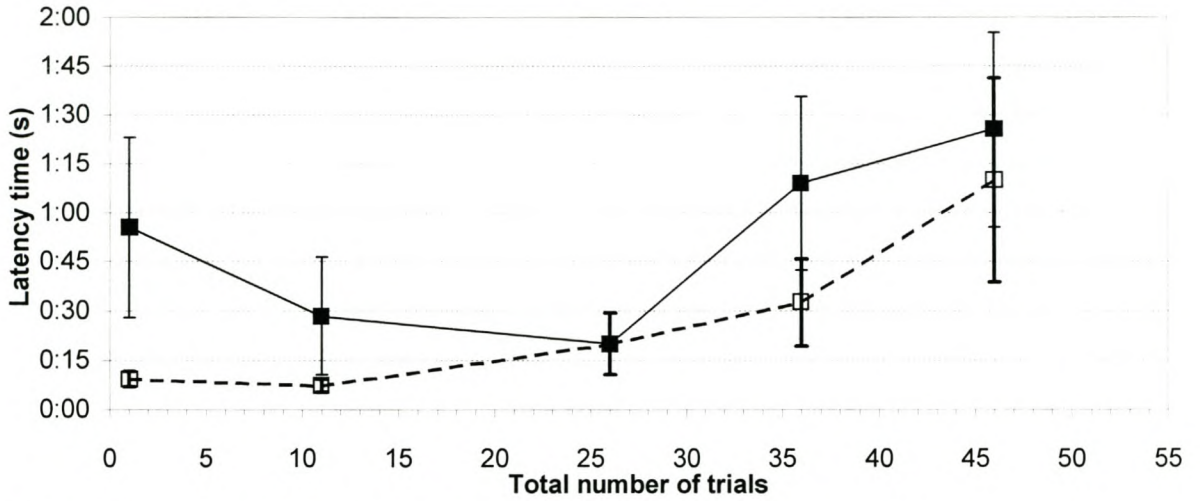


Figure 2.2. Daily and best daily mean latency times of compound CS control of male *M. mustelus*, B_c .

feeding area on the 1st day of testing (Fig. 2.3, 2.4).

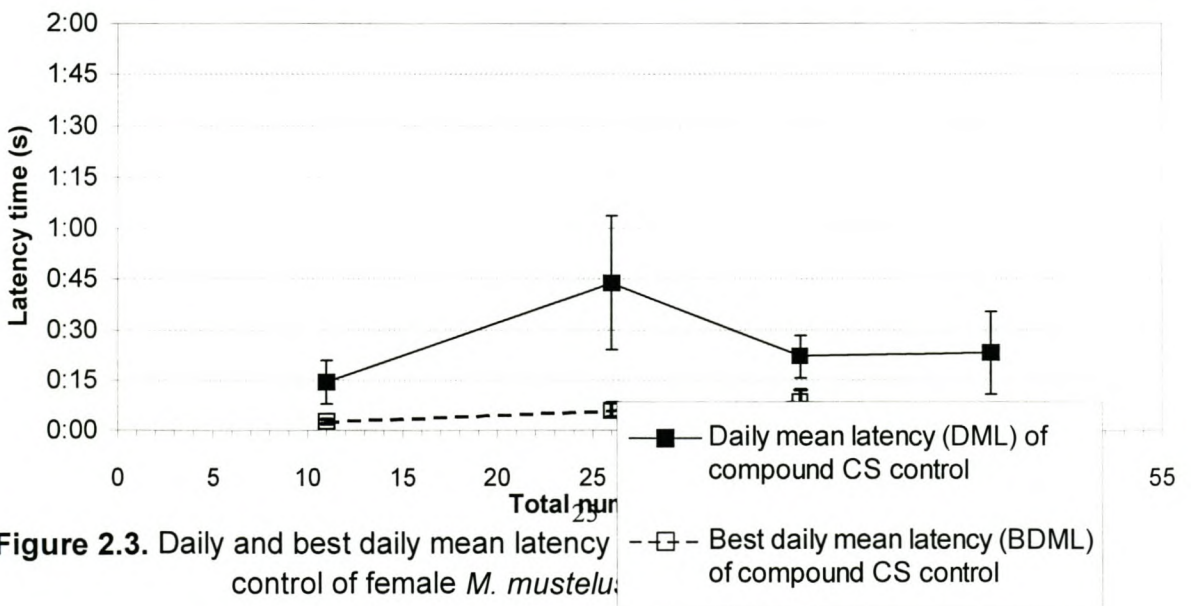


Figure 2.3. Daily and best daily mean latency times of compound CS control of female *M. mustelus*.

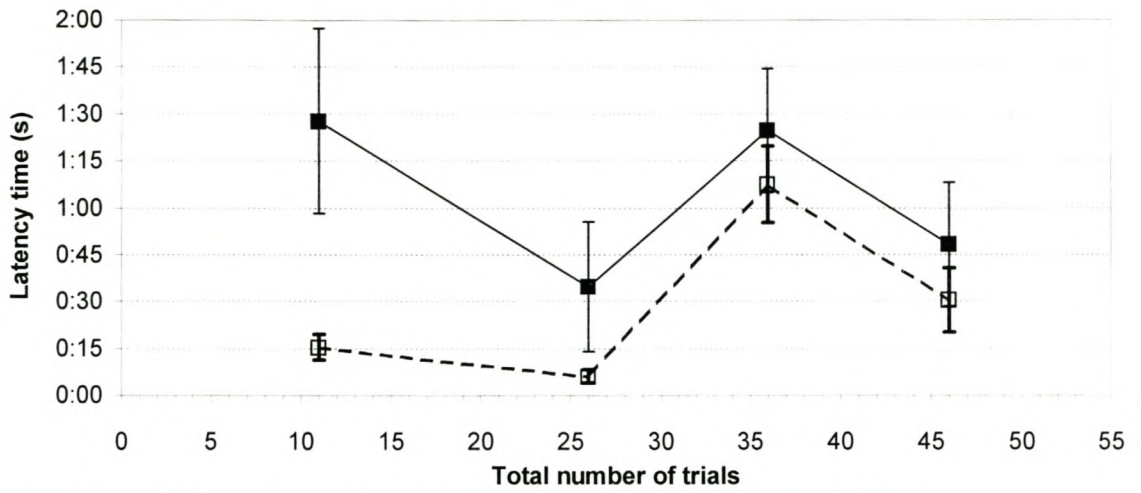


Figure 2.4. Daily and best daily mean latency times of compound CS control of female *M. mustelus*, RY_c .

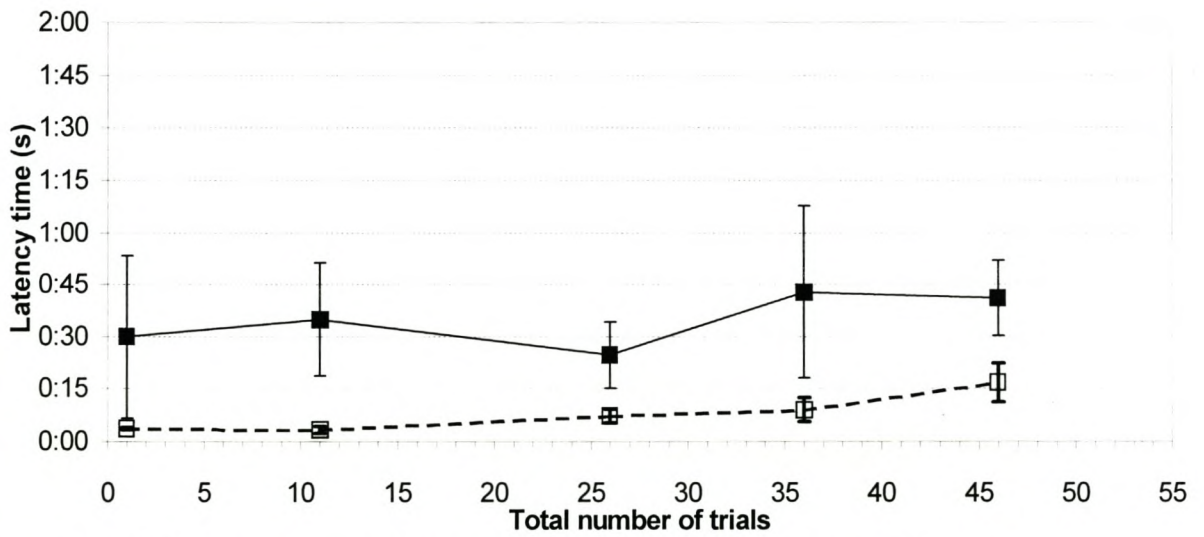
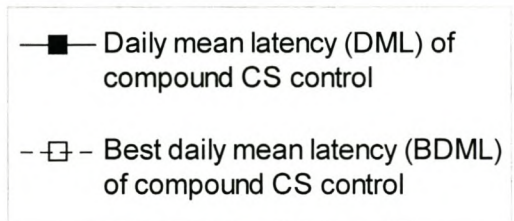


Figure 2.5. Daily and best daily mean latency times of compound CS control of female *M. mustelus*, Y_c .



In comparison with the shark behaviour in the CS experiments, individual shark behaviour during the first 50 trials was compared. A Friedman ANOVA found no significant differences among the *c*-values averaged over 10 trials of individual sharks in the CS control during the 10th to 50th trial ($F = 5.013$, $p = 0.286$, $n = 4$). Therefore, the combined *c*-values of all the sharks in the control were used for the comparison of shark behaviour under different experimental conditions.

2.4.2 *CS experiment, replicate 1:*

a) *Initial training* - The performance of sharks during initial training and the 2nd training period is illustrated in Fig. 2.6a to 2.9a. All the sharks displayed conditioned responses after the presentation of the CS. The CR consisted of entering of the feeding area and vertical circling of the area where the food was or would be presented. The daily mean latency time of all of these sharks decreased as training progressed, but this decrease was only significant for two of the sharks (P_1 and Y_1) after the fifth day of conditioning (Kruskal-Wallis ANOVA's - P_1 : $H = 12.306$, $p = 0.031$; Y_1 : $H = 13.322$, $p = 0.021$) (Fig. 2.6a, 2.9a). This variance was due to differences in performance on *condit*₁ (day with the highest daily mean latency) and *condit*₃ (Wilcoxon Mann-Whitney test: $U = 19$, $p = 0.012$) and *condit*₅ (Wilcoxon Mann-Whitney test: $U = 35$, $p = 0.019$) for P_1 and between pre-train and all the other days of training (Wilcoxon Mann-Whitney tests, significance level: $\alpha < 0.05$) for Y_1 . The best daily mean latencies also show a downward trend for these two sharks (P_1 and Y_1), but are significant only for P_1 (Kruskal-Wallis ANOVA's - P_1 : $H = 12.710$, $p = 0.026$; Y_1 : $H = 8.856$, $p = 0.115$). The variance in the performance of P_1 was due to differences between pre-train and *condit*₃ (Kolmogorov-Smirnov: $D = 1$, $p < 0.100$), *condit*₄ (Kolmogorov-Smirnov: $D = 1$, $p < 0.100$) and *condit*₅ (Kolmogorov-Smirnov: $D = 1$, $p < 0.100$). In addition, the best daily mean latency was

under 15 s from condit₁, for both the sharks. These sharks entered the feeding area after the presentation of the CS, but even before the presentation of food (US).

Therefore, two of the four sharks (P₁ and Y₁) associated the presentation of food with the CS after five days of conditioning.

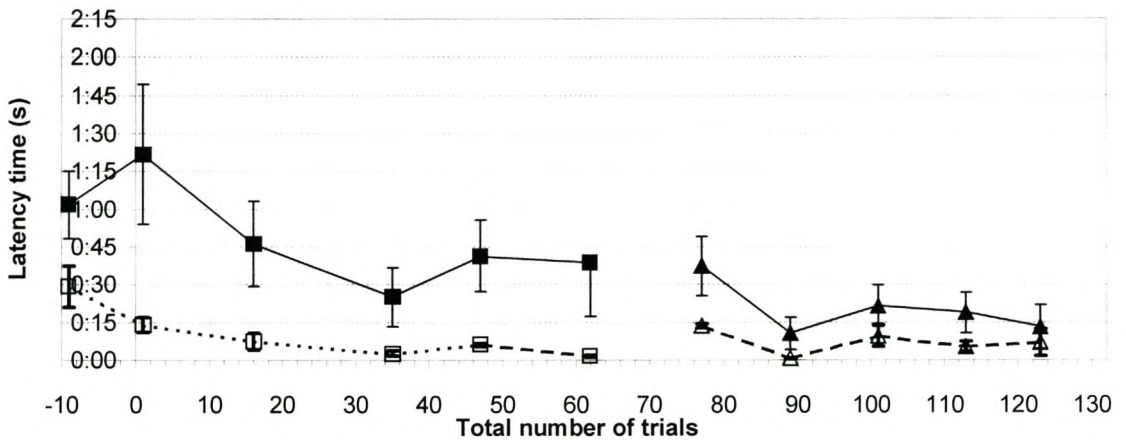
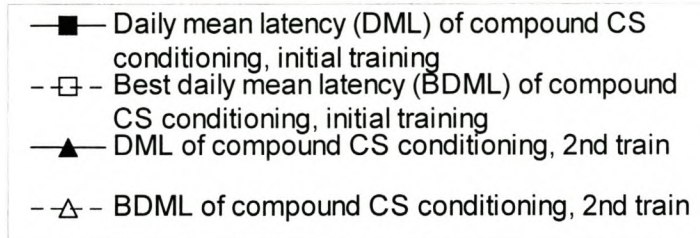


Figure 2.6 a) Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, P₁ (1st replicate).



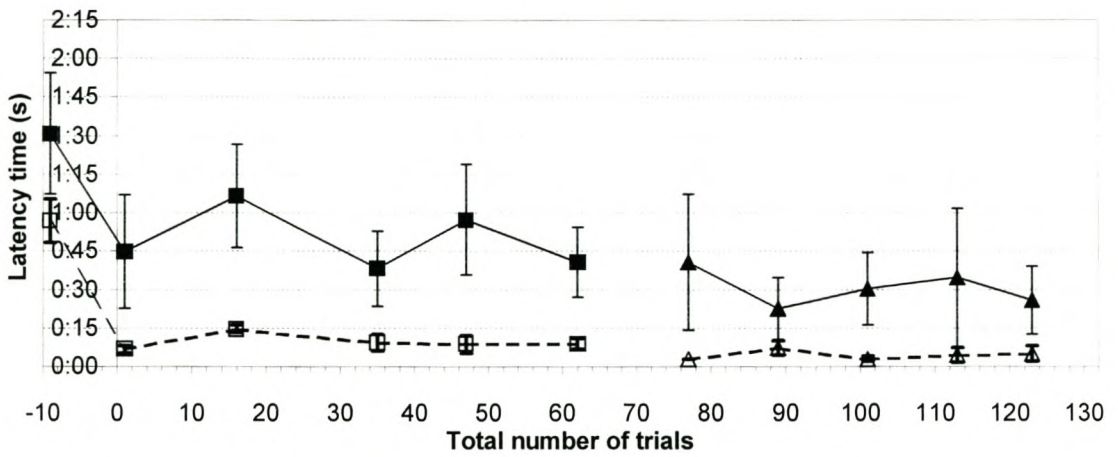


Figure 2.7 a) Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, RY₁ (1st replicate).

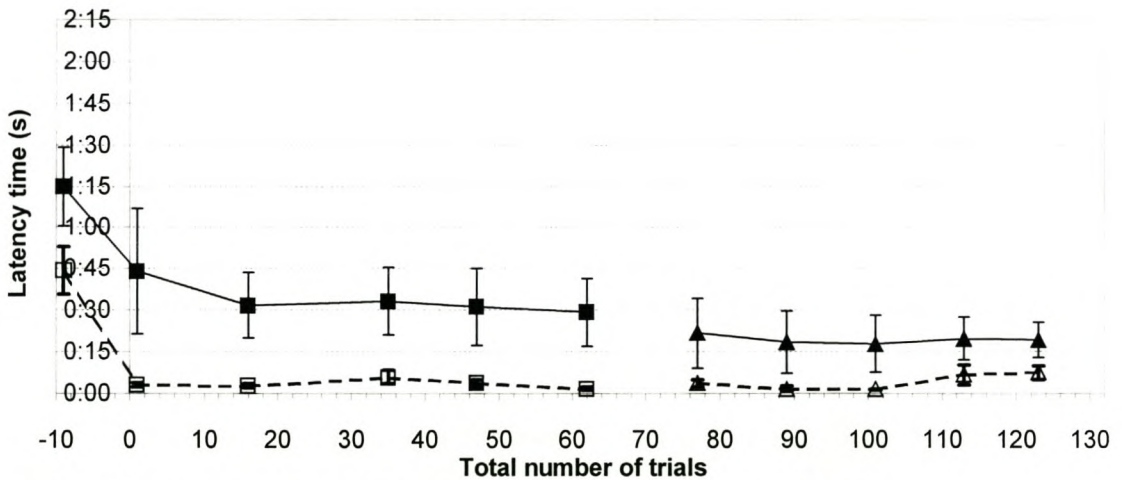


Figure 2.8 a) Daily and best daily mean latency times of compound CS conditioning of male *M. mustelus*, Y₁ (1st replicate).

- Daily mean latency (DML) of compound CS conditioning, initial training
- □ - Best daily mean latency (BDML) of compound CS conditioning, initial training
- ▲— DML of compound CS conditioning, 2nd train
- △ - BDML of compound CS conditioning, 2nd train

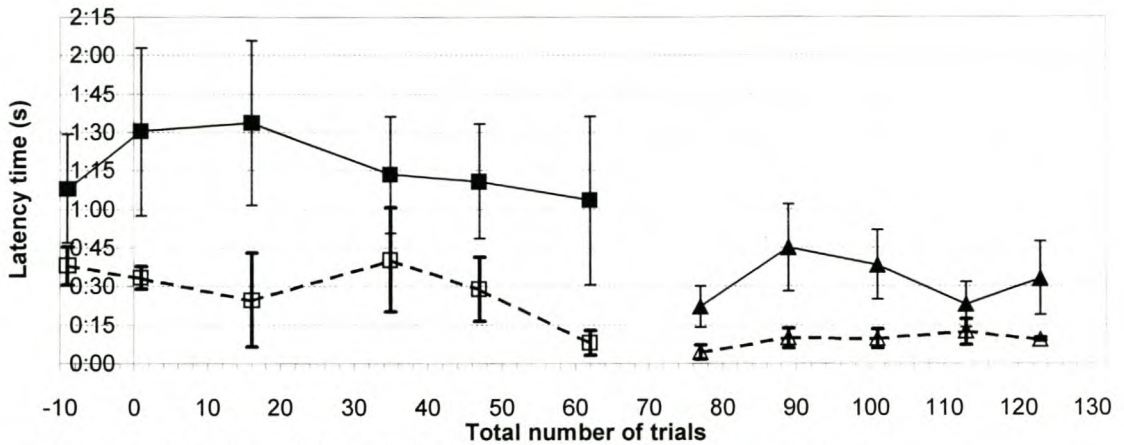
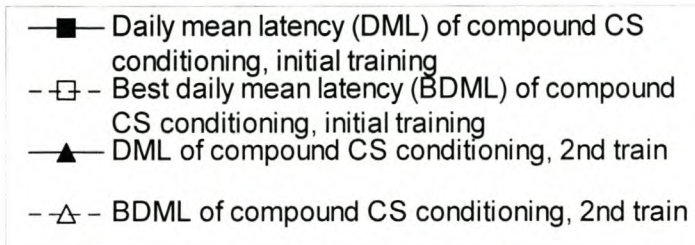


Figure 2.9 a) Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, YP₁ (1st replicate).



