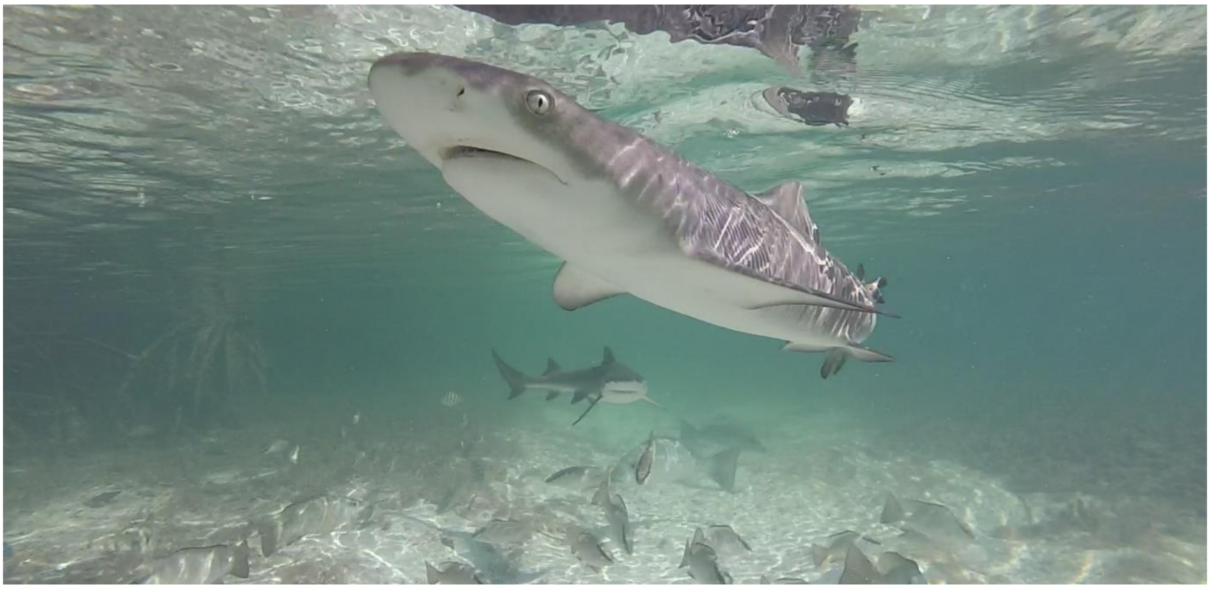
Learning capabilities of sharks in the context of behavioural changes caused by wildlife tourism



By

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Thesis summary

Learning enables animals to develop adaptive responses to novel situations and changing environmental conditions based on individual experience. Although the cognitive abilities of birds, mammals, and bony fishes have been intensely studied, little research has focused on the learning capacity of Chondrichthyans (sharks, rays, chimaeras). The early divergence of sharks and rays from other vertebrates makes sharks an ideal model system to explore the evolution of cognition in vertebrates. The need for an understanding of the cognitive ability of chondrichthyans has also recently been highlighted in relation to the effects of wildlife tourism. Recent studies have documented changes in behaviour, feeding ecology, and body condition in response to tourism related feeding activities. Therefore, the overall objective of my thesis is to improve our understanding of the cognitive abilities of sharks in relation to shark tourism.

Habituation acts as a fundamental filtering mechanism that can free up neuronal resources. Understanding its limitations in sharks will help to better understand its evolutionary origin. Following the daily exposure to the smell of squid, the response of Port Jackson sharks rapidly decreased. This suggests that the use of smell as a daily attractant for tourism operators could result in sharks becoming less likely to respond over time. Building on findings from this study on a fundamental cognitive capacity of sharks, I tested the effects of reward frequency and magnitude on learning rates. Sharks were trained in a simple spatial cognitive task consisting of the choice between two potential foraging pathways. Findings from this study suggest that the frequency at which sharks were trained had greater influence on learning performance than the amount of food they received as a reward.

Tracking food resources through time and space is vital for maximising fitness. Learning about novel food patches often requires animals to make an association between temporal and spatial information. To assess whether juvenile lemon sharks are able to learn such time-place associations I used semi-captive experiments, where sharks were fed daily over 41 days at two distinct times and locations inside a 45 m^2 enclosure. I found no evidence of time-place learning in this species; rather, juvenile lemon shark movement was more affected by tidal fluctuations than daily feedings. To determine if juvenile lemon sharks are able to anticipate feedings under natural conditions, I initiated a daily feeding regime over 27 days at a novel location. Sharks started to anticipate feeding events within ~11 days, as shown by a change in their fine-scale movement patterns and activity. While activity was affected by feeding, it was not sufficient to affect the sharks' field metabolic rates or energy requirements.

Overall, my PhD thesis advances our understanding of the cognitive capacities and limitations of elasmobranchs, which helps to fill a knowledge gap in the fields of biology, neurophysiology, ethology, and ecology. My thesis further provides experimental evidence that supports the view that cognitive capacities of elasmobranchs are similar to those found in other vertebrate groups. Results from my thesis also provide empirical information for decisions and policies of wildlife tourism management that involve feeding sharks. Based on my findings, I emphasise the importance of managing the frequency of shark feeding operations as a more sustainable approach compared to the amount of food used per feeding event. While these data are applicable to wildlife tourism and conservation planning for these and other benthic and epipelagic shark species, future studies should aim to determine species-specific implications and investigate ontogenetic changes in cognitive abilities.

Declaration

I certify that this thesis:

1. does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and
2. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed…

Date: 21.02.2021

Author contributions, permits, and funding

The following outlines the author contribution and acknowledgements for each data chapter:

Chapter 2

This study was designed by Dennis Heinrich, Culum Brown and Charlie Huveneers. DH was responsible for setting up and carrying out the experimental trials with the assistance of a number of volunteers, analysed the video recordings and ran the statistical analyses with the assistance of CB and CH, Félicie Dhellemmes and Tom Houslay. DH wrote the manuscript with the advice of CB and CH.

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Chapter 3

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All authors designed the study. DH and CVP carried out the experimental trials. DH performed the video and statistical analyses and wrote the manuscript with the advice of CVP, CB, and CH.

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Chapter 5

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All authors designed the study. DH, FD and TLG were responsible for the tagging of the sharks with the assistance of the BBFS staff and volunteers. DH carried out the daily feedings with the help of the BBFS staff and volunteers. The statistical analyses and write-up of the manuscript were done by DH with the advice of FD, TLG, MS, CB, JR, CH and Vinay Udyawer.