Conditioning was less clearly shown for the other two sharks, RY₁ and YP₁. The downward trend in daily mean latencies of RY₁ was marginally significant (Kruskal-Wallis ANOVA: $H = 10.057$, $p = 0.074$) after initial training (Fig. 2.7a). However the association between the presentation of the CS and food (US) did begin to develop as the best daily mean latencies exhibited a downward trend (Kruskal-Wallis ANOVA: $H = 10.712$, $p = 0.057$) and was under 15 s from $condit_1$. YP₁ also began to show a slight downward trend from $condit_3$ in both the daily (Kruskal-Wallis ANOVA: $H = 2.173$, $p = 0.825$) and the best daily mean latencies (Kruskal-Wallis ANOVA: $H = 3.780$, $p = 0.581$), but this was not significant (Fig. 2.9a). The best daily mean latency was under 15 s only on $condit_5$, indicating that the shark, YP₁, began entering the feeding area only before the presentation of food (US), on $condit_5$.

For the comparison of shark behaviour in this replicate to the CS control, the *c*-values averaged over 10 trials of individual sharks during the first 50 exposures to the compound CS, was compared. A Friedman ANOVA demonstrated significant variance among the behaviour of the four sharks during the 10th to the 50th exposure to the compound CS ($F = 10.2$, $p = 0.017$, $n = 4$). Wilcoxon matched pairs tests found differences in behaviour between the shark P_1 and all the other sharks, except YP_1 ($Z = 1.826$, $p = 0.068$); between RY_1 and all the other sharks, except Y_1 ($Z = 1.826$, $p = 0.068$); and between Y_1 and YP_1 ($Z = 1.826$, $p = 0.068$). Only Y_1 and RY_1 showed any conditioning on $condit_3$ (after 50 exposures to CS). Accordingly, only the combined *c*-values of the sharks, RY_1 and Y_1 were used in the comparison of shark behaviour with the CS control.

b) Second training period - After the two-month rest period, all the sharks demonstrated the CR. Sharks entered the feeding area after the presentation of the CS, but even before the US. Conditioning could be demonstrated in the second training period for the two sharks (RY_1 and YP_1), which did not show conditioning clearly after initial training. The downward trend of daily mean latencies (Fig. 2.7a) over pre-train, initial training and $condit_1$ and $condit_2$ of 2nd train was significant for RY_1 (Kruskal-Wallis ANOVA: $H = 15.385$, $p = 0.031$). Similarly the downward trend (Fig. 2.9a) over pre-train, initial training and $condit_1$ of 2nd train was significant for YP_1 (Kruskal-Wallis ANOVA: $H = 13.046$, $p = 0.042$).

All the sharks were at the same level of performance on $condit_1$ of 2nd train than they were before the two-month rest period (Fig. 2.6a – 2.9a). For three of the four sharks (P_1 , RY_1 and Y_1), a difference in daily mean latencies was found over pre-train and 2nd

train (Kruskal-Wallis ANOVA's - P₁: H = 27.047, p < 0.001; RY₁: H = 21.844, p = 0.009; Y₁: H = 20.396, p = 0.001), but no difference was found over the last day of initial training and 2nd train (Kruskal-Wallis ANOVA's - P₁: H = 10.194, p = 0.070; RY₁: H = 5.750, p = 0.331; Y₁: H = 4.539, p = 0.475; YP₁: H = 7.378, p = 0.194). Wilcoxon Mann-Whitney tests indicated the variance was due to differences between pre-train and condit₂ (U = 4, p < 0.001); condit₃ (U = 12.5, p = 0.002), condit₄ (U = 7, p = 0.001) and condit₅ (U = 7, p < 0.001) of 2nd train for P₁; and between pre-train and condit₂ (U = 4, p = 0.008), condit₃ (U = 8.5, p = 0.010), condit₄ (U = 7, p = 0.011) and condit₅ (U = 5, p = 0.013) of 2nd train for RY₁; and between pre-train and all the days of 2nd train for Y₁. Although only a marginally significant difference was found between pre-train and 2nd train for the shark, YP₁ (Kruskal-Wallis ANOVA: H = 16.469, p = 0.063), Wilcoxon Mann-Whitney tests found significant differences in performance between pre-train and condit₁ (U = 6, p = 0.009) and condit₄ (U = 13.5, p = 0.014) of 2nd train. Thus, all the sharks responded significantly faster to the CS during 2nd train than at the beginning of initial training and the CR was maintained at the same level as before the rest period.

The best daily mean latencies also support this finding. Kruskal-Wallis ANOVA's found significant differences between pre-train and 2nd train for Y₁ (H = 11.185, p = 0.048) and no significant differences between the last day of initial training and 2nd train for all the sharks (P₁: H = 9.480, p = 0.091; RY₁: H = 8.557, p = 0.128; Y₁: H = 6.925, p = 0.226; YP₁: H = 4.364, p = 0.498). The variance in Y₁ was due to difference in performance between pre-train and all the days of 2nd train (Kolmogorov-Smirnov: D = 1, p < 0.100). Even though no variance was found between pre-train and

2nd train for the three sharks, P₁, RY₁ and YP₁; Kolmogorov-Smirnov tests found a significant difference between pre-train and all the days of 2nd train for RY₁ (D = 1, p < 0.100) and YP₁ (D = 1, p < 0.100). For the shark, P₁, Kolmogorov-Smirnov tests found significant differences between pre-train and condit₂ (D = 1, p < 0.100) and condit₄ (D = 1, p < 0.100) of 2nd train. In addition, the best daily mean latencies of all the sharks stayed under 15 s throughout 2nd train. Thus, all the sharks anticipated the presentation of food to follow the CS.

Performance during 2nd train stayed fairly constant. Kruskal-Wallis ANOVA's on the daily mean latencies of three of the sharks (RY₁, Y₁ and YP₁) (RY₁: H = 1.462, p = 0.833; Y₁: H = 3.823, p = 0.431; YP₁: H = 6.883, p = 0.142) found no significant differences. Variance was found in the performance of the shark, P₁ (Kruskal-Wallis ANOVA: H = 11.273, p = 0.024) that was due to differences between performance on condit₁ and condit₂ (Wilcoxon Mann-Whitney test: U = 21.5, p = 0.009) and condit₅ (Wilcoxon Mann-Whitney test: U = 25, p = 0.021) of 2nd train (Fig. 2.6a). No variance was found in the best daily mean data of any of the sharks (Kruskal-Wallis ANOVA's - P₁: H = 7.399, p = 0.116; RY₁: H = 5.229, p = 0.265; Y₁: H = 5.720, p = 0.221; YP₁: H = 10.548, p = 0.308). Thus, sharks reached their individual threshold latency and it is unlikely that further training would have improved their performance. However further training would have slowed down the rate of extinction as was found by Breuning and Wolach (1981) with goldfish.

c) *Extinction* - Fig. 2.6b to 2.9b illustrate the performance of individual sharks during extinction testing. The data from ext₁ provides further evidence for the conditioning of the

sharks. On the first day of extinction trials all the sharks continued to enter the feeding area after the presentation of the CS, even though the US (food) was not presented. No differences were found for any of the sharks in the daily (Wilcoxon Mann-Whitney tests – P_1 : $U = 15.5$, $p = 0.118$; RY_1 : $H = 28$, $p = 0.315$; Y_1 : $H = 40$, $p = 1.035$; YP_1 : $H = 18$, $p = 0.606$) and best daily mean latencies (Kolmogorov-Smirnov tests: P_1 : $D = -0.333$, $p > 0.100$; RY_1 : $D = -0.333$, $p > 0.100$; Y_1 : $D = -0.667$, $p > 0.100$; YP_1 : $D = 0.667$, $p > 0.100$) between the last day of conditioning (condit₅ of 2nd train) and ext₁.

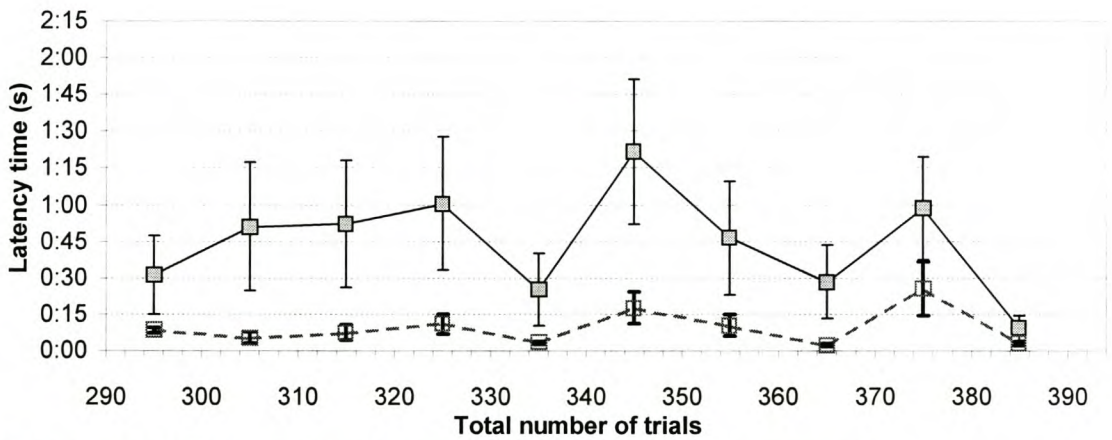
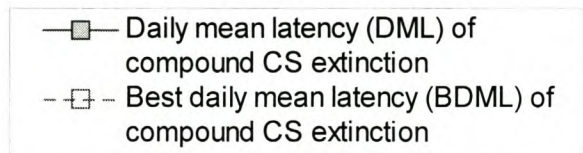


Figure 2.6 b) Daily and best daily mean latency times of compound CS extinction training of female *M. mustelus*, P_1 (1st replicate).



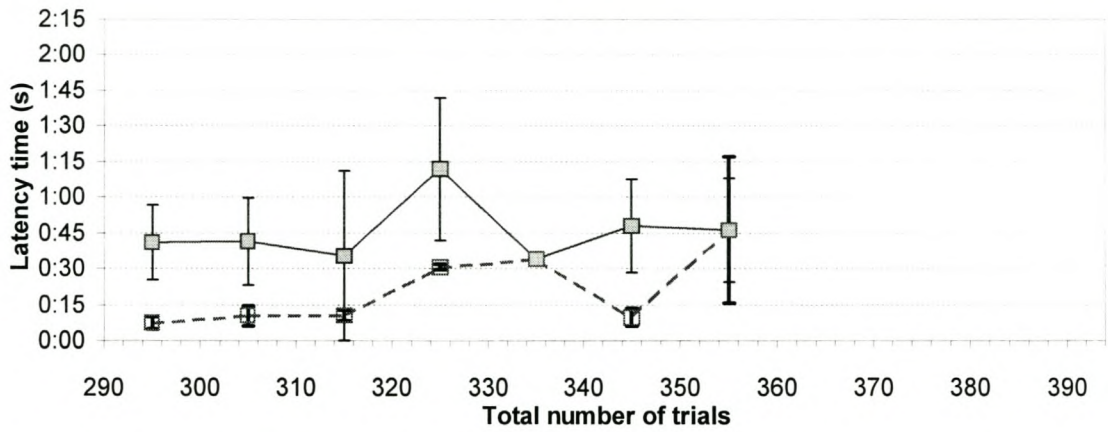


Figure 2.7 b) Daily and best daily mean latency times of compound CS extinction training of female *M. mustelus*, RY_1 (1st replicate).

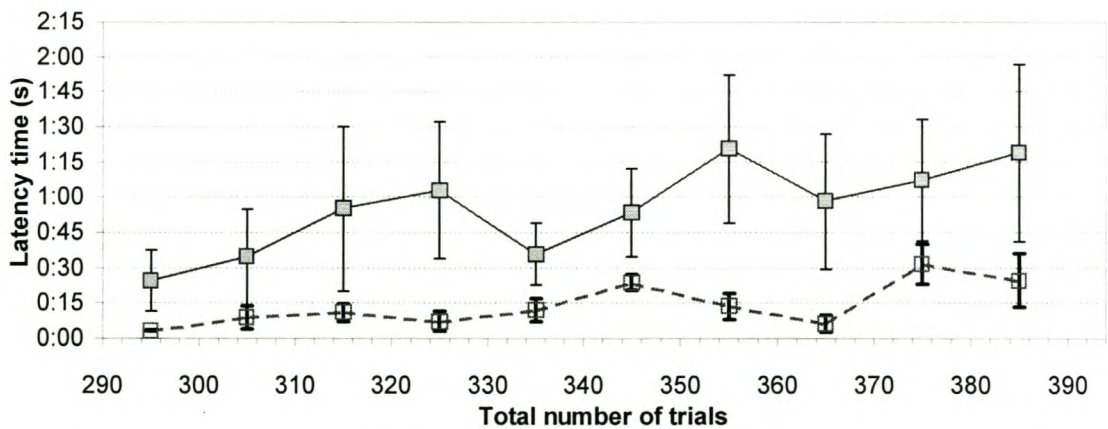
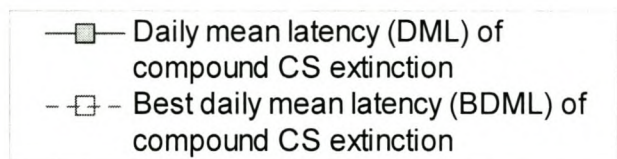


Figure 2.8 b) Daily and best daily mean latency times of compound CS extinction training of male *M. mustelus*, Y_1 (1st replicate).



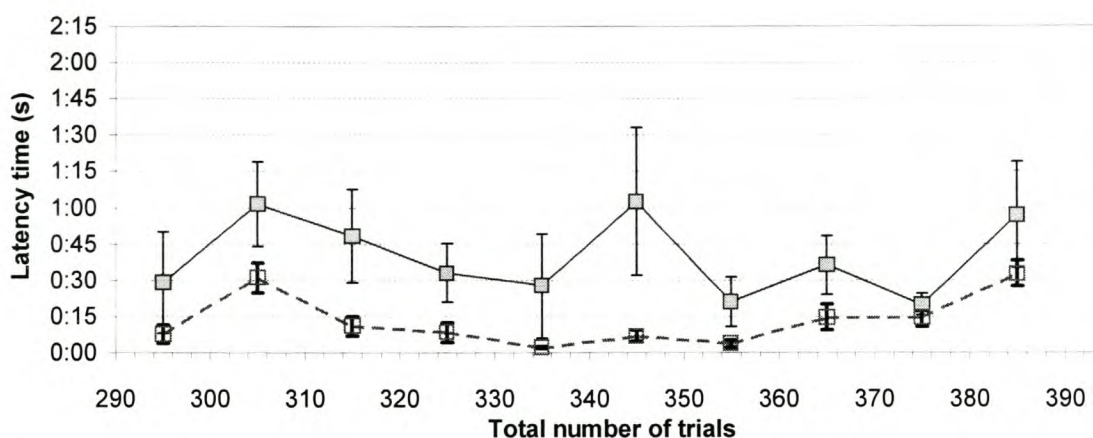
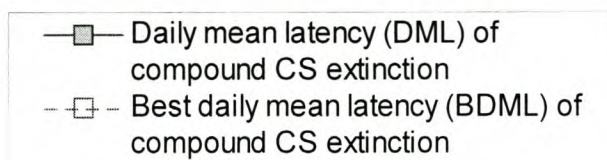


Figure 2.9 b) Daily and best daily mean latency times of compound CS extinction training of female *M. mustelus*, YP₁ (1st replicate).



Performance varied greatly during extinction. Sharks responded badly to the CS on some days, but the CR could be maintained at the same level than at the end of conditioning training, on subsequent days. The daily mean latencies of the shark, P₁, (Fig. 2.6b) were maintained at the same level on ext₁, ext₅, ext₇, ext₈ and ext₁₀, than on condit₅ of 2nd train (Wilcoxon Mann-Whitney tests - ext₂: U = 20.5, p = 0.043; ext₃: U = 20.5, p = 0.023; ext₄: U = 13.5, p = 0.039; ext₆: U = 10, p = 0.002; ext₉: U = 8.5, p = 0.016). The shark, RY₁ died after ext₇, but throughout extinction, except on ext₄, the CR was maintained at the same level than on condit₅ of 2nd train (Wilcoxon Mann-Whitney test on daily mean latencies- ext₄: U = 6, p = 0.046) (Fig. 2.7b). The shark, Y₁ maintained conditioned responses at the same level as on the last day of conditioning (condit₅ of 2nd train) on ext₁ through to ext₅ and again on ext₈ and ext₁₀ (Fig. 2.8b). Wilcoxon Mann-Whitney tests on the daily mean latencies found significant differences

between performance on condit_5 of 2nd train and ext_6 ($U = 9$, $p = 0.022$), ext_7 ($U = 17$, $p = 0.022$) and ext_9 ($U = 7$, $p = 0.028$). This is reflected in the best daily mean latency data. Kolmogorov-Smirnov tests found significant differences between performance on condit_5 of 2nd train and ext_6 ($D = -1$, $p < 0.100$) and ext_9 ($D = -1$, $p < 0.100$). The shark, YP₁ (Fig. 2.9b) maintained daily mean latencies at the same level throughout extinction (Wilcoxon Mann-Whitney tests, significance level: $\alpha < 0.05$). However, the best daily mean latencies were significantly higher on ext_2 and ext_{10} than on condit_5 of 2nd train (Kolmogorov-Smirnov tests: $D = -1$, $p < 0.100$). Thus, YP₁ maintained conditioned responses at the same level as at the end of conditioning training throughout extinction training, except on ext_2 and ext_{10} .