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Conference presentation relevant to this thesis

International Conference on Fish Telemetry, Arendal, Norway 2019. “Spatial distribution and activity of juvenile lemon sharks (*Negaprion brevirostris*) following the establishment of a novel feeding site”

Australian Society for Fish Biology and Oceania Chondrichthyan Society Joint Conference, Wrest Point, Tasmania, Australia 2016. “Shark cognition and its implication towards wildlife tourism”

Other publications or contributions

Vila Pouca, C., **Heirnich, D**., Huveneers, C., Brown, C. (2020). Social learning in solitary juvenile sharks. Animal Behaviour, 159, 21-27.

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Chapter 1

General Introduction

1.1 Animal cognition

The field of animal cognition attempts to understand the mental capabilities of animals, including the means by which an animal acquires, processes, and acts upon information gathered from its environment (Shettleworth, 2010, Brown et al., 2011). Although it was previously believed that animals acted autonomously until the late 1800s, Darwin and Romanes recognised cognitive abilities in animals (Darwin, 1871, Romanes, 2015), which was the first step towards modern experimental animal psychology. The work of Thorndike and Skinner (Skinner, 1948, Skinner, 1963, Thorndike, 1898) further progressed this field of research through the development of repeatable and quantifiable experimental protocols. By the mid-1950s, cognitive psychologists started to compare known human mental processes to those in birds and mammals. Since then, birds and mammals have been demonstrated to excel at a wide variety of cognitive abilities, ranging from habituation to constructing a cognitive map and the ability to use tools for a range of tasks (Hansell and Ruxton, 2008, Harten et al., 2020, Taylor et al., 2009, Mbise et al., 2020, Uchida et al., 2019). Many of these processes are based on phenotypic plasticity that allows for short- and long-term adaptive changes in behaviour (Brown, 2012, Guttridge et al., 2009b, Schluessel, 2015). Learning can be a way to achieve these behavioural changes, allowing animals to develop adaptive responses to novel situations based on experience within an individual's lifetime (Guttridge and Brown, 2013, Kawecki, 2010, Dill, 1983, Pearce and Bouton, 2001).

1.2 Fish and shark cognition

To this day most studies within the field of animal cognition have been focusing on birds and mammals (Brown et al., 2011). The early approach taken by cognitive psychologists in the 1950s is partly responsible for this bias, as they began to compare mental processes in humans to other species that shared a similar neocortex organisation. The widely spread misconception that teleosts and elasmobranchs were automatons with their behaviour being almost exclusively controlled by unlearnt predispositions further fuelled this bias (Northcutt, 2011, Schluessel, 2015, Brown et al., 2011), as did the deeply rooted belief that the cognitive evolution of vertebrates follows a linear progression from inferior to superior forms (Brown, 2015, Brown et al., 2011). With about 32,000 known extant species of teleosts and over 1,000 species of elasmobranchs, fishes are the most speciose group of vertebrates (Brown, 2015, Dulvy et al., 2014). They occupy almost every environmental niche available with the natural environment shaping the species morphology, physiology, and behaviour. Therefore, it comes as no surprise that bony fishes also show the widest range of variations in brain functions in all vertebrates (Nieuwenhuys et al., 2014) . Despite their overwhelming diversity and their evolutionary success, which has been supported by the adaptation of brain structures and higher cognitive abilities, cognition still remains poorly studied in bony fishes (Schluessel, 2015). However, an upsurge in the number of studies conducted on the cognitive abilities of teleosts has occurred over the last 25 years, acknowledging the significance of understanding cognitive processes in ancient vertebrate groups to reveal the evolutionary history of vertebrate brains. More recently, studies have investigated the cognitive abilities of elasmobranchs (sharks and rays). Being the most ancient group of jawed vertebrates, they have evolved and adapted to a variety of environmental conditions (Compagno, 1999).Therefore, they hold a key phylogenetic position to understanding the evolution of brain structures in jawed vertebrates (Schluessel, 2015, Yopak et al., 2007, Yopak et al., 2010). Although sharks and rays have been demonstrated to show sophisticated behaviours and to have a complex biology with sensory systems that are well adapted to life underwater (Hueter et al., 2004), knowledge on their cognitive abilities, such as learning and memory, and the corresponding brain areas is still limited.

Despite the early interest in shark cognition between the 1950s and 1970s, research on this topic decreased during the 1980s and 1990s, likely due to the logistical difficulties working with sharks and the negative public perception of these animals at the time. The early studies focused mainly on associative learning during operant and classical conditioning regimes. Classical conditioning occurs when two distinct events overlap in space and time, allowing for an originally neutral stimulus to be associated with an aversive or rewarding stimulus (Lieberman, 1999). In contrast, operant conditioning describes the learning process of a certain behaviour using a reward or punishment (Staddon and Cerutti, 2003). Among the first attempts to find evidence for associative conditioning in elasmobranchs, Eugenie Clark trained adult lemon sharks (*Negaprion brevirostris*) to bump into an underwater target to receive a remotely placed food reward. This was also one of the first studies indicating long-term memory capacities in sharks with the two trained lemon sharks retaining the conditioned response 10 weeks of inactivity (Clark, 1959). A few years later, the same experimental design was used to test and compare the learning rates of lemon sharks and bull sharks (*Carcharhinus leucas*). Whilst learning rates were similar among conspecifics, they differed between species, with lemon sharks learning faster than bull sharks (Wright and Jackson, 1964).

These basic findings were later confirmed during further operant conditioning studies using both positive and negative reinforcement regimes, and extending the results to include acoustic (Kritzler and Wood, 1961, Nelson, 1967) and visual discrimination learning (Aronson et al., 1967, Tester and Kato, 1966). A comparison between the learning rates in different taxa of vertebrates (mouse, teleost, and shark) suggested that sharks are able to learn discrimination tasks as quickly as other vertebrates (Aronson et al., 1967, Bitterman, 1965, Schneirla, 1962). Although most studies carried out during this period took a purely behavioural approach, some researchers broadened their discrimination experiments to incorporate behaviour, brain function, and brain structure. For instance, a study by Graeber et al. (1973) investigated the visual discrimination learning in six juvenile nurse sharks (*Ginglymostoma cirratum*) following the surgical removal of the optic tectum in three individuals. The optic tectum is the primary visual center in nonmammalian vertebrates. It controls the mechanics involved in visuomotor behaviours. The three sharks subjected to the surgery were able to learn the visual discrimination task within a similar period of time compared to the three non-operated individuals. This indicates that some type of recovery of the visual function occurred and contradicts the traditional view of exclusive tectal control over visual behaviour in lower vertebrates (Graeber et al., 1973). Building on these first experiments on operant conditioning, Gruber and Schneiderman (1975) conducted the first controlled study using a classical conditioning approach. During their study Gruber and Schneiderman (1975) paired a conditioned stimulus (light flash) with an unconditioned stimulus (electric shock), producing an eye-blink response in lemon sharks. The 20 juvenile lemon sharks tested were each exposed to 100 trials per day for seven consecutive days. Using this technique, they found that classical conditioning of the nictitating membrane (protective membrane covering the eye) response occurred reliably in 95% of the tested individuals (Gruber and Schneiderman, 1975). These results were among the first to demonstrate the similarities in classical conditioning responses between sharks and mammals.

Learning in general can be either non-associative or associate. Whilst the latter results in an association between two distinct events, the former is based on a repeated stimulation with a single stimulus (Lieberman, 1999). Non-associative learning can further be divided into three different forms: habituation, dishabituation, and sensitisation (Rankin et al., 2009, Thompson and Spencer, 1966). In contrast, associative learning is more variable, with forms including the recognition, discrimination, and categorisation of objects (Fuss et al., 2018, Gierszewski et al., 2013, Schluessel and Duengen, 2015, Schluessel et al., 2012), perception of symmetry and illusory contours (Agrillo et al., 2013, Fuss et al., 2014a, Merry and Morris, 2001, Schluessel et al., 2014a), acquisition of spatial cognitive tasks (Portavella and Vargas, 2005, Schluessel and Bleckmann, 2012, Schluessel and Ober, 2018, Sovrano et al., 2005), social learning (Guttridge et al., 2013, Vila Pouca et al., 2020), memory retention (Fuss and Schluessel, 2015, Guttridge and Brown, 2013), and tool use (Kuba et al., 2010). Each of these types of associative learning has been demonstrated in both teleosts and elasmobranchs (Table 1.1). My thesis primarily focuses on habituation, spatial learning, and memory retention with some aspects of object recognition in sharks. Therefore, the remainder of this introduction will provide background information on each of these four types of learning.

Table 1.1 Summary of previous studies investigating cognitive abilities of elasmobranchs. The list includes the species, age group, ecological niche and the type of conditioning that was tested.

Species	**Age group**	**Ecological niche**	**Type of conditioning**	**Paper**
Negaprion brevirostris	Adult	Epipelagic	Object recognition	Clark 1959
Carcharhinus leucas	Adult	Epipelagic	Auditive discrimination	Kritzler and Wood 1961
Negaprion brevirostris	Juvenile	Epipelagic	Object recognition	Wright and Jackson 1964
Carcharhinus leucas	Juvenile	Epipelagic		
Carcharhinus melanopterus	Juvenile	Epipelagic	Object discrimination	Tester and Kato 1966
Carcharhinus menisorrah	Juvenile	Epipelagic		
Ginglymostoma cirratum	Juvenile	Benthic	Object recognition and discrimination	Aronson et al. 1967
Negaprion brevirostris	Juvenile	Epipelagic	Auditive discrimination	Nelson 1967
Ginglymostoma cirratum	Juvenile	Benthic	Object discrimination	Graeber et al. 1973
Negaprion brevirostris	Juvenile	Epipelagic	Nictitating membrane response	Gruber and Schneiderman 1975
Potamotrygon castexi	Subadult	Benthic	Tool use	Kuba et al. 2010

Chiloscyllium griseum	Juvenile	Benthic	Spatial learning and memory retention	Schluessel and Bleckmann 2012
Heterodontus portusjacksoni	Juvenile	Benthic	Conditioned learning and memory retention	Guttridge and Brown 2013
Negaprion brevirostris	Juvenile	Epipelagic	Social learning	Guttridge et al. 2013
Chiloscyllium griseum	Juvenile	Benthic	Object discrimination	Fuss et al. 2014
Chiloscyllium griseum	Juvenile	Benthic	Memory retention	Fuss et al. 2015
Chiloscyllium griseum	Juvenile	Benthic	Object discrimination	Schluessel and Duengen 2015
Chiloscyllium griseum	Juvenile	Benthic	Object discrimination	Fuss et al. 2018
Potamotrygon motoro	Juvenile	Benthic	Spatial learning	Schluessel and Ober 2018
Heterodontus portusjacksoni	Juvenile	Benthic	Social learning	Vila Pouca et al. 2020

1.2.1 Habituation

Habituation describes the decrementing response following repeated stimulation of a stimulus (Rankin et al., 2009, Thompson and Spencer, 1966, Marcus et al., 1988). It has been demonstrated in a wide range of phyla, including nematodes (Hilliard et al., 2005, Rankin and Broster, 1992), insects (Baracchi et al., 2018, Das et al., 2011, Haupt and Klemt, 2005), birds (Dong and Clayton, 2009, Mbise et al., 2020, Petrinovich and Peeke, 1973), teleosts (Baenninger, 1970, Figler, 1972, Peeke and Peeke, 1970, Randlett et al., 2019, Staven et al., 2019), and mammals (Bolivar, 2009, Kirmani et al., 2010, Uchida et al., 2019), and is commonly referred to as the simplest, most elementary form of behavioural plasticity

(Lieberman, 1999, Thompson, 2009). By allowing animals to ignore recurring irrelevant stimuli, habituation acts as a fundamental filter mechanism and becomes prerequisite for other forms of learning (Rankin et al., 2009, Thompson, 2009, Thompson and Spencer, 1966). Ignoring irrelevant stimuli frees up neuronal resources, which in turn can be allocated to other, more complex cognitive tasks. Therefore, habituation is also considered to be a cognitive building block (Rankin et al., 2009, Wilson and Linster, 2008, Fabiani et al., 2006, Sinding et al., 2017).