2.4.3 CS experiment, replicate 2:

a) *Initial training* – The performance during conditioning and extinction training of individual sharks is shown in Fig. 2.10 to 2.13. As in the first replicate, all the sharks entered the feeding area and circled the area where food was or would be presented, after the presentation of the CS. However only two of the four sharks, B₂ and RYR₂ (Fig. 2.10, 2.12) were conditioned after six days of conditioning training. This could be seen in the downward trend of the daily mean latencies (Kruskal-Wallis ANOVA's - B₂: $H = 14.937$, $p = 0.021$; RYR₂: $H = 16.709$, $p = 0.010$). For B₂, Wilcoxon Mann-Whitney tests demonstrated some of the variance was due to differences between pre-train and condit_6 ($U = 8.5$, $p < 0.001$); and for RYR₂, the variance was due to differences between pre-train and condit_4 ($U = 40$, $p = 0.040$), condit_5 ($U = 13$, $p < 0.001$) and condit_6 ($U = 21.5$, $p = 0.005$). The best daily mean latencies also show a downward trend for these two sharks, being marginally significant for B₂ and significant for RYR₂

(Kruskal-Wallis ANOVA's - B_2 : $H = 12.190$, $p = 0.058$; R_{YR_2} : $H = 21.704$, $p = 0.048$). In addition, the best daily mean latency stayed under 15 s from $condit_1$ for both of the sharks. These sharks entered the feeding area before food was presented, indicating that they anticipated the presentation of food after the CS.

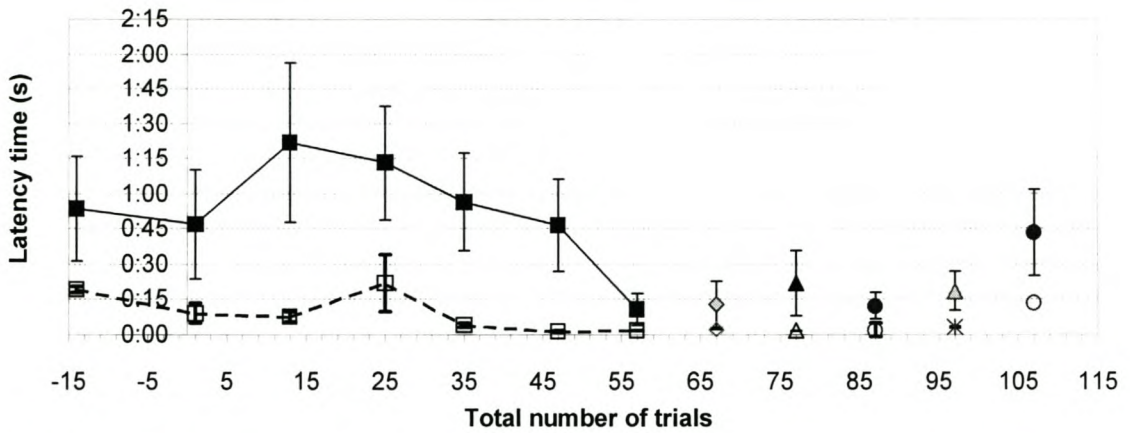
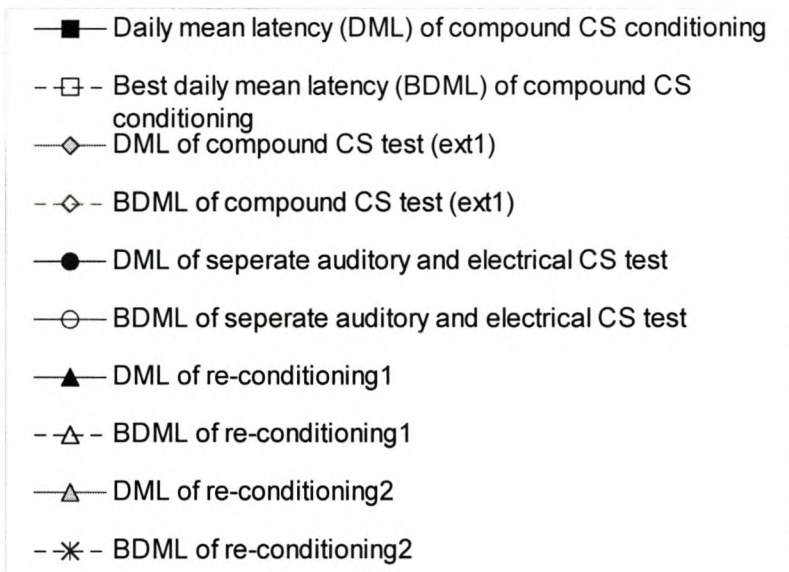


Figure 2.10. Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, B_2 (2nd replicate).



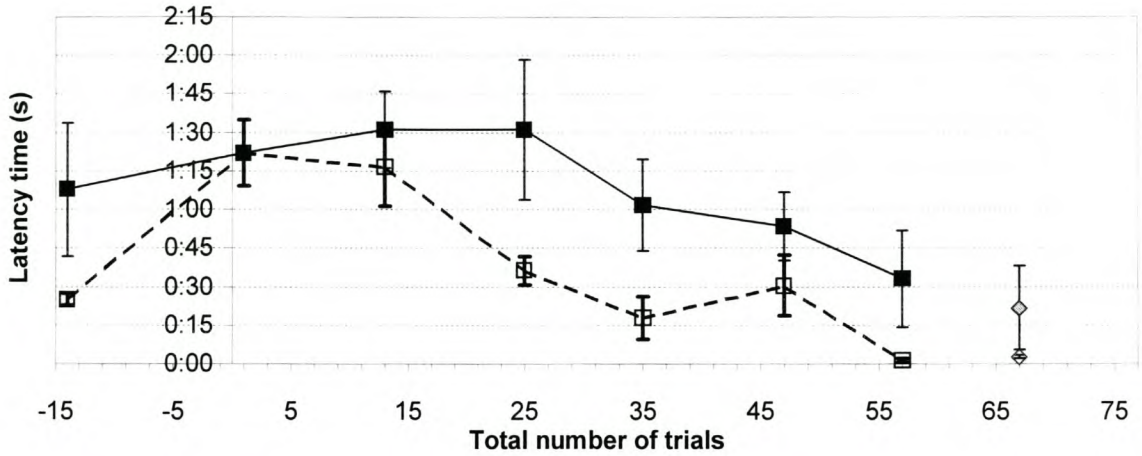


Figure 2.11. Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, RY_2 (2nd replicate).

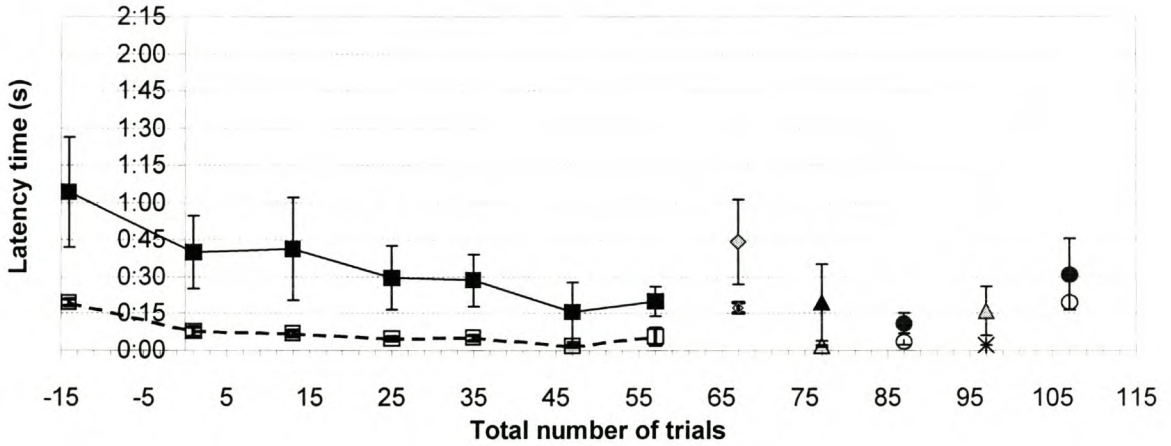
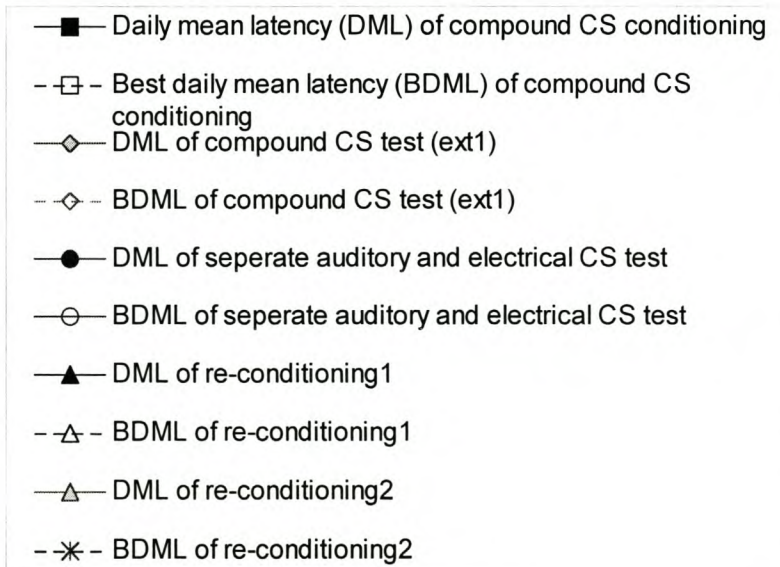


Figure 2.12. Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, RYR_2 (2nd replicate).



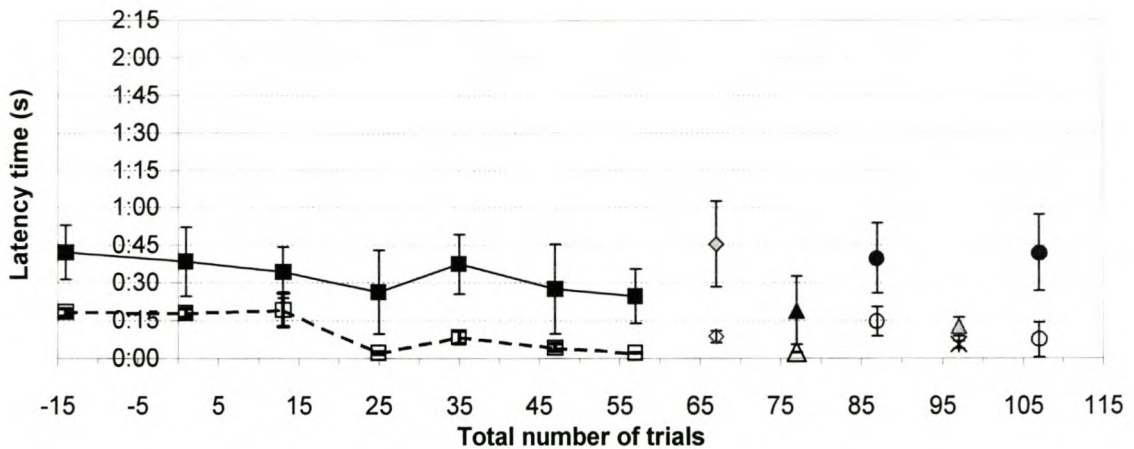
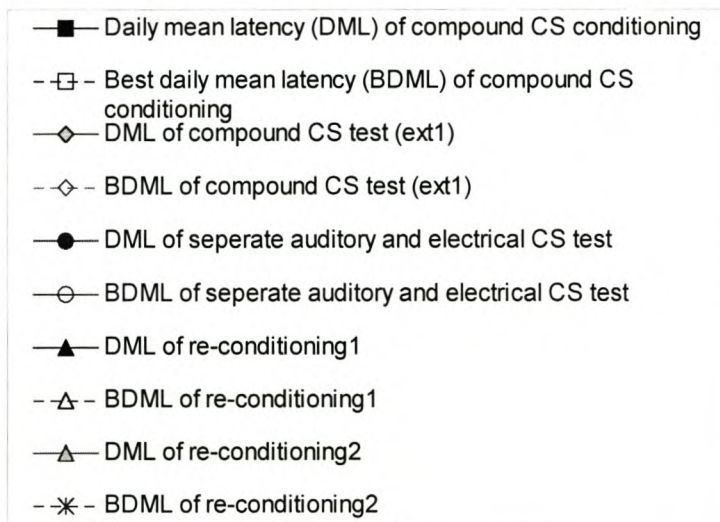


Figure 2.13. Daily and best daily mean latency times of compound CS conditioning of female *M. mustelus*, Y₂ (2nd replicate).



The sharks, RY₂ and Y₂, (Fig. 2.11, 2.13) also showed a downward trend in daily mean latencies (Kruskal-Wallis ANOVA's - RY₂: H = 10.248, p = 0.115; Y₂: H = 9.047, p = 0.171) from condit₃, but this was not significant. However an association between the CS and the presentation of food (US) did begin to develop, as the downward trend in best daily mean latencies of both sharks was significant (Kruskal-Wallis ANOVA's – RY₂: H = 15.902, p = 0.014; Y₂: H = 14.903, p = 0.021). The best daily mean latencies

of RY₂ and Y₂ were also under 15 s from condit₆ (Fig. 2.11) and condit₃ (Fig. 2.13), respectively.

For the comparison of shark behaviour in the CS experiment and control, the shark behaviour of individual sharks was compared during the first 50 exposures to the compound CS. A Friedman ANOVA found significant differences among the *c*-values averaged over every 10 trials of the sharks in this replicate ($F = 9.900$, $p = 0.019$, $n = 4$). Wilcoxon matched pairs tests found slight differences in behaviour between B₂ and all the sharks, except Y₂ ($Z = 1.826$, $p = 0.068$); between RYR₂ and all the other sharks; and between RY₂ and Y₂ ($Z = 1.826$, $p = 0.068$). Since, only RYR₂ demonstrated any conditioning on condit₄ (after 50 exposures to the compound CS), only RYR₂ was used in the comparison of shark behaviour in the CS control and experiment.

b) Extinction - On ext₁, the test day, all four sharks maintained conditioned responses on the same level as on the last day of conditioning training (condit₆). Sharks continued to enter the feeding area after the CS even though the US (food) was not presented. Wilcoxon Mann-Whitney tests on the daily mean latencies showed no significant difference between performance of all the sharks on condit₆ and ext₁ (B₂: $U = 41.5$, $p = 0.780$; RY₂: $U = 18.5$, $p = 0.220$; RYR₂: $U = 34.5$, $p = 0.888$; Y₂: $U = 31.5$, $p = 0.165$). Furthermore, the best daily mean latencies was under 15 s on ext₁ for all the sharks, indicating that sharks anticipated the presentation of food after the CS (Fig. 2.10 – 2.13).

For two of the sharks, B₂ and RY₂, (Fig. 2.10, 2.11) Wilcoxon Mann-Whitney tests showed significant differences in performance between pre-train and ext₁ (B₂: $U = 14.5$,

$p < 0.001$; RY_2 : $U = 12$, $p = 0.021$). This was reflected in the best daily mean data. Kolmogorov-Smirnov tests found no significant difference between performance on $condit_6$ and ext_1 (B_2 : $D = 0.333$, $p > 0.100$; RY_2 : $D = -0.667$, $p > 0.100$) and significant difference between pre-train and $condit_6$ (B_2 : $D = 1$, $p < 0.100$; RY_2 : $D = 1$, $p < 0.100$) for both sharks. This further confirms that these sharks were conditioned after six days of conditioning.

For two of the sharks, RYR_2 and Y_2 , (Fig. 2.12, 2.13) conditioning could only be shown after additional training. The shark, RYR_2 , (Fig. 2.12) which showed conditioning after six days of training, did not perform differently from pre-train on ext_1 (Wilcoxon Mann-Whitney test: $U = 33.5$, $p = 0.218$), but did again on re- $condit_1$ (Wilcoxon Mann-Whitney test: $U = 19.5$, $p = 0.005$) and on re- $condit_2$ (Wilcoxon Mann-Whitney test: $U = 10$, $p < 0.000$). Thus, the association between the CS and the presentation of food, may not have been strong enough on ext_1 , but was reinforced on re- $condit_1$ and re- $condit_2$. The daily mean latencies of one shark, Y_2 , (Fig. 2.13) differed only on re- $condit_1$ (Wilcoxon Mann-Whitney test: $U = 38.5$, $p = 0.041$) and re- $condit_2$ (Wilcoxon Mann-Whitney test: $U = 12.5$, $p < 0.001$) from pre-train. However, Kolmogorov-Smirnov tests found significant differences between pre-train and ext_1 ($D = 1$, $p < 0.100$), re- $condit_1$ ($D = 1$, $p < 0.100$) and re- $condit_2$ ($D = 1$, $p < 0.100$). Thus, Y_2 was conditioned after re- $condit_1$ and re- $condit_2$.

The results show all the sharks developed some association between the CS and the presentation of food; the weakest association formed by the shark, Y_2 (Fig. 2.13) and the strongest association by the shark B_2 (Fig. 2.10).

2.4.4 Comparison of CS control and CS experiment replicates:

All the sharks in the CS control (Fig. 2.2 to 2.5), two of the sharks, (RY₁ and Y₁: Fig. 2.7a, 2.8a) in the 1st replicate and one shark, RYR₂, (Fig. 2.12) in the 2nd replicate was used for the comparison of the behaviour of the sharks in these treatments. The behaviour of the sharks in the CS control, stayed relatively constant while that of the sharks in the 1st and 2nd replicate changed during the first 50 exposures to the compound CS (Fig. 2.14). The sharks in the CS experiments responded faster to the compound CS after the 50th exposure than at the beginning of training. The only significant difference in the *c*-values of sharks in the 1st and 2nd replicate of the CS experiment and control, was found during the 41st – 50th exposure to the CS (Kruskal-Wallis ANOVA's - 11th – 20th: $H = 1.029$, $p = 0.598$; 21st – 30th: $H = 3.043$, $p = 0.218$; 31st – 40th: $H = 4.281$, $p = 0.118$; 41st – 50th: $H = 14.665$, $p = 0.007$). Wilcoxon Mann Whitney tests found no significant differences during the 11th - 20th and the 21st - 30th exposure to the CS between the *c*-values of sharks in the control and in the 1st replicate (11th - 20th: $U = 38$, $p = 0.393$; 21st - 30th: $U = 30$, $p = 0.143$) and the 2nd replicate (11th - 20th: $U = 39$, $p = 0.436$; 21st - 30th: $U = 27$, $p = 0.156$). During the 31st - 40th exposures, a difference was found in behaviour between the control and the 1st replicate sharks (Wilcoxon Mann-Whitney test: $U = 23$, $p = 0.043$), but not with the 2nd replicate shark (Wilcoxon Mann-Whitney test: $U = 30$, $p = 0.143$). Wilcoxon Mann Whitney tests found significant differences during the 41st - 50th exposures to the CS between the *c*-values of sharks in the CS control and the 1st ($U = 4$, $p < 0.001$) and the 2nd replicate ($U = 9$, $p = 0.001$). No significant difference was found between the behaviour of sharks in the 1st and 2nd replicate of the CS experiment at any time of the experiment (Wilcoxon Mann Whitney tests, significance level: $\alpha < 0.05$). Clearly, shark behaviour in the 1st and 2nd

replicate was only significantly different from that of sharks in the CS control from the 31st and the 41st exposure to the CS, respectively.