In teleosts, habituation has been demonstrated to occur in multiple species and in response to a variety of stimuli targeting different sensory modalities. Habituation has further been tested in bony fishes across different temporal scales, including short-term (i.e., an individual's response decrements within a single day or training session) and long-term habituation (i.e. an individual's response decrements over multiple days or training sessions). Both short- and long-term habituation have been shown in female Trinidadian guppies (*Poecilia reticulata*) in response to recurring male colour patterns during courtship, thereby selecting a more diverse gene pool (Daniel et al., 2019). Long-term habituation in response to a social stimulus has also been reported in zebrafish that were repeatedly exposed to a group of conspecifics (de Almeida Moura and Luchiari, 2016). In accordance with the principals of habituation, the rate of habituation in bony fishes can vary significantly with stimulus type, intensity, and frequency (Daniel et al., 2019, Post and von der Emde, 1999, Wong et al., 2010, Staven et al., 2019). This was demonstrated in the electrogenic fish *Gnathonemus petersij* in response to different sensory modalities, including acoustic (frequency: 500 Hz for 100 ms), visual (red photodiode for 10 ms), and electrical (electrical properties of a "dipole object") (Post and von der Emde, 1999). Both stimulus intensity and interstimulus intervals (time between stimulations within a single training session) had a significant effect on the rate of habituation with fish habituating quicker when stimulus intensities were low and the interstimulus intervals were short (Post and von der Emde, 1999).

While the number of studies on habituation in teleosts continues to grow (Daniel et al., 2019, Post and von der Emde, 1999, Wong et al., 2010, Staven et al., 2019), we know little about this form of learning in elasmobranchs. Investigating the limitations of habituation in elasmobranchs will help us understand how well sharks and rays can deal with an overflow of irrelevant stimuli to free up limited cognitive resources that are needed elsewhere. Furthermore, animals tend to use more than one type of learning simultaneously, unless tested in isolation. Therefore, it is crucial to understand each form of learning and their limits in elasmobranchs and other taxonomic groups in isolation to get a better understanding on how they influence shark behaviour in the wild. To date, habituation has been observed in relation to shark hearing and electro-magnetic field sensing capabilities. For instance, lemon sharks habituate to attractive low frequency underwater sounds during prolonged testing (Myrberg et al., 1969). Silky sharks (*Carcharhinus falciformis*) were later reported to habituate to abrupt changes in underwater sounds, resulting in a rapid withdrawal from the area (Myrberg et al., 1978). Small-spotted catsharks (*Scyliorhinus canicula*) show signs of habituation when repeatedly stimulated with a weak electric current that resembled their natural prey items (Kimber et al., 2014). Electro-magnetic fields can also act as a deterrent to sharks, and has been used to develop bycatch reduction and personal shark deterrent devices (Huveneers et al., 2018b, Robbins et al., 2011). Shark response to such deterring electro-magnetic fields was variable with some individuals showing signs of habituation (O'Connell et al., 2011, Gauthier et al., 2020). Investigating the capacity of sharks to habituate to irrelevant deterrents and attractants will provide important information on the effectiveness of these bycatch reduction and personal shark deterrent devices during long-term applications. However, habituation was rarely the main focus of these studies, limiting the amount of details provided. Furthermore, habituation in sharks still needs to be tested in response to other sensory modalities, besides acoustic and electro-magnetic. Sharks use a range of senses to detect and orient themselves to their prey, with olfaction being the first sense used when sharks are furthest from potential prey, followed by hearing, vision, electroreception, and touch as sharks get closer (Gardiner et al., 2014).

Testing habituation in sharks toward different sensory modalities will not only help us better understand this form of learning in elasmobranchs but will also help develop sustainable strategies for wildlife tourism management. Using habituation to the presence of humans and avoiding habituation to attractive stimuli through intermittent feedings could provide a sustainable strategy to manage shark feeding operations.

1.2.2 Object recognition and discrimination

Following a lack of studies in the 1980s and 1990s, research on elasmobranch cognition witnessed an upsurge in the 2000s with studies beginning to focus on object recognition and discrimination abilities of sharks (Fuss et al., 2014d, Guttridge et al., 2009a, Schluessel et al., 2012, Schluessel et al., 2014b). These skills are essential for a wide array of behaviours, including selection of food sources, prey and predator identification, and recognition of territories, conspecifics, heterospecifics and potential mates (Schluessel, 2015).

Bony fishes visually detect and recognise 2D and 3D shapes (Gierszewski et al., 2013, Schluessel et al., 2012, 2014b, Siebeck et al., 2009, Wyzisk and Neumeyer, 2007) and discriminate between objects using active electrolocation (Von der Emde, 1999). Furthermore, bony fishes are able to categorise and distinguish images from their mirror image counterparts (Gierszewski et al., 2013), and are potentially capable of individual recognition (Kohda et al., 2019). Although no matching-to-sample ability was found using geometric symbols in cichlids (Gierszewski et al., 2013), it has been demonstrated in goldfish using coloured lights (Zerbolio and Royalty, 1983). The visual cues that can be identified by fish to single out individuals or objects under natural conditions are extremely diverse and range from postural changes and subtle colour and shape variations, to facial and body patterns, and differences in size (Altbäcker and Csányi, 1990, Csanyi, 1985, 1986, Karplus and Algom, 1981, Karplus et al., 1982).

In comparison, the ability of sharks and rays to recognise and discriminate between objects has been shown to closely match that of teleosts, even though the subject has yet to be investigated to the same extent (Schluessel, 2015, Guttridge et al., 2009b). For example, simple visual discrimination of geometric symbols has been shown on a range of elasmobranch species (Schluessel, 2015). Ocellate river stingrays (*Potamotrygon motoro*) and sharks (*C. punctatum* and *Chiloscyllium griseum*) rely on their visual sensory system in various place learning and object discrimination tasks (Fuss et al., 2014b, 2014c, Schluessel and Bleckmann, 2005, 2012, Fuss et al., 2014d). Gray bamboo sharks, for example, can discriminate between two-dimensional geometric stimuli. They can learn to distinguish between a square (rewarded) and several unrewarded shapes at a rate comparable to that of teleosts (Fuss et al., 2014d, Schluessel et al., 2012, Wyzisk and Neumeyer, 2007). The recognition of and discrimination between sensory cues is, however, not always based in visual stimuli. For instance, Kimber et al. (2011) found small-spotted catsharks could discriminate between electric fields of different magnitudes with a significant preference for stronger fields. Sharks also showed a preference toward alternating currents (AC) when provided with a choice between an AC and a direct current (DC) (Kimber et al., 2011). In another study, Port Jackson sharks (*Heterodontus portusjacksoni*) were successfully trained to associate a jazz song with a food reward (Vila Pouca and Brown, 2018). However, during the follow-up discrimination task, sharks were unable to distinguish between the familiar jazz and a novel classical song. Taken together, the data to date suggests that sharks show fantastic recognition and discrimination abilities across a wide range of senses, but relatively few species have been studied.