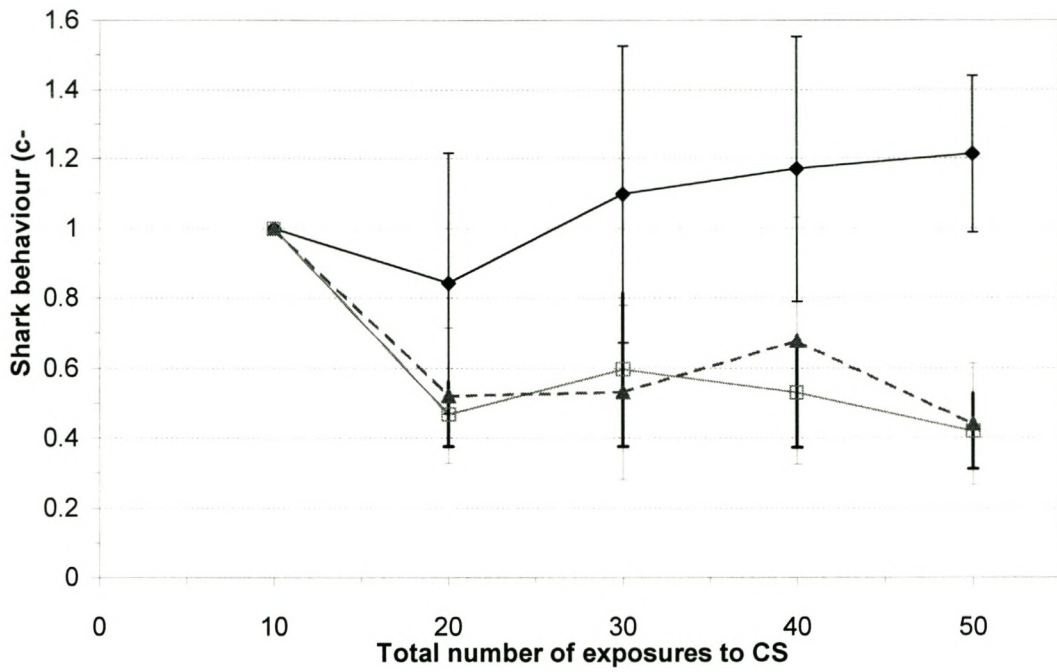


Figure 2.14. Shark behaviour during compound CS control (B_c , R_c , RY_c , YR_c) and compound CS experiment replicates (1st: RY_1 and Y_1 ; 2nd: RYR_2) expressed as c-values.



2.5 Discussion

The results show a contrast in the behaviour of sharks in the CS control and those in the CS experiment replicates. Although, two of the sharks in the CS control, R_c and RY_c (Fig. 2.3, 2.4: best daily mean data) began to show signs of habituation to the stimulus and further testing may have resulted in habituation, shark behaviour did not change convincingly after five days of training or 55 control trials. Thus, these sharks learnt nothing about the compound stimulus.

In contrast, shark behaviour of all the sharks undergoing CS conditioning changed significantly from the pre-training day to the end of conditioning. These sharks were conditioned to associate a compound auditory and electrical CS with the presentation of food (US). In both the 1st and 2nd replicate, half of the sharks were conditioned after five (Fig. 2.6a, 2.8a: 62 conditioning trials) and six (Fig. 2.10, 2.12: 57 conditioning trials) days of conditioning training, respectively. The rest of the sharks were conditioned after additional training (1st replicate: Fig. 2.7a, 2.9a; 2nd replicate: Fig. 2.11, 2.13). This supports results from previous conditioning studies done on sharks. In several operant conditioning experiments with lemon sharks, an auditory CS was paired successfully with a response to obtain food (Aronson *et al.* 1967; Clark 1959; Wright & Jackson 1964). In a conditioning study with stingrays by (Kalmijn 1982; Kalmijn & Kalmijn 1981), two out of three stingrays learnt to make the association between the food reward and the induced magnetic pole.

The strong conditioned association formed in this study lends support to the adaptive approach. This association in replicate 1 was strong enough for the conditioned

responses to be retained at the same level after a two-month rest period than before the rest period (Fig. 2.6a to 2.9a). This supports previous studies involving the retention of a conditioned association between an auditory CS and the presentation of food. After a two-month rest period, two conditioned lemon sharks resumed striking of a ringing target to obtain food (Clark 1959) and conditioned red sea bream approached an auditory CS to receive food (Fujiya *et al.* 1980).

During extinction training in replicate 1, when the conditioned association was not reinforced by the US, the CR was still maintained at the same level than at the end of conditioning training. In some cases when the CR deteriorated significantly (Fig. 2.6b, ext₂; Fig. 2.8b, ext₆), it would reappear again at the same level than before extinction began (Fig. 2.6b, ext₆; Fig. 2.8b, ext₁₀). This conforms to other extinction data for fishes. Takahashi *et al.* (1985) taught bluegill sunfish *Lepomis macrochirus* to make a response for food to a light signal. When the light signal was given, responses were rewarded with food but the periods (inter-signal period) between light signals were not rewarded. The researchers found that responses decreased during an inter-signal period, but could recover again in the next inter-signal period. This retention and reappearance of the CR, observed in this study, when it no longer served a purpose can be explained by the adaptive approach. Through classical conditioning the conditioned association became part of the feeding behaviour of the shark, affording it some advantage in utilising a new food source (Hollis 1982). Therefore, the conditioned association became difficult to eliminate even when this behaviour no longer served a purpose (Hollis 1982).

The results suggest all smoothhound sharks can be taught to make a simple association between a compound auditory and electrical CS and the presentation of

food, but the rate of learning and performance level varies. This individual variation in learning is not unusual. In a study where pumpkinseed sunfish *Lepomis gibbosus* were taught to feed on novel food items, the time it took for 16 individual fish to make their first capture of the novel food item varied significantly: $F_{(14, 253)} = 4.56$, $p < 0.0001$ (Kieffer & Colgan 1991). The reason for this variation was attributed to differences in changes in physiological conditions, as the rate of digestion may reflect motivational differences, i.e. how hungry a fish is. Hunger was also shown to increase the rate of learning of sticklebacks *Spinachia spinachia* and cause individual variation in learning ability (Croy & Hughes 1991).

In this study, for a shark to feed, its hunger should be a more powerful factor than the fear of the speaker and the feeding rod. Only if a shark comes to feed, does the possibility exist for conditioning to occur. Thus, the sharks that learned the fastest should have responded more often than the sharks that the learnt the slowest. In the 1st replicate, the shark, which learnt the association the fastest, Y_1 (after only 15 trials) responded on 69 trials and the slowest learner, Y_{P2} on only 45 trials during initial training. Thus, individual variation observed in this study could also be explained by differences in motivational drives, such as hunger and fear.

Despite individual differences in learning, all of the sharks displayed the CR. In several instances, the CR was elicited even before the US was presented. This can be most clearly seen in the best daily mean data. Sharks would approach and circle the area where food would be given shortly after the CS was presented, even before the US was presented, but would only feed between 1 and 2 minutes after the presentation of the CS (pers. obs.). Enough food was presented at a time so there would be little

competition between the sharks for food. Thus, it would serve the sharks just as well to pay no attention to the CS and wait until the food was lowered into the water to approach the feeding area. However, the CS became a signal for the presentation of food and the CR was anticipatory to the US. In a study by Davies *et al.* (1963) where several shark species were conditioned to associate auditory stimuli with food, this anticipatory CR was also observed. Sharks turned sharply and swam to the feeding area after the presentation of the CS before the US was presented. This phenomenon can be explained by the adaptive approach. According to the adaptive approach, the CR prepares the shark to optimise interaction with the US (food) (Domjan 1992; Hollis 1982). Thus, through classical conditioning, smoothhound sharks can adapt to utilise new food sources, effectively.

This mechanism may be of especial importance in the lifestyle of smoothhounds. The diets of smoothhound sharks change with growth as well as the current depth and location of the shark, and seem to be governed mainly by the availability of prey and the ability of smoothhound sharks to take prey successfully (Smale & Compagno 1997). A part of their diet is also scavenged (Smale & Compagno 1997). Thus, these sharks are opportunistic feeders. Clearly, the cues signalling food would change as the sharks matured or smoothhound and prey populations migrated. Thus, an adaptable learning mechanism would be invaluable to smoothhound sharks in utilising food sources.

To conclude, smoothhound sharks do have the ability to learn the association between a compound auditory and electrical CS and the presentation of food, but individual variation in the learning capabilities due to differences in motivational drives, such as hunger, will determine the time required for and success of conditioning. Genetic differences or differences between the sexes may also account for the observed

variation in learning. Learning through classical conditioning provides a mechanism for the shark to change the cues to which it responds, to locate food as it grows or migrates. In this study, it was not clear which cue, the auditory or electrical stimulus, was the main signal for the presentation of food. Therefore, it was not possible to determine whether the auditory or electrical sense of smoothhound sharks was more important in natural feeding behaviour. Additional conditioning experiments with separate auditory and electrical conditioned stimuli would be necessary to determine the relative importance of each sense in the feeding behaviour of smoothhound sharks.

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CHAPTER 3

BIOLOGICAL RELEVANCE OF FOOD-ASSOCIATED CONDITIONING IN THE SMOOTHHOUND SHARK *MUSTELUS MUSTELUS* WITH AUDITORY AND ELECTRICAL CONDITIONED STIMULI

3.1 Summary

The biological relevance of auditory and electrical cues in signalling the location of prey for the smoothhound shark *Mustelus mustelus* was investigated. According to the “belongingness” phenomenon, the conditioned stimulus (CS) should be biologically relevant to the unconditioned stimulus (US). Thus, by comparing the conditioning success with different conditioned stimuli, the biological relevance of each CS can be determined. In separate experiments, an auditory CS or an electrical CS was paired with the presentation of food (US). The conditioned CS-US association formed in the majority of the sharks after 60 and 57 conditioning trials in the auditory CS and electrical CS experiment, respectively. However, after five days of conditioning, conditioning with the electrical CS had proceeded further and the electrical CS-US association was stronger than the auditory CS-US association. These results suggest both auditory and electrical cues are biologically relevant signallers of the presence of food, facilitating the opportunistic feeding behaviour of smoothhound sharks. However, electrical cues may be more biologically relevant due to past feeding experiences. This illustrates how

classical conditioning during past feeding experiences can influence the feeding behaviour of smoothhound sharks.

3.2 Introduction

Not all conditioned associations are formed with equal ease. For conditioning to be rapid and effective, the CS should be biologically relevant or “belong” to the US (Domjan 1992; Hollis 1997). This phenomenon of “belongingness” was observed by Garcia and Koelling (1966) in food-aversion conditioning with rats, and was initially thought to be unique to food-aversion conditioning. Later “belongingness” was demonstrated in food and shock-associated conditioning in pigeons by Shapiro *et al.* in 1980. However, no study with sharks has investigated the effect of biological relevance of the CS to the US on food-associated conditioning.

In food-aversion studies by Garcia & Koelling (1966) and Domjan & Wilson (1972), rats were presented with two conditioned stimuli, a taste CS and audio-visual CS, and two unconditioned stimuli, shock and sickness. The rats formed conditioned associations only between the sickness US and the taste CS, and between the shock US and the audio-visual CS (Garcia & Koelling 1966; Domjan & Wilson 1972). From this it was inferred, that for animals with a highly developed sense of taste, taste is a better cue for food toxicity than an external event, such as shock (Garcia & Koelling 1966). However, external cues may be more reliable signallers of physical pain (Garcia *et al.* 1974). Rozin & Kalat (1971) extended this, by suggesting food-aversions would be formed effectively with any eating-related cues (cues a species use to identify food). This was confirmed by food-aversion studies with birds. Visual cues may be more important for the identification

of food for birds than taste cues due to a poorly developed gustatory system, and as expected, it was demonstrated that chickens *Gallus gallus* formed food-aversions more readily with visual than taste cues (Gillette *et al.* 1980). Thus, the success of the formation of the CS-US association depends on the biological relevance of the CS to the US (Domjan 1992; Hollis 1997).

This phenomenon of “belongingness” was also demonstrated in other studies than food-aversion conditioning. In operant conditioning studies with pigeons (Foree & LoLordo 1973; LoLordo & Furrow 1976) it was demonstrated that a conditioned response (CR) to obtain food was controlled by the visual component of a compound CS, whereas a CR to avoid electrical shock was controlled by the auditory component. Subsequently Shapiro *et al.* (1980) demonstrated the importance of biological relevance of the CS, once again in food and shock-associated classical conditioning studies with pigeons. As was expected, pigeons formed a strong conditioned association between food and the visual CS, but not with the auditory CS in the study (Shapiro *et al.* 1980). Thus, by comparing the conditioning success of conditioning with different conditioned stimuli, the biological relevance of a particular CS to the US can be determined.

In a previous study on smoothhound sharks, by the author, a compound auditory and electrical CS was successfully paired with the presentation of food (Chapter 2). However, it was not clear which component of the compound CS the sharks attended to most, and therefore which component was biologically more relevant in locating food. The modality of the more biologically relevant CS would reflect the sense detecting the more important cues used by smoothhound sharks in locating their prey.

Two of the senses that might be of some importance in locating the prey of sharks are the auditory and electrical senses. Both these senses are highly developed and are used to detect prey. Sharks detect sounds with the inner ear, lateral line and general cutaneous sense (Bleckmann & Hofmann 1999) and are especially sensitive to low-frequency underwater sounds (less than 1000 Hz) (Bleckmann & Hofmann 1999). Evidence suggests sharks use auditory cues to locate food. In several studies carcharhinid and sphyrnid shark species have been attracted by recordings of sounds similar to those made by struggling fish and the prey of these sharks (Nelson & Gruber 1963; Banner 1972). Furthermore, Davies *et al.* (1963) demonstrated several shark species could associate auditory cues with the presentation of food in a classical conditioning study.

Sharks can detect electrical fields as small as $2\mu\text{V/m}$ with the ampullae of Lorenzini. Several studies have demonstrated that elasmobranchs use electrical cues to locate prey. Electrical fields from real prey and electrical fields simulating prey have elicited feeding responses from several shark and ray species (Kalmijn 1966, 1971, 1982). Furthermore, it was demonstrated that California round stingrays *Urobatis halleri* could associate electrical cues with a food reward in a conditioning study. The stingrays were conditioned to expect a food reward at a specific induced magnetic pole (Kalmijn 1982; Kalmijn & Kalmijn 1981). Elasmobranchs are thought to orient themselves in a magnetic field by detecting voltage gradients when swimming through the field (Kalmijn 1984). Thus, the stingrays used electrical cues to locate the food reward. Clearly conditioning studies with the auditory and electrical CS and the presentation of food as the US are needed to illustrate the role of these senses in the feeding behaviour of sharks.

In this study it was attempted to condition smoothhound sharks *Mustelus mustelus* (Chondrichthyes: Triakidae) to associate either an auditory or an electrical CS with the presentation of food (US). These sharks are epibenthic bottom-dwelling sharks (Smale & Compagno 1997), can easily be kept in captivity and are relatively easily obtainable. The conditioning success with each CS was compared. The more biologically relevant CS signalling the presence of food to smoothhounds would condition with more success, according to the “belongingness” phenomenon. Thus, the relative importance of the auditory and electrical sense in the natural feeding behaviour of smoothhound sharks could be inferred. Furthermore, Gillette *et al.* (1980) postulated that the biological relevance of a CS to the US might be influenced by past feeding experiences, possibly by learning through classical conditioning. Consequently, the role of classical conditioning in the formation of natural feeding behaviour could be determined.

3.3 Method and Materials

3.3.1 Specimens and holding

In this study smoothhound sharks were used to compare conditioning and food-association with either an auditory or electrical CS. Five smoothhound sharks, two females (R_a and Y_a) and three males (B_a , RY_a and YR_a) were used in the auditory CS experiment and five specimens, three females (R_e , RY_e , and Y_e) and two males (B_e and YR_e) in the electrical CS experiment. In the auditory CS control, two smoothhounds, two females (B_c and YR_c) were used and two sharks, one female (B_c) and one male (YR_c) in the electrical CS control. The use of only two animals in each of the control treatments was on account of limited availability of animals at the time. The letters in the name of

the sharks refer to the colour code of their tag, i.e. P = pink, R = red, and the subscript letters refer to the experiment, i.e. c = control, a = auditory CS experiment and e = electrical CS experiment. Different sharks were used for the auditory and electrical CS controls as well as the compound CS control (Chapter 2), although only subscript c was used throughout to indicate these animals. All the sharks were collected at Fish Hoek, Western Cape Province, South Africa (34 ° E; 18 ° S) by trek fishermen from February to March 2001. According to size at maturity data (Smale & Compagno 1997), these sharks were all immature, as the total length of each was between 400 and 700 mm.

Sharks were housed at the Sea Fisheries Research Institute (Marine and Coastal Management) research aquarium at Sea Point, Cape Town, where the experiments were conducted. The sharks were kept in a circular tank, 7 m deep and 3 m in diameter with several teleost fish species, including sardine *Sardinops sagax*, white steenbras *Lithognathus lithognathus*, red roman *Chrysoblephus laticeps* and white stumpnose *Rhabdosargus globiceps*. Seawater in the tank was aerated and circulated; and a 12h: 12h light: dark cycle was maintained. Sharks were fed sardines when experiments were not conducted, three times a week during daylight hours.

3.3.2 Apparatus

A specialised feeding rod, an auditory and electrical CS were custom-made for the experiments. The feeding rod was used during experiments to feed the sharks. It consisted of a plastic tube that was suspended in the tank, approximately 0.600 m under the water surface against the tank wall during experiments. Pieces of sardines were hung on fishing line from the rod and the rod was weighed down with two pieces of lead. In so doing, the feeding area and the amount of feeding could be manipulated.

The auditory CS was a recording of two pieces of wood being hit together, producing several tones between 300 and 1,100 Hz. The recording was played on a tape-recorder attached to a speaker enclosed in a plastic container. The speaker could be suspended into the water, approximately 1.350 m from the tank wall where the food was presented on the feeding rod. The speaker, when in contact with the water induced an electrical field of 1mV/cm. When the auditory CS experiment was conducted, the speaker was sealed watertight, thus eliminating the electrical component of the CS. During the electrical CS experiment, the speaker was not sealed and was directly in contact with water. The tape was removed from the tape-recorder and the volume turned down so there would be no auditory component to the CS.

During the experiments, shark behaviour was recorded by a video camera through a circular window in the side of the tank, with a diameter of 0.500 m. The camera was positioned opposite from the tank wall where the food was presented. All the windows on the same level were covered with black plastic to eliminate extraneous stimuli. The position of the conditioned stimuli, feeding rod and camera is shown in Fig. 3.1a and 3.1b.