1.2.3 Spatial learning and orientation

The scales of movement in teleosts and elasmobranchs can range from just a few meters covered on a day-to-day basis to thousands of kilometres during long distance migrations that can take several months to complete, such as those made by salmon (*Oncorhynchus* spp.) (Dittman and Quinn, 1996), hammerhead sharks (*Sphyrna lewini*)

(Klimley, 1993), and white sharks (*Carcharodon carcharias*) (Bradford et al., 2020). Navigation and orientation during any migration, regardless of its scale requires some form of spatial learning. This type of learning, in general, can be described as the formation of memories that permit later discrimination of position, i.e. the relative orientation of the learning individual, and place using surroundings as reference (Bitterman, 1996, Schluessel, 2015). Some species have been reported to follow sensory gradients or selected sensory cues, especially during long distance migrations. For example, salmon (*Oncorhynchus* spp.) use olfactory cues for homing (Dittman and Quinn, 1996) and the scalloped hammerhead shark (*S. lewini*) has been suggested to use the earth’s magnetic field for navigation (Klimley, 1993, Meyer et al., 2005). Others may learn and remember the relationship between different environmental cues using spatial memory to construct mental maps (O'Keefe and Nadel, 1978). This strategy is referred to as using a cognitive map, which is a form of spatial learning that provides a certain degree of flexibility with regard to the presence or absence of single environmental features. It also enables animals to reach a goal using shortcuts and novel routes even when released from unfamiliar sites (Odling-Smee and Braithwaite, 2003, Schluessel, 2015). Alternative strategies may include taxon or egocentric strategies during which the animal uses either simple visual cues (beacon or guidance learning) or a body-centered reference system (cue or turn procedure) to orient and to approach or avoid certain locations (Schluessel, 2015). Fish from varying habitats differ in the cue hierarchies they employ for navigation (e.g. sand v rocky shores; White and Brown, 2015). These strategies, however, are easily disrupted by habitat alterations and landmark depletions (O'Keefe and Nadel, 1978).

Earlier studies on the spatial learning and memory capabilities on elasmobranchs investigated the spatial memory of Port Jackson sharks and the homing ability of displaced lemon sharks (Edrén and Gruber, 2005, O'Gower, 1995). Whilst the repeated visitation of specific resting sites indicates the presence of some form of spatial memory in Port Jackson sharks, lemon sharks demonstrated an innate sense of direction and possibly imprinting, with 31 out of 32 displaced individuals returning to their original home ranges within 8 days, (Edrén and Gruber, 2005, Guttridge et al., 2009b, O'Gower, 1995). The mechanisms used for navigation and orientation were not investigated, but preliminary homing studies performed on juvenile lemon sharks at Bimini demonstrated their ability to return home with their nostrils blocked or their eyes patched (Sundström et al., 2001). These results do not rule out the use of these sensory cues in homing, but rather demonstrate that sharks do not require them (Sundström et al., 2001), indicating the use of multiple sensory cues and the formation of cognitive spatial maps during homeward orientation. Besides vision and smell, sharks may also use the earth's electromagnetic field to orientate themselves (Kalmijn, 1982, Kalmijn, 1988, Klimley, 1993, Meyer et al., 2005). Further evidence, indicating that sharks are capable of constructing cognitive spatial maps was found in tiger sharks (*Galeocerdo cuvier*) and thresher sharks (*Alopias vulpinus*) (Meyer et al., 2010, Papastamatiou et al., 2011). Both species perform 'directed walks', which suggests intimate knowledge of their surroundings (Meyer et al., 2010, Papastamatiou et al., 2011).

An experimental study under controlled laboratory conditions on freshwater stingrays (*Potamotrygon motoro*) provides conclusive support for the use of some form of spatial learning, and suggests that elasmobranchs are able to construct a visual cognitive map of their surroundings (Schluessel and Bleckmann, 2005). Twelve juvenile freshwater stingrays were trained to either use visual cues or body-centred turns to retrieve a food reward. Results demonstrated that rays were capable of reaching the goal even from unfamiliar starting locations in the presence of consistent visual cues, strongly indicating the construction of a visual cognitive map (Schluessel and Bleckmann, 2005). Freshwater stingrays have been shown to use landmark and directional cues to learn their way through a maze and apply spatial memory. However, transfer test demonstrated that the freshwater stingrays placed more importance on directional information than on landmark cues (Schluessel and Ober, 2018). Similar experiments performed on grey bamboo sharks further revealed that they too are able to use visual landmarks and directional information to learn a spatial task, further indicating the construction of visual cognitive maps in elasmobranchs (Fuss et al., 2014b, 2014c, Schluessel and Bleckmann, 2012).

Locating resources, such as food, habitat, and mates often requires spatial and temporal navigation. Learning about relevant spatio-temporal interactions is known as time-place learning. It describes an association formed between important events that vary in both time and place and is critical for animals that live in a circadian changing environment to optimise resource localisation and predator avoidance (Mulder et al., 2013). The foundation of these associations is built upon endogenous circadian clocks (Gómez-Laplaza and Morgan, 2005). For an individual to learn the association between a time of day and a location, its circadian clock needs to maintain a continuous influence on said individual's behaviour (Reebs, 1996). Bony fishes, like the cichlid angelfish (*Pterophyllum scalare*) and the golden shiners (*Notemigonus crysoleucas*), have been demonstrated to successfully learn the association between time and location of daily food sources within 3–4 weeks (Gómez-Laplaza and Morgan, 2005, Reebs, 1996). More recently, zebrafish were found to successfully learn time-place associations between two different times and locations using social reinforcement instead of the more commonly used food incentive (de Almeida Moura and Luchiari, 2016). The fish learnt the time and location of the daily introduction of a group of conspecifics to the aquarium (de Almeida Moura and Luchiari, 2016). However, when reared under constant light or dark conditions, zebrafish were unable to learn this time-place association, suggesting that the successful learning of such associations requires the circadian clock to be synchronised using environmental factors (de Almeida Moura et al., 2017). In sharks, some species have been demonstrated to align their long-distance migrations with the seasonal peak abundances of resources (Hammerschlag et al., 2016, Meyer et al., 2010, Sims et al., 2003, Sims et al., 2005). For instance, basking sharks (*Cetorhinus maximus*) engage in extensive horizontal migrations (up to 3400 km) along productive continental-shelf and shelf-edge habitats, successfully locating temporally discrete productivity hotspots (Sims et al., 2003). Another example shows tiger sharks