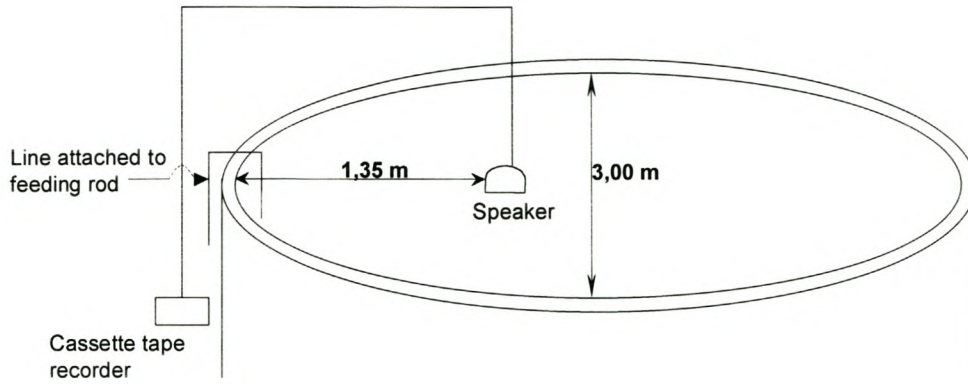


Figure 3.1a) Diagram of side-view of experimental tank with position of conditioned stimulus and feeding rod during training, indicated.

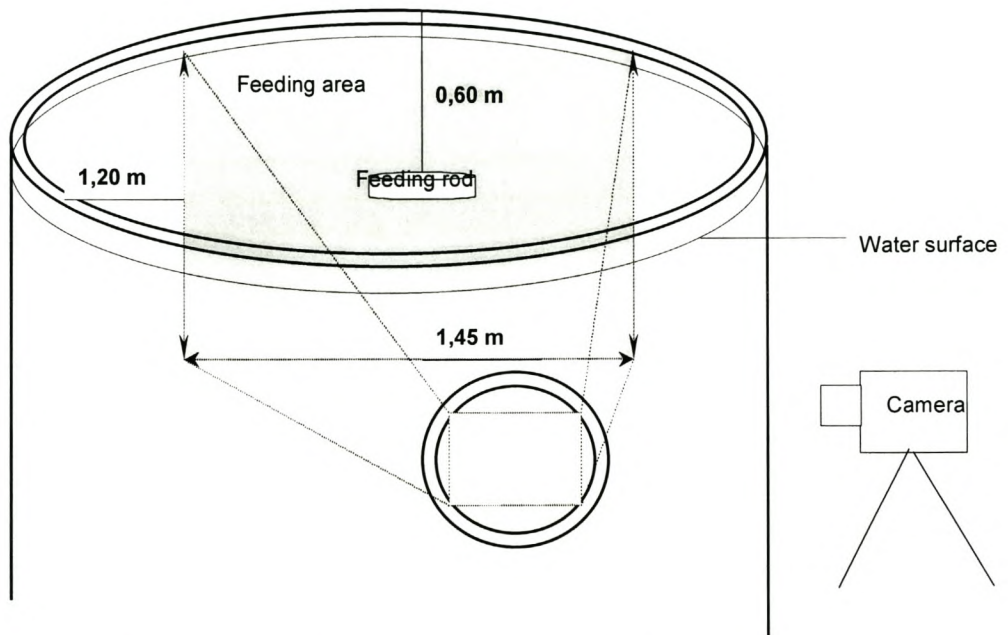


Figure 3.1 b) Diagram of side-view of experimental tank showing position of camera, feeding area and feeding rod.

3.3.3 Experimental procedure

The CS controls were conducted to determine inherent attractiveness of the CS and compare shark behaviour with those in the CS experiments. The aim of the CS experiments was to determine if sharks could be conditioned to associate either an auditory or electrical CS with the presentation of food (US) and to compare the rate and success of conditioning with different conditioned stimuli.

Training was done in a group-situation in the home-tank. At least one hour prior to the first trial, the other fish in the tank were fed small scraps of sardine. These pieces of food were too small for the sharks to eat. The speaker was also placed in the tank and the windows covered.

A control trial in the CS control consisted of the presentation of only the stimulus used as the CS in the CS experiment for 15 s. No US was presented and shark behaviour was recorded for three minutes after the presentation of the stimulus. These trials were conducted ten times on a training day with an inter-trial period of approximately 10 minutes.

In the CS experiments, a training trial in these experiments began with the presentation of the CS for 15s, followed by the presentation of food (US) on the feeding rod in the feeding area. The feeding area was defined as a square area of 1.450 m x 1.200 m around the area where the food was presented on the feeding rod.

The CS was then presented for another 5s with the US. Sharks were allowed two to three minutes to feed. At the end of the trial, all the uneaten food was removed. This was followed by an inter-trial period of approximately 10 minutes. Trials were repeated on a

training day until each shark received three pieces of food. No fewer than ten and no more than 20 trials were conducted on any particular training day.

During the experiments, the latency time of each shark on each trial was recorded. The latency time was defined as the time elapsed from the start of the presentation of the CS until the shark displayed the CR. The CR was the entering of the feeding area and circling of the area where food was presented.

During test and extinction trials, the CS was presented as in the training trials, but was not followed by the presentation of food (US). Shark behaviour was observed for two to three minutes after the presentation of the CS. Ten of these trials were conducted on each training day with inter-trial periods of 10 min. At least an hour after the last test or extinction trial, the sharks were fed by throwing sardine pieces into the water.

3.3.3.1 CS control:

The auditory CS control with the auditory CS was conducted from 10/03/01 to 18/03/01 with the sharks, B_c and YR_c; and the electrical CS control with the electrical CS was conducted from 19/03/01 to 27/03/01 with the sharks, B_c and YR_c. During the CS controls, shark behaviour to the CS was observed to be compared to behaviour observed in the experiments. Training was done every second day, for five training days. Trials were conducted as mentioned.

3.3.3.2 CS experiments:

The auditory CS experiment was conducted with the auditory CS from 17/02/01 to 05/03/01 with the sharks, B_a, R_a, RY_a, Y_a and YR_a; and the electrical CS experiment with

the electrical CS, from 06/04/01 to 22/04/01 with the sharks, B_e , R_e , RY_e , Y_e and YR_e .

Each experiment consisted of an initial training period, and extinction training. During the initial training period, smoothhounds were conditioned to associate the CS with the presentation of food. Training was done every second day. To obtain base-line data with which performance on the following training days could be compared, training began with a pre-training day (labelled pre-train). On this day, the speaker was kept in the tank and food was presented during training, but the CS was not presented. The pre-training day was followed by five days of conditioning training (condit₁ to condit₅) during which trials were conducted as described.

During extinction, it was attempted to extinguish the conditioned association formed during initial training. Testing was done on five successive days (ext₁ to ext₅). Trials were conducted as mentioned with the CS used in initial training, except on ext₂ and ext₃. Ext₁ served as the test day to determine if conditioning occurred with the CS used in initial training (conditioned stimulus) and ext₂ and ext₃ served to compare responses to the conditioned CS and the non-conditioned stimulus. On ext₂, the first five trials were conducted with the conditioned stimulus used in initial training and after two hours, the 2nd five trials were conducted with the non-conditioned stimulus (e. g. electrical CS, if the auditory CS was used during initial training, and vice versa). On ext₃, the first five trials were conducted with the non-conditioned stimulus and after two hours, the second five trials with the conditioned stimulus.

3.3.4 Analyses

The data from each experiment and control were analysed separately. For each shark, the mean latency time (daily mean latency time) and the three shortest latency

times on a training day (best daily mean latency times) were plotted against the number of trials to obtain learning curves for each shark. Kruskal-Wallis analyses of variance by ranks (Kruskal-Wallis ANOVA's) were used to statistically analyse the difference in performance over days of training (Siegel 1956). The source of variance was further analysed with Wilcoxon Mann-Whitney tests, except with the best daily mean latency data. Kolmogorov Smirnov two-sample tests were used for this data, as it is more accurate with small sample sizes than the Wilcoxon Mann-Whitney test (Siegel 1956). For each training day, $n \leq 3$, as a result, probabilities could only be calculated to a significance level of $\alpha \leq 0.100$ (see Table L_{II} in Siegel & Castellan 1988). Consequently a p -value < 0.1 was taken as significant in this particular case.

The daily mean latency time of each shark was standardised to compare the performance of different sharks and under different experimental conditions, by the following calculation:

$$c = a / b$$

where c = latency on trial x of training as fraction of daily mean latency on day 1 of training; a = latency time on trial x and b = daily mean latency time on first day of training. The differences in performance between sharks were calculated with Friedman one way analyses of variance (Friedman ANOVA's) and Wilcoxon matched pairs tests using the c -values averaged over every 10 trials (Siegel 1956). The pooled c -values for all the sharks in a particular experimental condition were used to compare performance under different experimental conditions, calculated with Kruskal-Wallis ANOVA's and Wilcoxon Mann-Whitney tests. Differences in performance were termed significant at the 95 % confidence limit. All statistical analyses were done on the computer program, STATISTICA.

3.4 Results

3.4.1 Auditory CS results

3.4.1.1 Auditory CS control:

Both sharks eventually entered the feeding area after the presentation of the stimulus (Fig. 3.2, 3.3). However, on day 2 and 3 of the control, the shark, YR_c, never entered the feeding area. The auditory stimulus had some inherent attractiveness, as the best daily mean latency on the day 1 of training was under 15 s, but on subsequent days it was always higher than 15 s. No change in the response time of the sharks to the auditory stimulus occurred during the four days (40 trials) of control training. Kruskal-Wallis ANOVA found no significant differences in either the daily (B_c: H = 2.293, p = 0.514; YR_c: H = 0, p = 1.000) or the best daily mean latencies (B_c: H = 6.012, p = 0.111; YR_c: H = 0, p = 1.000) during control trials. Thus, these sharks did not learn anything about the auditory stimulus.

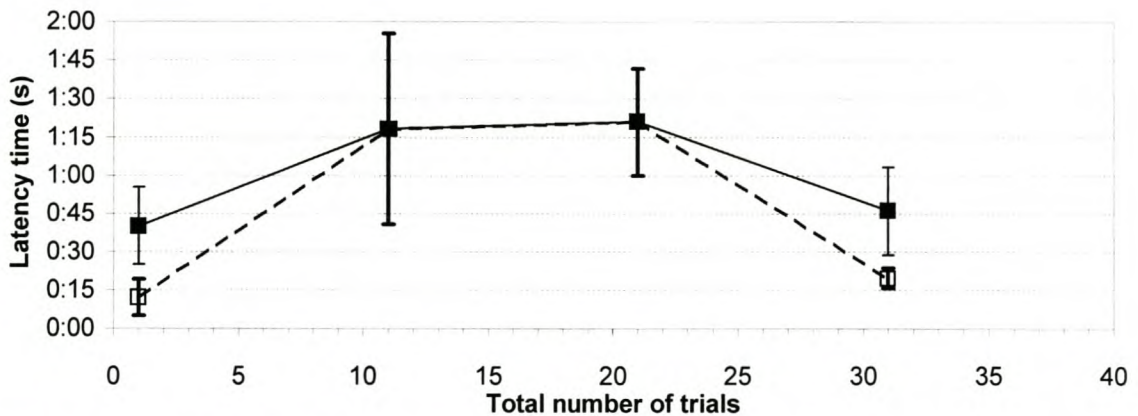
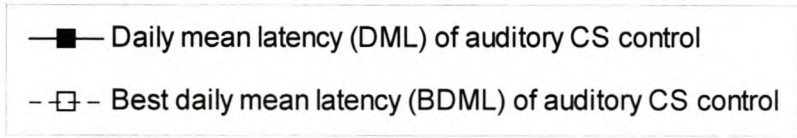


Figure 3.2 Daily and best daily mean latencies of auditory CS control of female *M. mustelus*, B_c.



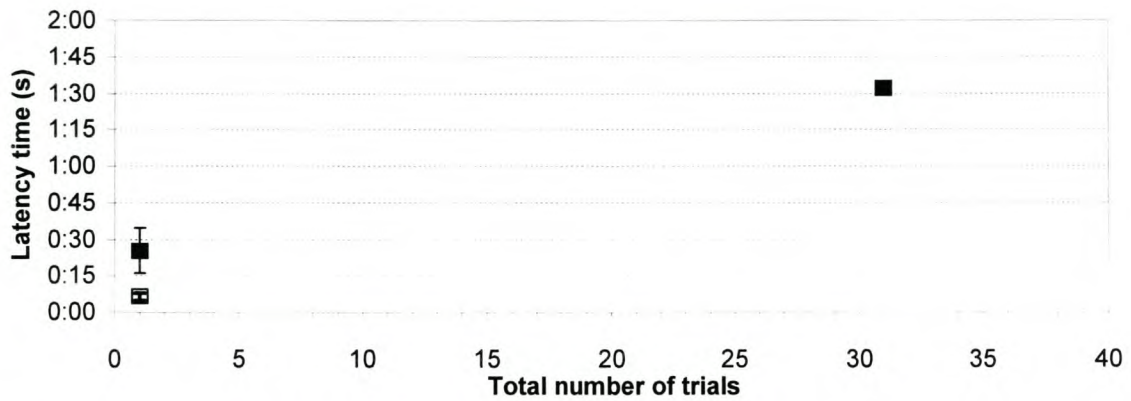


Figure 3.3 Daily and best daily mean latencies of auditory CS control of female *M. mustelus*, YR_c.

—■— Daily mean latency (DML) of auditory CS control
 - □ - Best daily mean latency (BDML) of auditory CS control

As the shark, YR_c did not enter the feeding area on the 2nd and 3rd day of testing (Fig. 3.3), shark behaviour, expressed as *c*- values, in the CS control could not be compared. Thus, only the *c*-values obtained from the shark, B_c, were used to compare the behaviour of sharks during the CS control with those in the CS experiment.

3.4.1.2 Auditory CS experiment:

a) *Initial training* - The shark, R_a, entered the feeding area only once on pre-train, making statistical analyses with pre-train impossible. Thus, for this shark, performance on *condit*₁ was used as a base-line performance with which the performances on other training days were compared. All the sharks entered the feeding area (CR) after the presentation of the auditory CS (Fig. 3.4 – 3.8). This was demonstrated in the significant downward trend in the daily mean latencies over initial training of four of the five sharks, B_a, R_a, R_{ya} and YR_a (Kruskal-Wallis ANOVA's – B_a: $H = 13.522$, $p = 0.019$; R_a: $H = 20.268$, $p = 0.016$; R_Y_a: $H = 17.106$, $p = 0.004$; YR_a: $H = 7.669$, $p = 0.018$).

Wilcoxon Mann-Whitney tests demonstrated the variance was due to differences in performance between pre-train and condit_4 ($U = 23.5$, $p = 0.006$) for the shark, B_a (Fig. 3.4); between condit_1 and condit_2 ($U = 8$, $p = 0.005$), condit_3 ($U = 5.5$, $p = 0.003$), condit_4 ($U = 1$, $p = 0.001$) and condit_5 ($U = 3$, $p = 0.008$) for R_a (Fig. 3.5); on pre-train and condit_4 ($U = 9$, $p < 0.000$) for RY_a (Fig. 3.6); and on pre-train and condit_3 ($U = 20$, $p = 0.009$) for the shark, YR_a (Fig. 3.8). In addition, a downward trend was observed in the best daily mean latencies for these sharks, but was only significant for R_a and RY_a (Kruskal-Wallis ANOVA's – B_a : $H = 11.051$, $p = 0.050$; R_a : $H = 11.205$, $p = 0.048$; RY_a : $H = 13.326$, $p = 0.021$; YR_a : $H = 9.756$, $p = 0.083$). Kolmogorov Smirnov two-sample tests found differences in performance between pre-train and condit_4 ($D = 1$, $p < 0.100$) and condit_5 ($D = 1$, $p < 0.1000$) for the shark, B_a (Fig. 3.4); between condit_1 and condit_2 ($D = 1$, $p < 0.100$), condit_3 ($D = 1$, $p < 0.100$), condit_4 ($D = 1$, $p < 0.100$) and condit_5 ($D = 1$, $p < 0.100$) for R_a (Fig. 3.5); on pre-train and condit_3 ($D = 1$, $p < 0.100$), condit_4 ($D = 1$, $p < 0.100$) and condit_5 ($D = 1$, $p < 0.100$) for the shark, RY_a (Fig. 3.6); and on pre-train and condit_1 ($D = 1$, $p < 0.100$), condit_3 ($D = 1$, $p < 0.100$) and condit_5 ($D = 1$, $p < 0.100$) for YR_a (Fig. 3.8). Thus, conditioning could be demonstrated for four (B_a , R_a , RY_a and YR_a) of the five sharks after initial training.

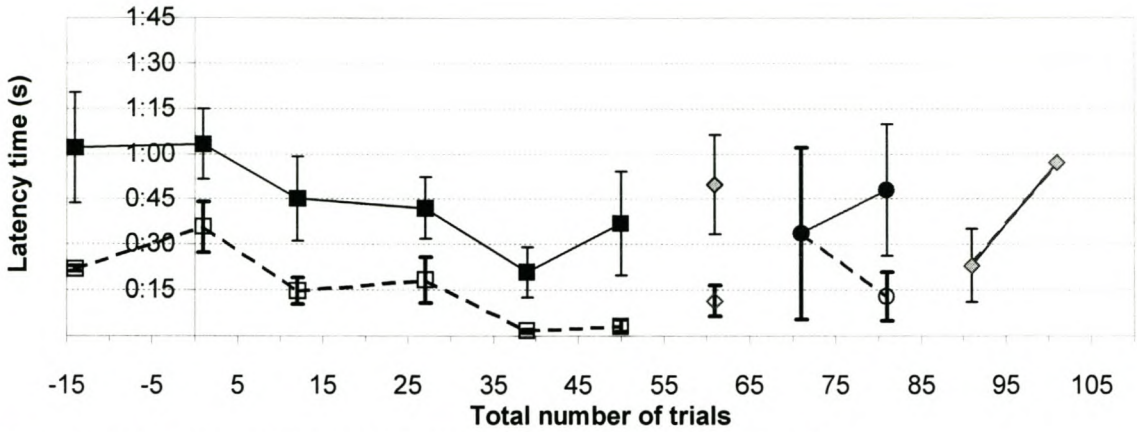


Figure 3.4 Daily and best daily mean latencies of auditory CS conditioning of male *M. mustelus*, **B_a**.

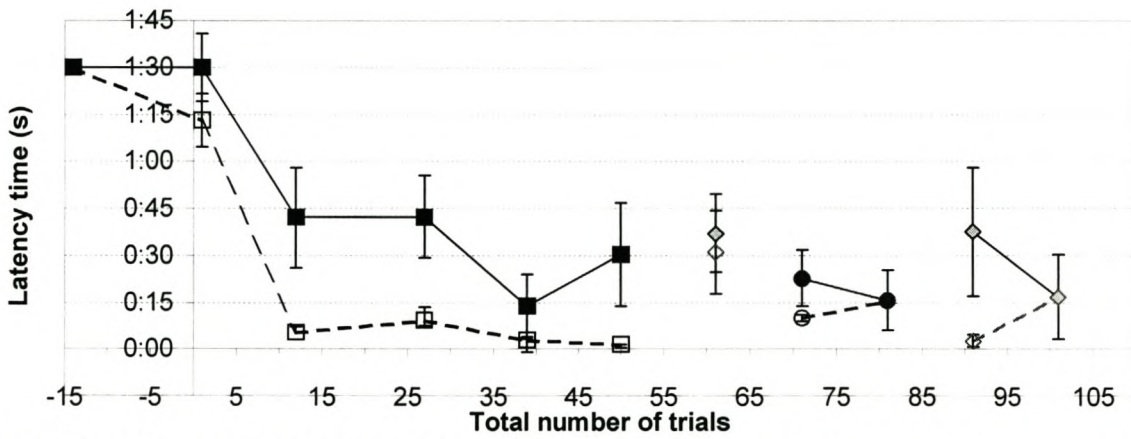
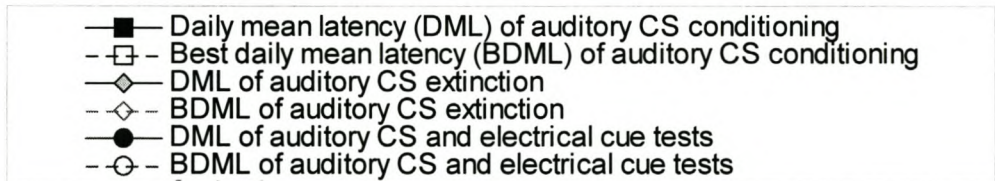


Figure 3.5 Daily and best daily mean latencies of auditory CS conditioning of female *M. mustelus*, **R_a**.



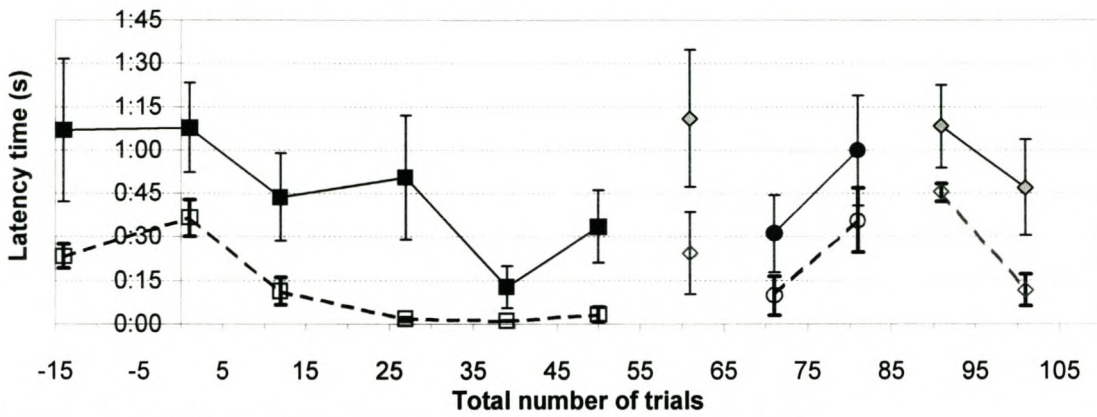


Figure 3.6 Daily and best daily mean latencies of auditory CS conditioning of male *M. mustelus*, RY_a.

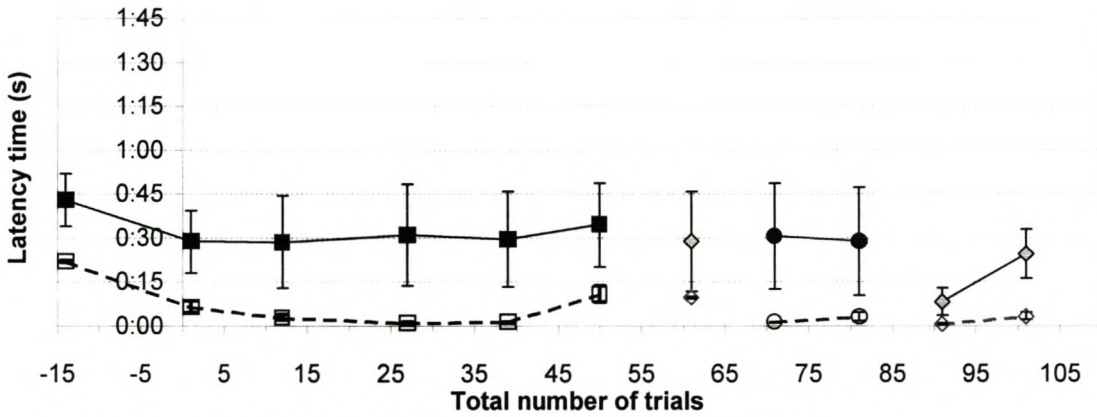
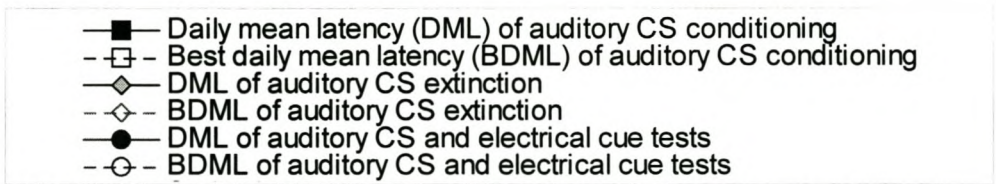


Figure 3.7 Daily and best daily mean latencies of auditory CS conditioning of female *M. mustelus*, Y_a.



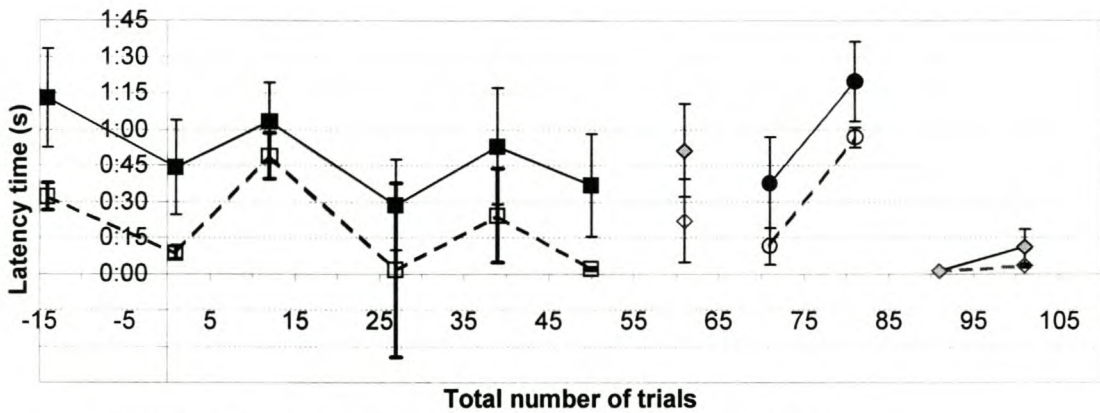
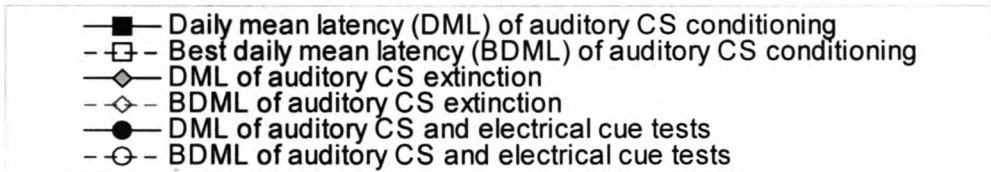


Figure 3.8 Daily and best daily mean latencies of auditory CS conditioning of male *M. mustelus*, YR_a.



Conditioning was not as evident for the shark, Y_a (Fig. 3.7). A slight downward trend in the daily mean latency times of this shark was observed, but was not significant (Kruskal-Wallis ANOVA's – Y_a: H = 7.052, p = 0.217). Despite this, Wilcoxon Mann Whitney tests found significant differences in performance between pre-train and condit₂ (U = 5.5, p = 0.016) for the shark Y_a. However, some association between the CS and the presentation of food did develop in the shark, Y_a. The downward trend in the best daily mean latencies was significant (Kruskal-Wallis ANOVA: H = 14.931, p = 0.010) and the variance was due to differences in performance on pre-train and all the other days of initial training (D = 1, p < 0.100). In addition, the best daily mean latencies were under 15 s even from condit₁ (Fig. 3.7). This shark entered the feeding area even before the US (food) was presented. Thus, this shark expected the presentation of food after the CS from condit₁.

Thus, conditioning of the association between an auditory CS and the presentation of food could be shown clearly after five days of conditioning training (60 conditioning trials) for only four of the five sharks B_a , R_a , RY_a and YR_a . However, the shark, Y_a also began to demonstrate signs of conditioning.

For the comparison of shark behaviour in the CS experiment to the control, the c -values averaged over every 10 trials of individual sharks during the first 40 exposures to the auditory CS, was compared. A Friedman ANOVA demonstrated no significant variance among the behaviour of the five sharks during the first 40 exposures to the auditory CS ($F = 1.100$, $p = 0.809$, $n = 5$). Therefore, the combined c -values of all the sharks in the CS experiment, B_a , R_a , RY_a , Y_a and YR_a , were used in the comparison of shark behaviour with the control.

b) Extinction - Although no food was presented during extinction testing, sharks continued to enter the feeding area (CR) after the presentation of the CS. The level of conditioned responses of all the sharks, on ext_1 , the test for conditioning with the auditory CS, was the same as at the end of initial training (Fig. 3.4 – 3.8). Wilcoxon Mann Whitney tests on the daily mean latencies found no significant difference in performance between $condit_5$ and ext_1 for any of the sharks (B_a : $U = 38.8$, $p = 0.393$; R_a : $U = 13.5$, $p = 0.927$; RY_a : $U = 80.5$, $p = 0.088$; Y_a : $U = 39$, $p = 0.435$; YR_a : $U = 17$, $p = 0.937$). However, significant differences in performance between pre-train and ext_1 could only be demonstrated for the shark, R_a (Wilcoxon Mann Whitney tests - B_a : $U = 60$, $p = 0.585$; R_a : $U = 1$, $p = 0.019$; RY_a : $U = 43$, $p = 0.877$; Y_a : $U = 44$, $p = 0.091$; YR_a : $U = 54$, $p = 0.39$). The best daily mean data reflects the daily mean data. Even though Kolmogorov Smirnov tests found no significant differences between $condit_5$

and ext₁ for any of the sharks (B_a : $D = -0.333$, $p > 0.100$; R_a : $D = -0.667$, $p > 0.100$; RY_a : $D = -0.667$, $p > 0.100$; Y_a : $D = 0.333$, $p > 0.100$; YR_a : $D = -0.333$, $p > 0.100$), significant differences in performance between pre-train and ext₁ could only be shown for Y_a ($D = 1$, $p < 0.100$); and between condit₁ and ext₁, for R_a ($D = 1$, $p < 0.100$).

Therefore, the association formed during initial training between the auditory CS and the presentation of food may not have been strong enough for all of the sharks to be maintained when the CS-US association was not reinforced by food. The strength of association varied for the sharks, with a stronger association formed by R_a (Fig. 3.5) than by YR_a (Fig. 3.8).

The CR was maintained at the same level by the end of extinction testing, ext₅, for all the sharks, as at the end of initial training, condit₅. Wilcoxon Mann Whitney tests and Kolmogorov Smirnov tests found no significant differences in the daily (R_a : $U = 6$, $p = 0.383$; RY_a : $U = 29.5$, $p = 0.360$; Y_a : $U = 42.5$, $p = 0.579$; YR_a : $U = 10$, $p = 0.762$) or best daily mean latencies (R_a : $D = 0.333$, $p > 0.100$; RY_a : $D = -0.667$, $p > 0.100$; Y_a : $D = 0.667$, $p > 0.100$; YR_a : $D = -0.333$, $p > 0.100$) between condit₅ and ext₅, for four of the sharks, R_a , RY_a , Y_a and YR_a (Fig. 3.5 – 3.8). The shark B_a , entered the feeding area only once on ext₅, making analysis with ext₅ impossible (Fig. 3.4). However no significant difference was found in the daily (Wilcoxon Mann Whitney test: $U = 9$, $p = 0.909$) or best daily mean latencies (Kolmogorov Smirnov tests: $D = -0.667$, $p > 0.100$) between condit₅ and ext₄. Thus, after 40 trials, of non-pairing of the auditory CS and the presentation of food, for four sharks (R_a , RY_a , Y_a and YR_a) and 30 trials for one shark (B_a), the CS-US association formed during initial training, was as strong as before extinction trials.

3.4.1.3 Comparison of control and CS experiment:

For comparison of shark behaviour in the control and CS experiment, one shark, B_c (Fig. 3.2) in the control and all the sharks, B_a, R_a, RY_a, Y_a and YR_a (Fig. 3.4 – 3.8) in the CS experiment were used. The behaviour of the sharks in the control, stayed relatively constant while that of the sharks in the CS experiment changed during the first 40 exposures to the auditory CS (Fig. 3.9). Response time to the auditory CS after the 40th exposure was faster than at the beginning of training. A Wilcoxon Mann Whitney test found significant difference in the c-values during the 21st – 30th trials of sharks in the CS experiment and the control ($U = 0$, $p = 0.007$). However a Wilcoxon Mann Whitney test found no significant difference in c-values during the 31st – 40th trials ($U = 20$, $p = 0.161$). However, the conditioned association was only formed strongly after the 5th day (60 trials plus 15 exposures to CS on pre-train) of training in the CS experiment. Thus, the difference in behaviour would have been more marked after more exposures to the CS.

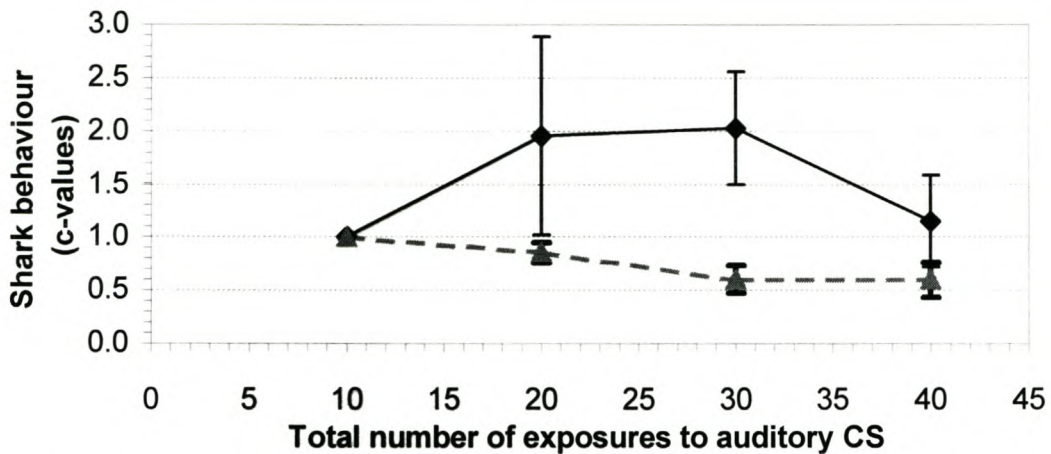


Figure 3.9 Shark behaviour during auditory CS control (B_c) and CS experiment (B_a , R_a , RY_a , Y_a , YR_a) expressed as c-values.



3.4.2 Electrical CS results

3.4.2.1 Electrical CS control:

All the sharks eventually entered the feeding area after the presentation of the electrical stimulus. The electrical stimulus is inherently attractive, as the best daily mean latencies of YR_c stay under 15 s throughout testing although no food was presented after the stimulus and of B_c stay under 15 s from day 2 of training (Fig. 3.10, 3.11). When the electrical stimulus was presented, sharks swam faster and subsequently entered the feeding area more frequently, but swimming was not directed towards the area where food was presented in the CS experiments.

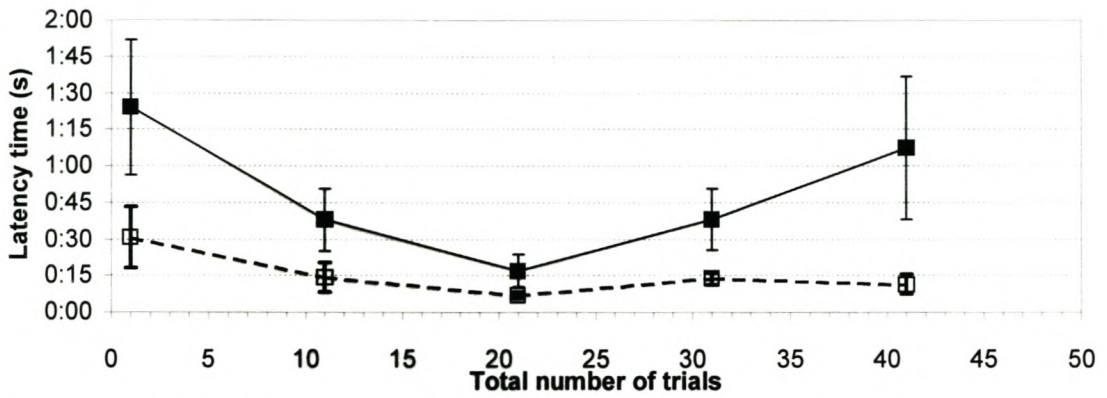


Figure 3.10 Daily and best daily mean latencies of electrical CS control of female *M. mustelus*, B_c.

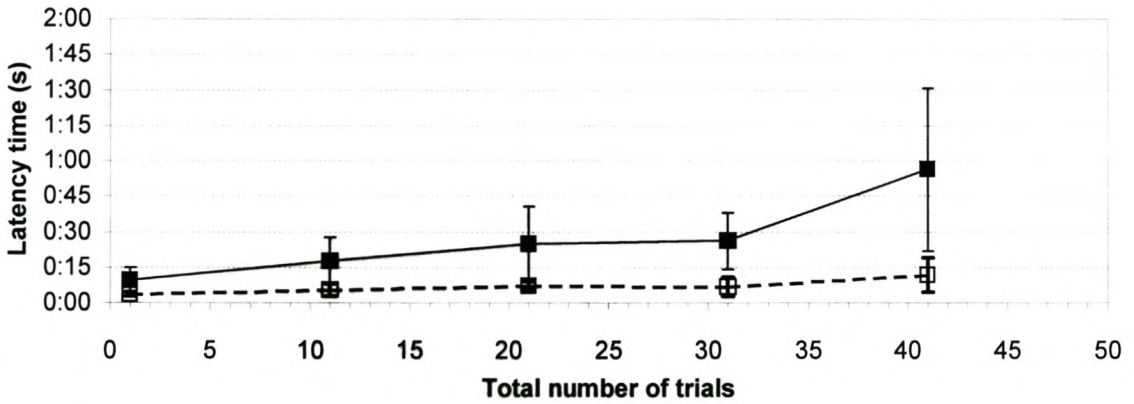
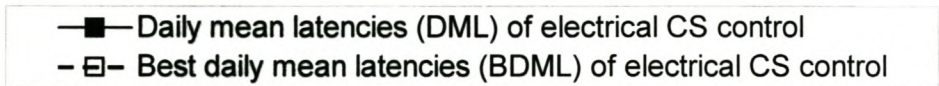


Figure 3.11 Daily and best daily mean latencies of electrical CS control of male *M. mustelus*, YR_c.