(*Galeocerdo cuvier*) travel long distances during the summer to prey on fledgling Albatross (*Phoebastria* spp.) at a remote atoll in Hawaii (Meyer et al., 2010). Tiger sharks also appear to line up their migrations with the breeding season of green turtles (*Chelonia mydas*) at a popular breeding site in Australia (Hammerschlag et al., 2016). On a finer spatial scale, short-tail stingrays have learnt a time-place association due to the incidental provisioning at a local fish cleaning station in Australia (Pini-Fitzsimmons et al., 2018). An increased visitation rate in the afternoon, irrespective of whether or not the cleaning station is used suggests that rays anticipate the feedings and learnt the association (Pini-Fitzsimmons et al., 2018). However, given that the cleaning station has been in use for >30 years there is no data available on the rays' behaviour prior to its establishment, making it difficult to determine how much of the observed behaviour is a result of the incidental provisioning and consequently time-place learning. Therefore, time-place learning in sharks based on endogenous circadian clocks needs to be investigated in a more controlled setup that will allow for a planned manipulation of feeding time and location.

1.2.4 Memory retention

Teleosts can learn novel tasks and behaviours at similar rates to birds and mammals (Brown et al., 2011, Schluessel, 2015). They also possess impressive memory retention capacities, which have been shown to vary significantly between species and context. For example, it may be beneficial for species living in highly fluctuating environments to forget certain skills and information relatively quickly given that the related information is likely to change frequently (Warburton, 2003). In contrast, species that live and forage in relatively stable environments likely benefit from long-term memories given the relative stability of the related information (Warburton, 2003, Hughes and Mackney, 1995). While forgetting learnt behaviours might be beneficial to adapt to changes in food patch profitability, the once assumed 'three-second memory of the goldfish' is truly fictional. Previous studies have shown that the memory windows in bony fishes can range from days to months (Fuss and Schluessel, 2015). Brown (2001), for example, demonstrated that rainbow fish

(*Melanotaenia duboulayi*) were able to remember a learnt escape response for up to 11 months. Similarly, a recent study on wild cleaner fish (*Labroides dimidiatus*) suggested that a single aversive event resulted in a memory retention of up to 11 months (Triki and Bshary, 2020). Other examples for the impressive long-term memories of bony fishes include the Atlantic cod (*Gadus morhua*), which was found to retain associations of time-separated events for more than three months (Nilsson et al., 2008a, Nilsson et al., 2008b), and the common carp (*Cyprinus carpio*) and sockeye salmon (*Oncorhynchus nerka*) that were trained to successfully avoid fishing hooks and remembered that information for more than a year(Tarrant, 1964, Beukema, 1969).

Memory retention in sharks was investigated in the late 1950s, when Clark (1959) reported that lemon sharks were able to memorise how to obtain food in a classical conditioning paradigm for up to 10 weeks. These results were, however, based on a single observation. More recent studies showed that Port Jackson sharks retained a learnt association between a LED light or a stream of bubbles with the release of food for up to 40 days. A similar memory window was demonstrated in grey bamboo sharks, which were able to remember two spatial learning tasks in the absence of reinforcement for up to 42 days (Schluessel and Bleckmann, 2012). Some contrasting results were provided by Kimber et al. (2014) who demonstrated that none of the learnt behaviours of small-spotted catsharks were remembered after three weeks. However, forgetting in this case may be advantageous due to the variable environment this benthic predator commonly lives in (Kimber et al., 2014, Warburton, 2003). To further understand the limitations of memory retention in elasmobranchs, we need to investigate memory windows in species from different ecological niches. This will provide a greater overlap with species from other taxonomic groups that experience similar environmental conditions, which will allow for more reliable comparisons between the memory capacities of sharks and other species. Reliable comparisons of cognitive abilities between species, including sharks will help to trace the evolutionary history of the vertebrate brain.

1.3 Implication for wildlife tourism

Gaining information on the cognitive capacities of elasmobranchs and their ability to learn not only advances our understanding of the evolution of cognition but may further help understand the mechanisms that drive behavioural alterations observed during wildlife tourism. These alterations may include changes in the movement patterns of focal species (Corcoran et al., 2013, Huveneers et al., 2013), increases in conspecific aggression (Semeniuk and Rothley, 2008), as well as increases in aggressive behaviours towards humans (Altmann and Muruthi, 1988, Burns and Howard, 2003). They have been reported to result in impaired fitness and survival in terrestrial (Orams, 2002), avian (Steven et al., 2011), and aquatic species (Williams and Crosbie, 2007, Semeniuk and Rothley, 2008). As one of the fastest growing sectors of the tourism industry, wildlife tourism attracts millions of participants globally each year (Orams, 2002, Trave et al., 2017, Moorhouse et al., 2015) and already contributes ~$100 billion dollars yearly to the global Gross Domestic Product (GDP) (WTTC, 2019). This specific type of tourism involves activities around wildlife encounters, such as organised Jeep safaris in Africa or white shark diving in South Australia. This essentially means that humans are entering the animals' natural habitats (Knight, 2009). Wildlife tourism has often been associated with several socio-economic and conservation benefits (Huveneers et al., 2017, Orams, 2002, Apps et al., 2018, Newsome et al., 2019) and may allow economies to transition from a primarily consumptive to a perceived non-consumptive, more sustainable use of the local wildlife (Newsome et al., 2019). For example, such transition has been successful in African national park where tourists observe lemur (Lemuroidea), chimpanzees (*Pan troglodytes),* and gorillas (*Gorilla beringei*) in their natural habitats (Newsome and Hassell, 2014, Newsome and Hughes, 2016). Wildlife tourism has created a strong financial incentive for local communities to protect and preserve their natural resources, providing opportunities to former hunters and poachers to work as guides or porters instead (Newsome and Hassell, 2014, Newsome and Hughes, 2016, Goodman and Benstead, 2005). Changes from consumptive to non-consumptive use has also occurred in marine tourism. In Fiji, visitors are charged a diver fee when diving in the Shark Reef Marine Reserve. This fee is distributed to the local villages that have exchanged their traditional fishing rights in the marine reserve for this new source of income (Brunnschweiler, 2010). These fees directly benefit the local economy, creating a strong incentive to maintain these protected areas (Spergel and Moye, 2004).