One of the sharks, YR_c, demonstrated no change in responses to the electrical CS (Fig. 3.11). Kruskal-Wallis ANOVA's found no significant differences in the daily (H = 4.76, p = 0.358) or best daily mean latencies (H = 0.842, p = 0.932) over the five days of control training (50 trials). A Kruskal-Wallis ANOVA found significant differences in the daily mean latencies (H = 10.328, p = 0.035) of the shark B_c and Wilcoxon Mann Whitney tests demonstrated this variance was due to differences between performance on day 3 and day 1 (U = 8, p = 0.007), day 2 (U = 20, p = 0.023) and day 4 (U = 22, p = 0.035) of training (Fig. 3.10). However no difference could be demonstrated between day 1 and day 5 of training (U = 21, p = 0.464). In addition, no significant difference could be found in the best daily mean latencies during the control (Kruskal-Wallis ANOVA: H = 4.909, p = 0.297). Therefore, these sharks, B_c and YR_c, did not learn anything about the electrical CS during the control.

To compare the behaviour of sharks in the CS control and the CS experiment the c-values averaged over every 10 trials of the two sharks in the CS control was analysed. A Friedman ANOVA found no significant differences in behaviour between sharks over the first 40 exposures to the CS (F = 1.826, p = 0.068, n = 2). Thus, the pooled c-values of B_e and YR_e were used in comparison to the CS control.

3.4.2.2 Electrical CS experiment:

a) *Initial training* - All the sharks entered the feeding area and swam around the area where food was presented (CR) after the presentation of the electrical CS, sometimes even before the presentation of the food (US). Tank water was too cloudy on condit₄ to accurately observe shark responses to the CS and data from condit₄ was excluded from statistical analyses. A conditioned association between the electrical CS and the US

could clearly be shown for four of the five sharks, B_e , R_e , RY_e and Y_e (Fig. 3.12 – 3.15). This was most evident in the downward trend of the daily mean latencies of these sharks. Kruskal-Wallis ANOVA's found this was significant (B_e : $H = 19.195$, $p = 0.001$; R_e : $H = 13.215$; $p = 0.010$; RY_e : $H = 18.549$, $p = 0.001$; Y_e : $H = 21.601$, $p = 0.000$).

Wilcoxon Mann Whitney tests demonstrated the variance found was due to differences in performance on pre-train and $condit_2$ ($U = 14$, $p = 0.010$), $condit_3$ ($U = 6.5$, $p = 0.007$) and $condit_5$ ($U = 2$, $p = 0.003$) for the shark, B_e (Fig. 3.12); on pre-train and $condit_5$ ($U = 0$, $p = 0.001$) for R_e (Fig. 3.13); on pre-train and $condit_2$ ($U = 18$, $p = 0.027$), $condit_3$ ($U = 5$, $p = 0.005$) and $condit_5$ ($U = 5$, $p = 0.000$) for RY_e (Fig. 3.14); and on pre-train and $condit_1$ ($U = 19.5$, $p = 0.006$), $condit_2$ ($U = 14$, $p = 0.000$), $condit_3$ ($U = 6$, $p = 0.000$) and $condit_5$ ($U = 7$, $p = 0.002$) for Y_e (Fig. 3.15). Furthermore, a downward trend in the best daily mean latencies was found for these sharks, but was significant only for the sharks, B_e , RY_e and Y_e (Kruskal -Wallis ANOVA's - B_e : $H = 11.472$, $p = 0.0212$; R_e : $H = 6.795$, $p = 0.147$; RY_e : $H = 11.498$, $p = 0.022$; Y_e : $H = 12.831$, $p = 0.012$). Kolmogorov Smirnov tests demonstrated the variance was due to differences in performance on pre-train and $condit_2$ ($D = 1$, $p < 0.100$), $condit_3$ ($D = 1$, $p < 0.100$) and $condit_5$ ($D = 1$, $p < 0.100$) for B_e (Fig. 3.12); on pre-train and $condit_1$ ($D = 1$, $p < 0.100$), $condit_2$ ($D = 1$, $p < 0.100$), $condit_3$ ($D = 1$, $p < 0.100$) and $condit_5$ ($D = 1$, $p < 0.100$) for RY_e (Fig. 3.14); and on pre-train and $condit_2$ ($D = 1$, $p < 0.100$), $condit_3$ ($D = 1$, $p < 0.100$) and $condit_5$ ($D = 1$, $p < 0.100$) for the shark, Y_e (Fig. 3.15). Although the downward trend in best daily mean latencies was not significant for the shark, R_e , Kolmogorov Smirnov tests found significant differences in performance on pre-train and $condit_2$ ($D = 1$, $p < 0.100$) and $condit_5$ ($D = 1$, $p < 0.100$) (Fig. 3.13). In addition, the best daily mean latencies was under 15 s from $condit_1$ for Y_e and $condit_2$ for B_e , R_e and RY_e . Therefore, Y_e ; and R_e , RY_e and B_e entered the feeding

area after the presentation of the CS, but even before the presentation of food from $condit_1$ and $condit_2$, respectively. Thus, these sharks anticipated the presentation of food after the CS.

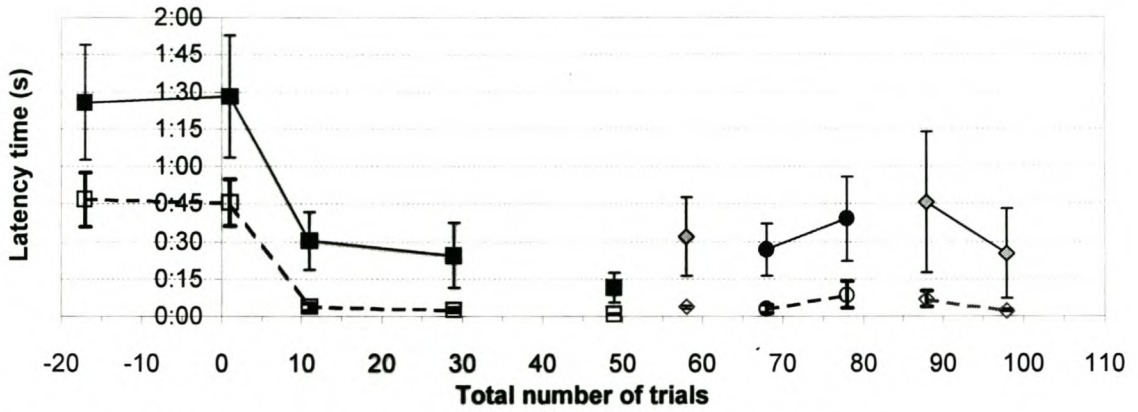


Figure 3.12 Daily and best daily mean latencies of electrical CS conditioning of male *M. mustelus*, B_e.

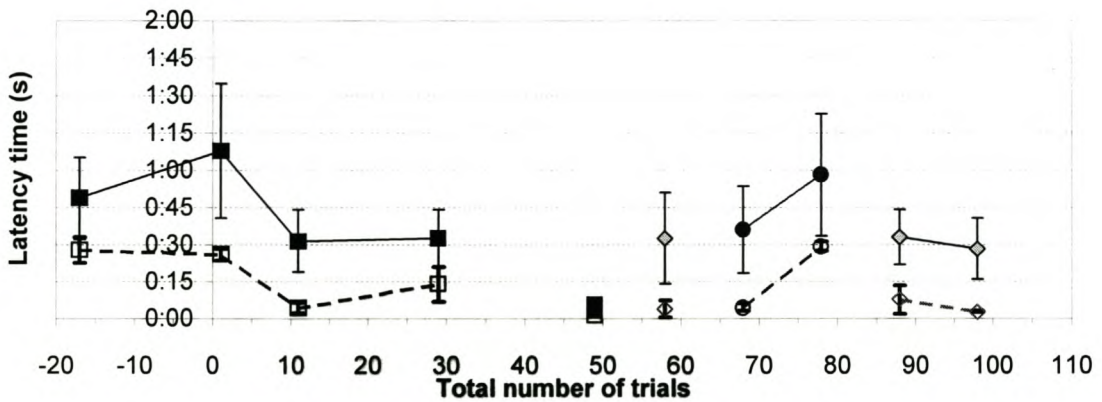
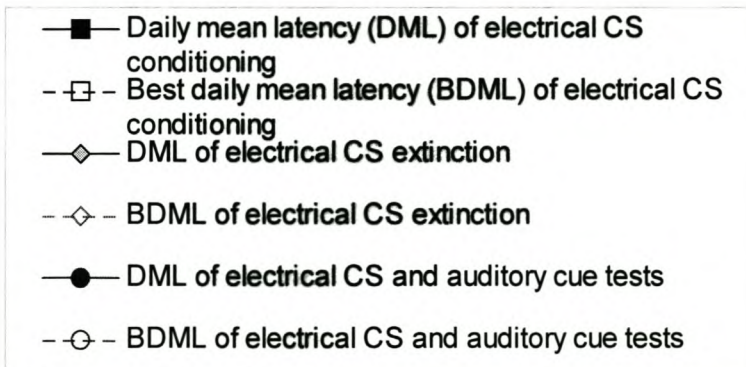


Figure 3.13 Daily and best daily mean latencies of electrical CS conditioning of female *M. mustelus*, R_e.



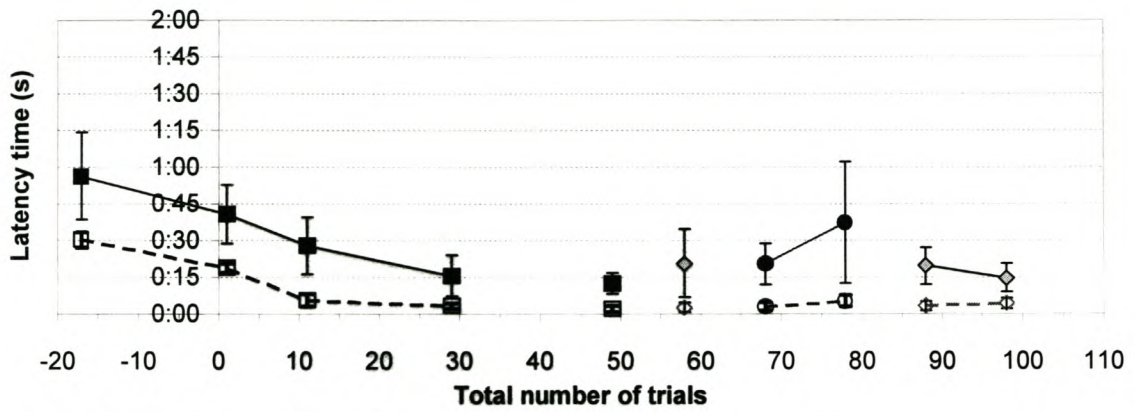


Figure 3.14 Daily and best daily mean latencies of electrical CS conditioning of female *M. mustelus*, RY_e.

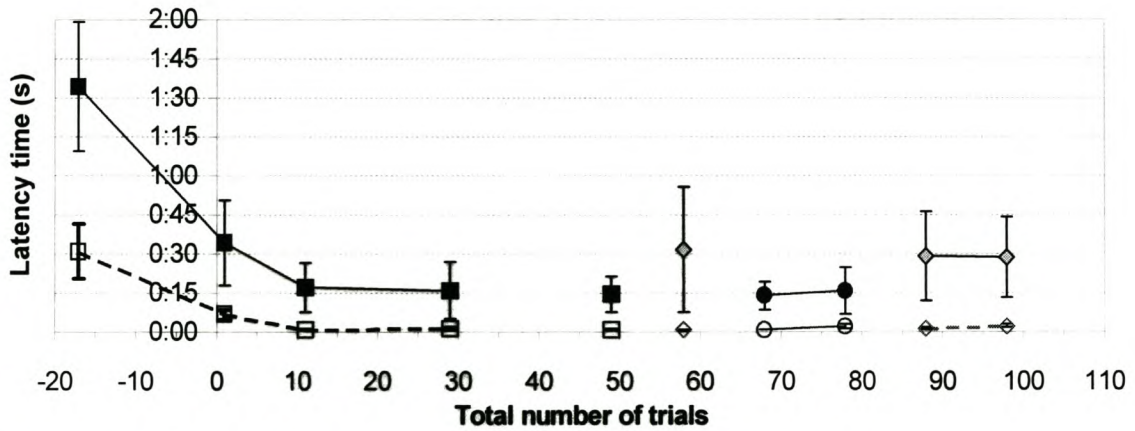
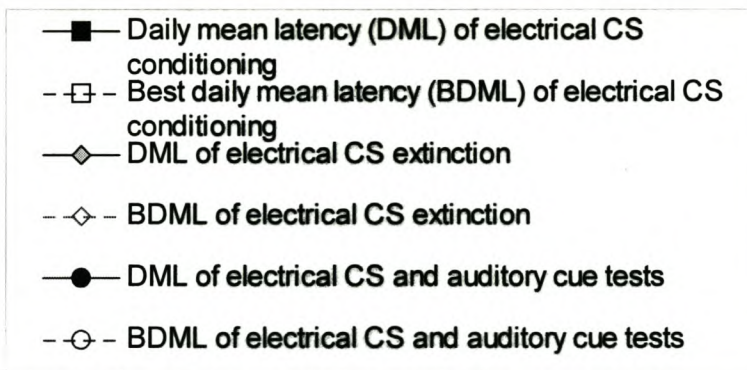


Figure 3.15 Daily and best daily mean latencies of electrical CS conditioning of female *M. mustelus*, Y_e.



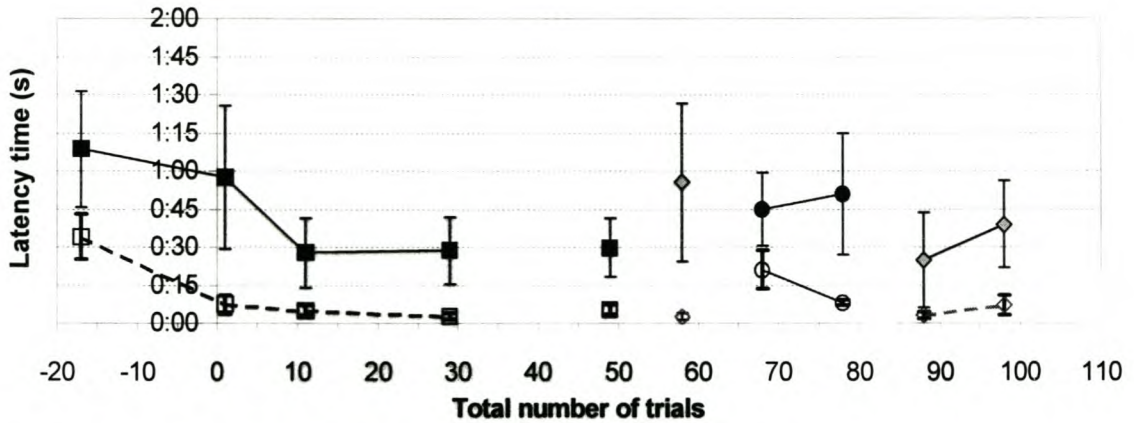
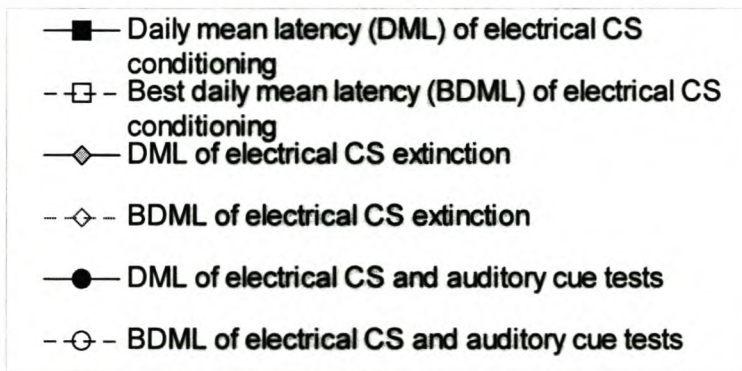


Figure 3.16 Daily and best daily mean latencies of electrical CS conditioning of male *M. mustelus*, YR_e.



For one shark, YR_e, conditioning was not as evident (Fig. 3.16). A downward trend in the daily mean latencies during conditioning training was observed for this shark, but it was not significant (Kruskal-Wallis ANOVA: $H = 5.997$, $p = 0.199$). However some association between the CS and the presentation of food did begin to develop as a Wilcoxon Mann Whitney test found a significant difference in performance on pre-train and condit₂ ($U = 16.5$, $p = 0.023$). Although the downward trend in the best daily mean latencies exhibited during conditioning training (Kruskal-Wallis ANOVA: $H = 7.975$, $p = 0.093$) was not significant, the downward trend from pre-train to ext₂ was significant (Kruskal-Wallis ANOVA: $H = 12.898$, $p = 0.045$). Kolmogorov Smirnov tests found significant differences between pre-train and condit₁ ($D = 1$, $p < 0.100$), condit₂ ($D = 1$, $p < 0.100$), condit₃ ($D = 1$, $p < 0.100$) and condit₅ ($D = 1$, $p < 0.100$). In addition, the best

daily mean latencies stayed under 15 s from $condit_2$. Consequently, this shark entered the feeding area after the presentation of the CS, even before the presentation of the US. Thus, this shark anticipated the presentation of food to follow the CS from $condit_2$.

Thus, conditioning could be demonstrated for four (B_e , R_e , RY_e and Y_e) of the five sharks after five days of conditioning training, or 57 conditioning trials. In addition, the conditioned association between the electrical CS and the presentation of food began to form in the 5th shark, YR_e , and conditioning in this shark could be demonstrated on ext_2 .