Up-close encounters with endangered and charismatic species can also result in an increased public awareness benefitting conservation efforts of the focal species and surrounding ecosystems (Apps et al., 2018, Newsome and Hassell, 2014, Newsome et al., 2019). However, these up-close encounters not only need to leave a lasting impression with the participating tourists but should also be provided during tourist excursion with reasonable consistency. Reliable encounters with focal species will ensure tourist satisfaction which is needed for wildlife tourism to be viable (Knight, 2009, Skibins et al., 2013). Whilst this is particularly challenging when targeting large, endangered, charismatic, and elusive marine animals, encounters with these species in their natural environments are particularly sought after (Giglio et al., 2015, Skibins et al., 2013, Tremblay, 2002). Tour operators use a range of different methods to attract and aggregate target species (Knight, 2009). While some operators only use bait, which often consists of fish remains stored in a bait box, others actively feed animals using natural and unnatural prey items or frozen chum blocks made up of minced offal (Brena et al., 2015, Patroni et al., 2018, Richards et al., 2015). The repeated exposure to these activities, especially those that involve the active feeding, provide opportunities for individuals to engage in associative learning, which may result in a permanent alteration of their behaviour and consequently impair their fitness and survival (Williams and Crosbie, 2007, Semeniuk and Rothley, 2008).

In sharks and rays, a variety of studies have shown behavioural and physiological alterations in response to wildlife tourism operations in both focal and non-focal species, including changes in residency, seasonality, and abundance (Meyer et al., 2009, Clarke et al., 2011), fine-scale movement patterns (Huveneers et al., 2013, Bruce and Bradford, 2013, Brunnschweiler and Barnett, 2013, Fitzpatrick et al., 2011), activity (Huveneers et al., 2018a, Corcoran et al., 2013, Barnett et al., 2016), diet (Meyer et al., 2020, Brunnschweiler et al., 2018), increased parasite loads (Semeniuk and Rothley, 2008), and an increased risk of disease transmissions (Semeniuk et al., 2009, Semeniuk and Rothley, 2008, Semeniuk et al., 2007). Bull sharks (*Carcharhinus leucas*), for example, exhibit changes in their fine-scale movement patterns at a popular feeding site in Fiji. Sharks tend to visit the feeding site for extended periods of time on feeding days compared to non-feeding days (Brunnschweiler and Barnett, 2013). Effects of wildlife tourism activities on the movement patterns of sharks have also been demonstrated in white sharks (*Carcharodon carcharias*) at the Neptune Islands in response to the local shark cage-diving operations (Bruce and Bradford, 2013, Huveneers et al., 2013). Following the doubling of cage-diving efforts in 2007, operators reported a significant increase in the number of sharks visiting the dive sites and an increase in the shark residency period (Bruce and Bradford, 2013). On days the shark cage-diving operators were on site, sharks were also reported to spend a significant amount of time in close proximity to the operators (Huveneers et al., 2013). Furthermore, interactions with wildlife tourism operators can affect the activity and possibly energy expenditure of focal species. Temporal increases in activity have been detected in white sharks during interaction with cage-diving operators, resulting in an overall dynamic body acceleration (ODBA) ~61% higher than times when no cage-diving operators are in the area (Huveneers et al., 2018a). While the field metabolic rates of white sharks remained unaffected by the short-lived increases in activity, the metabolic rates of whitetip reef sharks (*Triaenodon obesus*) at Osprey Reef, Australia increases by ~6% on feeding days compared to non-feeding days (Barnett et al., 2016, Huveneers et al., 2018a). Together with the misalignment of the diurnal rhythm of this predominantly nocturnal species, the increased metabolic rate could impact individual fitness or population viability (Barnett et al., 2016). There is one known case to date that demonstrates how changes in residency, behaviour, and activity in response to frequent wildlife tourism operations can result in detrimental effects on health and body condition, and consequently on individual fitness of the focal species. The normally solitary southern stingray (*Dasyatis americana*) is increasingly spending time in close proximity to other conspecifics as a result of daily feeding during tourism activities at the Cayman Islands. This has resulted in increased conspecific bites and parasite loads and a deterioration of southern stingrays' body condition (Corcoran et al., 2013, Semeniuk and Rothley, 2008).

In addition to the detrimental effects of wildlife tourism on the health and fitness of targeted and non-targeted species, behavioural modifications of potentially dangerous species, like sharks, due to learning occurring during wildlife tourism and feeding has also been identified as a concern (Burgess, 1998, Burns and Howard, 2003, Orams, 2002). For example, it has been suggested that sharks may associate humans with food, leading to higher probability of shark bites (Burgess, 1998, Orams, 2002). Therefore, given the fast-growing popularity of wildlife tourism activities, it is important to understand the underlying mechanisms that drive the behavioural alterations observed in focal species to assist in developing sustainable management strategies. Such strategies will protect both the focal species and humans from potential harm, benefiting conservation efforts through an increase in public awareness. Since learning is one of the major drivers of behavioural plasticity, allowing animals to adapt to novel situations and changing environmental conditions, it may play a role in the behavioural alterations observed in sharks in response to wildlife tourism. In particular, associative learning appears to be of most concern where sharks are intentionally fed as part of tourism activities. To ensure sustainable wildlife tourism, we need to determine the rate of associative learning in sharks based on different types of associations and how long these associations will be remembered for. This will enable us to develop management strategies that will prevent sharks from learning these associations, protecting sharks and humans alike from potentially harmful behavioural alterations.

1.4 Research objectives

The overarching objective of my thesis is to advance our understanding of the cognitive abilities of elasmobranchs and to provide further insights on how certain forms of learning will drive the behavioural alterations observed in shark species in response to the frequent exposure to wildlife tourism activities. The specific aims of this thesis are:

1. Investigate the viability of only using smell without feeding to attract sharks for wildlife tourism purposes;
2. Assess the limitations of associative learning in sharks and the effects of biotic factors on shark learning performance; and
3. Investigate the effects of regular feeding on the behaviour of sharks, to inform management strategies.

To achieve my objective and each of the three aims, I have compiled four thesis chapters (excluding the general introductory chapter 1 and the general discussion chapter 6). Each chapter has its own specific goals, which link back to the thesis objective and aims (Figure 1.1).

1.4.1 Thesis structure

Chapter 1 provides an introduction to shark cognition, outlining knowledge gaps in our understanding of the cognitive abilities of elasmobranchs and other taxonomic groups, including teleosts. It further introduces the impacts of shark tourism on the behaviour of focal species and outlines how research on elasmobranch cognition is linked to these behavioural alterations.