To compare shark behaviour during the CS experiment with that during the CS control, the c-values averaged over every 10 trials of individual sharks in the CS experiment was analysed. A Friedman ANOVA found significant differences in behaviour among sharks over the first 40 exposures to the CS ($F = 9.600$, $p = 0.048$, $n = 5$). This variance was due to differences in behaviour between the shark Y_e and all the other sharks ($Z = 1.604$, $p = 0.109$); and between R_e and all the other sharks ($Z = 1.604$, $p = 0.109$). Thus, only the c-values of B_e , RY_e and YR_e were combined for comparison with the CS control.

b) Extinction - During extinction training, all the sharks continued to enter the feeding area after the presentation of the CS even though no food was provided. On the first day of extinction training, ext_1 , the level of conditioned responses, was the same as at the end of conditioning, $condit_5$, for all the sharks, except B_e (Fig. 3.12 – 3.16). Wilcoxon Mann Whitney found no significant differences in the daily mean latencies between $condit_5$ and ext_1 for the sharks, R_e , RY_e , Y_e and YR_e (B_e : $U = 17.5$, $p = 0.043$; R_e : $U = 17$, $p = 0.074$; RY_e : $U = 40$, $p = 1.000$; Y_e : $U = 39.5$, $p = 0.965$; YR_e : $U = 41.5$,

$p = 0.780$). However, a significant difference in performance between pre-train and ext₁ was demonstrated only for three (B_e, RY_e and Y_e) of the sharks (Wilcoxon Mann Whitney tests - B_e: $U = 7$, $p = 0.011$; R_e: $U = 18.5$, $p = 0.328$; RY_e: $U = 6$, $p = 0.012$; Y_e: $U = 19$, $p = 0.006$; YR_e: $U = 21.5$, $p = 0.368$). These results were reflected in the best daily mean data. Kolmogorov Smirnov tests found no significant difference in performance on condit₅ and ext₁ for all the sharks, except B_e (B_e: $D = 1$, $p < 0.100$; R_e: $D = 0.667$, $p > 0.100$; RY_e: $D = -0.333$, $p > 0.100$; Y_e: $D = 0.333$, $p > 0.100$; YR_e: $D = 0.333$, $p > 0.100$) and a significant difference between pre-train and ext₁ for all the sharks (B_e: $D = 1$, $p < 0.100$; R_e: $D = 1$, $p < 0.100$; RY_e: $D = 1$, $p < 0.100$; Y_e: $D = 1$, $p < 0.100$; YR_e: $D = 1$, $p < 0.100$). Thus, the CS-US association was formed sufficiently strongly to be maintained on ext₁ when no reinforcement was received by three (B_e, RY_e and Y_e) of the sharks.

The CR was maintained until the end of extinction training, ext₅, for all the sharks (Fig. 3.12 – 3.16). Wilcoxon Mann Whitney and Kolmogorov Smirnov tests found no significant differences in the daily mean latencies between condit₅ and ext₅, for any of the sharks (B_e: $U = 8$, $p = 0.016$; R_e: $U = 19.5$, $p = 0.068$; RY_e: $U = 38$, $p = 0.863$; Y_e: $U = 28.5$, $p = 0.315$; YR_e: $U = 40$, $p = 0.720$) and none in the best daily mean latencies for any of the sharks, except for B_e (B_e: $D = -1$, $p < 0.100$; R_e: $D = 0.667$, $p > 0.100$; RY_e: $D = 0.667$, $p > 0.100$; Y_e: $D = -0.667$, $p > 0.100$; YR_e: $D = 0.333$, $p > 0.100$). However the best daily mean latency of the shark, B_e, was still significantly lower on ext₅ than on pre-train (Kolmogorov Smirnov: $D = 1$, $p < 0.100$). Thus, after 40 extinction trials, where the CS-US association was not reinforced by the presentation of

food, the CS-US association formed during initial training was as strong as before extinction training for all the sharks.

3.4.2.3 Comparison of CS control and CS experiment:

All the sharks in the CS control, B_c and YR_c , and the sharks, B_e , RY_e and YR_e , in the CS experiment were used for comparison of shark behaviour in these treatments. Shark behaviour during the CS control did not change during the first 40 control trials. In contrast, the behaviour of the sharks in the CS experiment changed during the first 40 exposures to the electrical CS (Fig. 3.17). These sharks responded faster to the electrical CS after the 40th exposure, than at the beginning of training. Wilcoxon Mann Whitney tests showed significant differences only between the c-values of the sharks in the CS control and the CS experiment during the 21st – 30th ($U = 21$, $p = 0.029$) and 31st – 40th exposure to the CS ($U = 18$, $p = 0.015$). Thus, from the 21st exposure to the electrical CS, shark behaviour in the CS experiment differed significantly from that in the CS control.

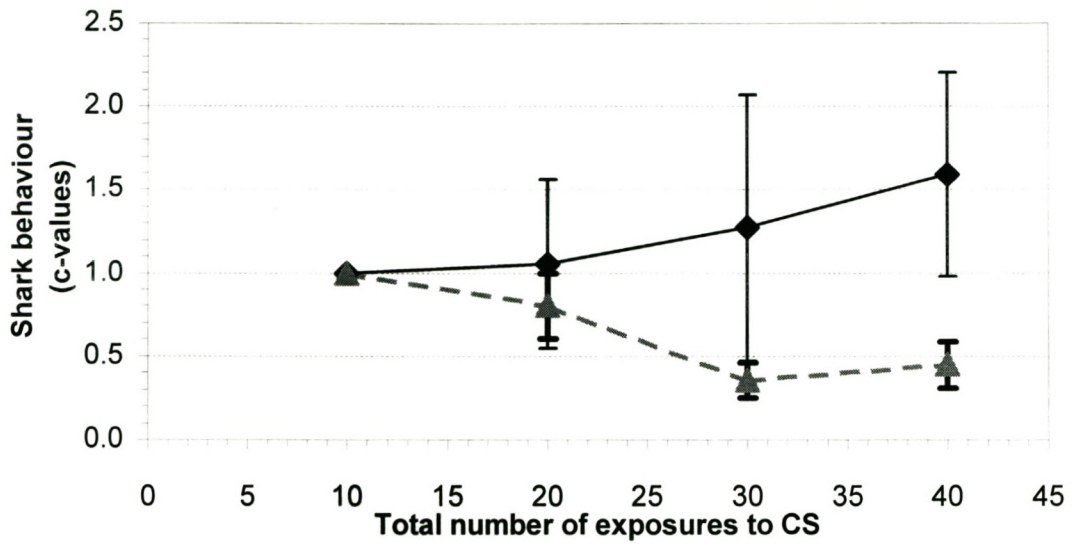


Figure 3.17 Shark behaviour during electrical CS control (B_c , YR_c) and CS experiment (B_e , RY_e , YR_e) expressed as c-values.



3.5 Discussion

The results suggest both the auditory and electrical conditioned stimuli were successful in signalling the presentation of food (US) for smoothhound sharks. Conditioning was evidenced by the differences in shark behaviour observed in the CS controls and CS experiments. As found in the previous chapter with a compound auditory and electrical CS, shark behaviour did not change significantly during control trials, whereas in the CS experiments, behaviour, in terms of response time, changed significantly for the majority of sharks (Chapter 2). In both CS experiments, response time to the particular CS decreased, indicating the formation of a conditioned association between the presentation of the CS and food (US). The conditioning rate and success of these CS experiments were similar to those in a chapter 2, in which three out of four smoothhounds were conditioned after five conditioning training days (69 conditioning trials) to associate a compound auditory and electrical CS with food.

In the auditory CS experiment, four (B_a , R_a , RY_a and Y_a) of the five sharks formed a strong conditioned association between the auditory CS and the presentation of food after five days of conditioning training or 60 conditioning trials (Fig. 3.4 – 3.7). This association also formed in the 5th shark (YR_a), but could only be demonstrated statistically on ext₄, after 60 conditioning trials and 30 extinction trials (Fig. 3.8). Conditioning of an auditory CS and food (US) association has been done successfully on other shark species. Davies *et al.* (1963) conditioned several shark species, *Carcharhinus obscurus* (Dusky shark), *C. brevipinna* (Spinner shark), *C. leucas* (Bull shark) and *Sphyrna lewini* (Scalloped hammerhead) to approach a feeding area when an auditory cue was presented after 12 training days over a period of a month.

Indirect evidence for the formation of a conditioned association between an auditory CS and the presentation of food can be found in several operant conditioning studies with lemonsharks (Aronson *et al.* 1967; Clark 1959; Wright & Jackson 1964) and nurse sharks (Aronson *et al.* 1967). These sharks were taught to press a target, causing a bell to ring, in order to obtain food. A food reward was given only when the target was pressed sufficiently hard to ring the bell. Thus, the sharks were rewarded with food only when the auditory cue was produced. Conditioning rate varied according to the age of test animals and experimental conditions. The conditioned association was formed faster by juvenile sharks than by adults of the same species, and was also faster when they were trained in isolation, rather than in a group. In a group situation, adult lemonsharks formed the conditioned association after 30 training days (Clark 1959) and juvenile lemonsharks after only 14 training days (Wright & Jackson 1964). For juvenile lemon and nurse sharks trained in isolation, training took only five days (Aronson *et al.* 1967). From these studies and the results from this study, it seems that several shark species can be conditioned to associate an auditory CS with the presentation of food (US).

As with the auditory CS, sharks were successfully conditioned to associate an electrical CS with the presentation of food. Four (B_e , R_e , RY_e and Y_e) of the five sharks in the electrical CS experiment formed a strong CS-US association after five days of conditioning training (Fig. 3.12 – 3.15: 57 conditioning trials). Conditioning could also be demonstrated for the fifth shark (YR_e) on ext_2 , after 57 conditioning trials and 10 extinction trials (Fig. 3.16). From this experiment, it seems that smoothhound sharks can be conditioned to associate an electrical CS with the presentation of food (US). Similarly, Kalmijn & Kalmijn (1981) found that California round stingrays used electrical cues to locate food in a conditioning study.

Although both the **auditory and electrical CS** signalled the presentation of food, these conditioned stimuli were not equally successful, as a slight qualitative difference in the conditioned associations was found. A stronger CS-US association was formed between the US and the **electrical CS** than with the auditory CS. In the auditory CS experiment, the response rate of only one (R_a) of the five sharks was significantly lower after conditioning (on ext_1) than before conditioning began, on pre-train (Fig. 3.5). In contrast, the response rates of three (B_e , RY_e and Y_e) of the five sharks in the electrical CS experiment were significantly lower after conditioning training than before (Fig. 3.12, 3.14, 3.15). Thus, the conditioned association between the electrical CS and the US was sufficiently strong to be maintained when the conditioned association was not reinforced by the presentation of food on ext_1 . Therefore the electrical CS was a slightly more effective signaller of the US. Furthermore, after 40 exposures to the CS, sharks in the electrical CS experiment demonstrated a greater difference in behaviour between experimental and control sharks than in those in the auditory CS experiment. Thus, conditioning had proceeded further with the electrical CS than with the auditory CS after 40 exposures to the CS (Fig. 3.9, 3.17).

In view of the “belongingness” phenomenon, this study demonstrates that both auditory and electrical cues are biologically relevant in signalling the presence of prey for smoothhound sharks. **However the electrical cue was biologically more relevant than the auditory cue in this study. This may be due to the previous feeding experiences of the smoothhound sharks used in this study. The prey taken by these sharks in the field before being caught, were likely to have been small Caridea and polychaetes (Smale & Compagno 1997) which can burrow into the substratum or be hidden among sea weeds (Branch *et al.* 1994). Few auditory cues would be obtained from this nature of prey.**

However all animals produce weak animate electrical fields - usually between 1 – 500 $\mu\text{V}/\text{cm}$, but reaching up to 1000 $\mu\text{V}/\text{cm}$ in wounded crustaceans (Kalmijn 1974). Consequently, smoothhound sharks can detect prey using electrical cues, although the prey may be visually hidden and producing few, if any, auditory cues. During previous encounters with these prey types in the field, sharks may have learnt through classical conditioning that electrical cues are reliable signallers of the presence of prey. By contrast, these sharks would seldom have used auditory cues to locate their prey. This may explain the observation that conditioning with the electrical CS was more successful than with the auditory CS.

However, the study found auditory cues also to be relevant signallers of prey. This may be due to the opportunistic feeding behaviour of smoothhounds. The diet of smoothhound sharks changes with age, larger sharks being able to take larger crustacean prey; as well as with depth and location due to the availability of different prey populations (Smale & Compagno 1997). Furthermore, part of their diet may comprise scavenged fisheries offal and the remains of the kills of other predators (Smale & Compagno 1997). As smoothhound diet change with age and location, the cues to which these sharks attend to, for the location of food would need to change. Consequently, it would be adaptive for smoothhound sharks to have evolved to respond to a variety of cues (auditory, electrical, olfactory and visual cues) that might signal the presence of food. By the mechanism of classical conditioning during feeding experiences, the more reliable signals of the location of prey may be incorporated into the feeding behavioural system and the unreinforced cues abandoned. This would increase feeding efficiency, which in turn translates into reproduction success and consequently increases fitness (McCleery 1978).

For sharks with different diets and feeding strategies, the interaction between the auditory and electrical sense may be different. Active hunters of pelagic fish, like carcharhinid and sphrynid sharks have been attracted by auditory cues of struggling fish and fish vocalisations (Nelson & Gruber 1963; Banner 1972). Morphologically carcharhinid sharks have a better developed inner ear structure than sedentary sharks feeding mainly on invertebrates (Bleckmann & Hofmann 1999). Thus, the auditory sense may be more important in the feeding behaviour of these sharks. Consequently in comparative studies with these sharks, little difference in the conditioning success may be observed between the auditory and electrical CS.

To conclude, smoothhound sharks can be conditioned to associate either an auditory or electrical CS with the presentation of food. The evolution of the possibility of the formation of connections between both auditory and electrical cues and the location of prey may have enabled smoothhounds to be opportunistic feeders. However conditioning with the electrical CS was more successful, indicating electrical cues are biologically more relevant for signalling the presence of food. During previous feeding experiences, by the mechanism of classical conditioning, electrical cues were incorporated into smoothhound feeding behaviour as reliable signallers of the presence of prey while auditory cues were less reliable. Comparative experiments with sharks with different diets may further demonstrate how classical conditioning during feeding experiences, influence the feeding behaviour of sharks. At least in smoothhound sharks, classical conditioning is a mechanism whereby smoothhound sharks can be maximally efficient by reacting only to reliable cues signalling prey.

3. 6 References

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CHAPTER 4

CONCLUSIONS

The role of classical conditioning, as well as that of the auditory and electrical senses in the feeding behaviour of the smoothhound shark *Mustelus mustelus* were investigated. This was done in the first instance by studying the general conditioning process with a compound auditory and electrical conditioned stimulus (CS) and the presentation of food as the unconditioned stimulus (US), in the context of the adaptive approach. Second, the relative conditioning success with separate auditory and electrical conditioned stimuli was compared, in order to determine the most biologically relevant cue signalling the presence of food.

Classical conditioning experiments demonstrated that smoothhound sharks have the ability to associate the presentation of a compound auditory and electrical CS with food (US). This CS-US association was formed in the majority of the sharks after only 57 to 60 conditioning trials, conforming to evidence from conditioning studies with several shark species. However, individual variation was observed in the time required and success of conditioning. This intraspecific variation in learning abilities corresponds with that found in other learning studies with fish, and was most likely due to differences in motivational drives, such as hunger.

Despite individual variation, the compound CS-US association formed strongly enough in the sharks to be retained over a two-month rest period, and was resistant to extinction. This retention of the conditioned response (CR) without reinforcement of the CS can be explained by the adaptive approach. During conditioning, the CR became

part of the feeding behaviour of these sharks, affording it adaptive value and it consequently became difficult to eliminate, even when it no longer served a purpose. The adaptive approach also explains the anticipatory nature of the observed CR. During classical conditioning, the CS came to signal the presentation of the US (food) and the CR prepared the sharks to optimise interaction with the US. Thus, classical conditioning provides a mechanism for smoothhound sharks to adapt to utilise new food sources effectively.

Adaptability may be vital in the lifestyle of smoothhound sharks, as their diet changes with growth, as well as with the depth at which they live, which changes from shallow-water prey (e.g. Three-spot swimming crab *Ovalipes trimaculatus* and Cape rock crab *Plagusia chabrus*) taken by sharks inshore, to mainly crustaceans taken between 50 and 100 m, and octopods taken by sharks deeper than 100 m. Furthermore, part of their diet may be scavenged. Thus, these sharks are opportunistic feeders. Consequently, the cues this species uses to find food would have to change as individuals grow or migrate. By the process of learning, through classical conditioning, smoothhound sharks can change the cues to which they respond and thereby adapt to changing prey populations during their lifetimes.

The importance of the separate auditory and electrical components of the compound CS to signal the presence of food could not be determined in the compound conditioning study. Subsequently, classical conditioning studies with separate auditory or electrical conditioning stimuli were conducted to determine the relative importance of these senses in the feeding behaviour of smoothhound sharks.

These experiments demonstrated that smoothhound sharks do have the ability to form an association between the presentation of food (US) and either an auditory or electrical CS. The majority of the sharks associated the presentation of food with the auditory CS after 60 conditioning trials and with the electrical CS after 57 conditioning trials. However, slight qualitative differences were found in the conditioned CS-US associations. A stronger CS-US association was formed with the electrical CS than with the auditory CS. Furthermore, shark behaviour in the experimental group differed more from that in the control in the electrical CS experiment than in the auditory CS experiment. Thus, conditioning had progressed further with the electrical CS than with the auditory CS. According to the "belongingness" phenomenon, this indicates that although both auditory and electrical cues can be biological relevant signals of the presence of food for smoothhound sharks, the electrical cue is biologically more relevant.

This superior biological relevance of the electrical cue is thought to be due to previous feeding experiences of the sharks used in this study. These sharks may have mainly taken invertebrate prey that produced reliable electrical cues, but few auditory cues. During encounters with prey, the sharks would have learnt through classical conditioning that electrical cues are reliable signallers of their location, but would have seldom used auditory cues to locate prey. Consequently, the electrical cue was shown to be more biologically relevant than the auditory cue. Clearly, classical conditioning can influence the formation of the feeding behaviour of smoothhound sharks.

The continued biological relevance of auditory cues to signal the presence of food, despite its unreliability, may be due to the opportunistic feeding behaviour of smoothhound sharks. It would be of adaptive value to have evolved to respond to a

variety of cues (auditory, electrical, olfactory and visual cues) that might possibly signal the presence of food. Possibly smoothhound sharks have the ability to form associations between a variety of cues. However during feeding experiences, the more reliable signals of the presence of prey may become incorporated in the feeding behavioural system and the unreinforced cues abandoned, by the mechanism of classical conditioning. This optimises the feeding behaviour of smoothhound sharks, by enabling sharks to react only to reliable cues signalling the presence of prey.

Future research focussing on comparative conditioning studies with sharks with different diets and feeding strategies is suggested. For more active hunters of pelagic prey, such as carcharhinid sharks, the auditory sense may be more important in detecting prey than sharks feeding on invertebrate prey. Consequently, conditioning with an auditory CS should be more successful with carcharhinid sharks than in species feeding on invertebrates. Furthermore, conditioning success with sharks caught in the field compared to those reared in aquaria, using several modalities of conditioned stimuli would illustrate the role of classical conditioning during feeding experiences on the formation of feeding behaviour.