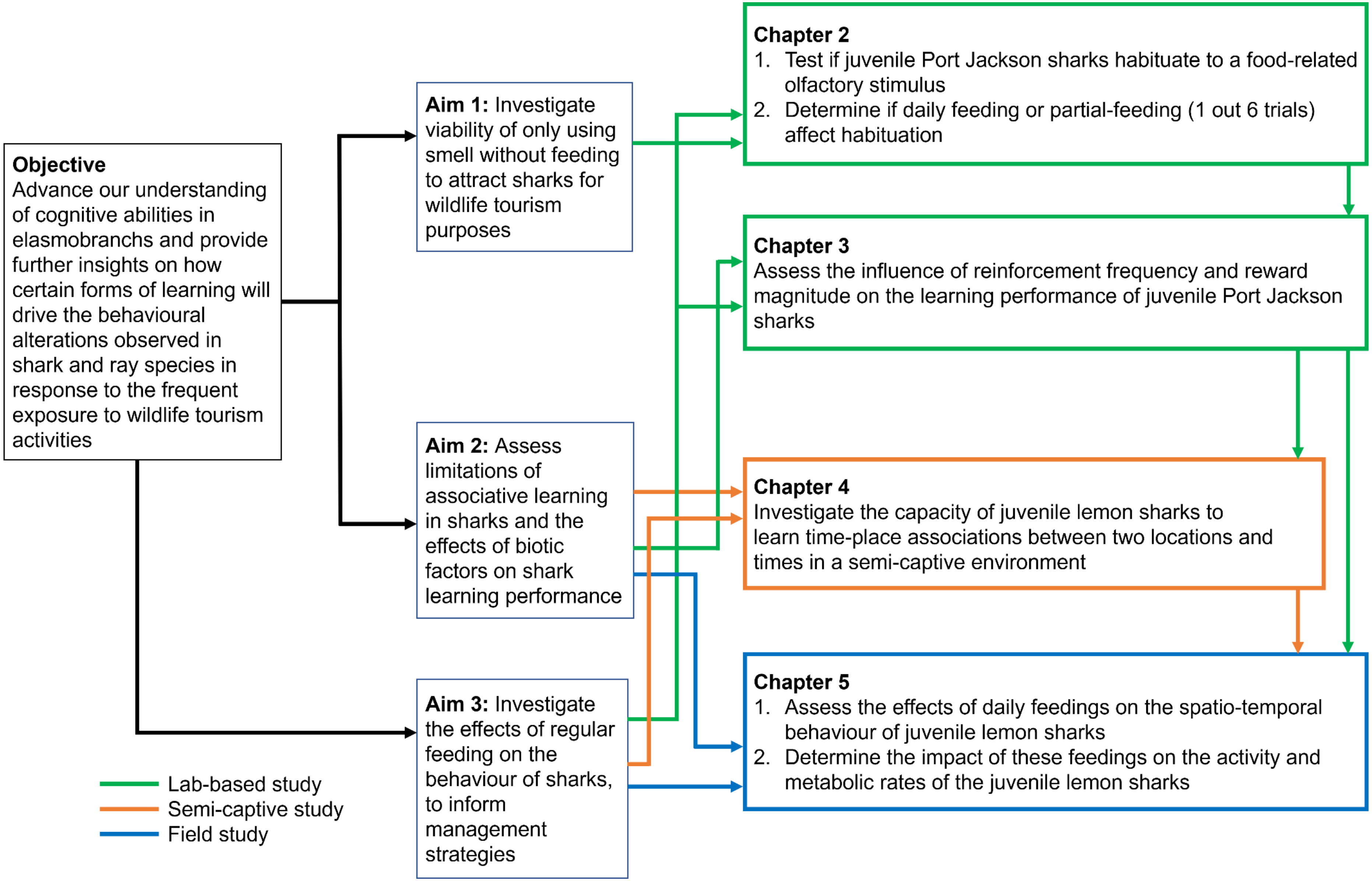
Chapter 2 investigated the habituation rates of juvenile Port Jackson sharks (*Heterodontus portusjacksoni*) to a food-related olfactory stimulus. This chapter relates back to Aims 1 and 2 of my thesis (Figure 1.1).

Chapter 3 assessed the influence of reinforcement frequency and reward magnitude on the learning success of juvenile Port Jackson sharks. This involved a simple spatial cognitive task within an operant conditioning regime in a controlled laboratory environment. This chapter relates back to Aims 2 and 3 of my thesis (Figure 1.1) and is already published in *Animal Cognition*. doi: 10.1007/s10071-020-01402-2

Chapter 4 investigated the capacity of juvenile lemon sharks (*Negaprion brevirostris*) to learn time-place associations between two locations and times of day inside a 45 m^2 semi-captive environment. This chapter relates back to Aims 2 and 3 of my thesis (Figure 1.1).

Chapter 5 investigated the effects of daily shark feedings on the spatio-temporal behaviour, activity, and field metabolic rates of free-living juvenile lemon sharks. This involved the collection of a baseline dataset prior to the establishment of the feeding site and the use of passive acoustic telemetry to track the movements and activities of the sharks. This chapter relates back to Aim 2 and 3 of my thesis (Figure 1.1) and is already published in *Animal Behaviour*. doi:10.1016/j.anbehav.2020.12.002

Chapter 6 discusses and synthesises the major findings and results of Chapters 2–5.



Objective
Advance our understanding of cognitive abilities in elasmobranchs and provide further insights on how certain forms of learning will drive the behavioural alterations observed in shark and ray species in response to the frequent exposure to wildlife tourism activities

Aim 1: Investigate viability of only using smell without feeding to attract sharks for wildlife tourism purposes

Aim 2: Assess limitations of associative learning in sharks and the effects of biotic factors on shark learning performance

Aim 3: Investigate the effects of regular feeding on the behaviour of sharks, to inform management strategies

Chapter 2
1. Test if juvenile Port Jackson sharks habituate to a food-related olfactory stimulus
2. Determine if daily feeding or partial-feeding (1 out 6 trials) affect habituation

Chapter 3
Assess the influence of reinforcement frequency and reward magnitude on the learning performance of juvenile Port Jackson sharks

Chapter 4
Investigate the capacity of juvenile lemon sharks to learn time-place associations between two locations and times in a semi-captive environment

Chapter 5
1. Assess the effects of daily feedings on the spatio-temporal behaviour of juvenile lemon sharks
2. Determine the impact of these feedings on the activity and metabolic rates of the juvenile lemon sharks

Figure 1.1 Overall objective and aims of the thesis and the contribution of each chapter to the individual aims and subsequent chapters. *Shark drawings have been removed due to Copyright restrictions.*

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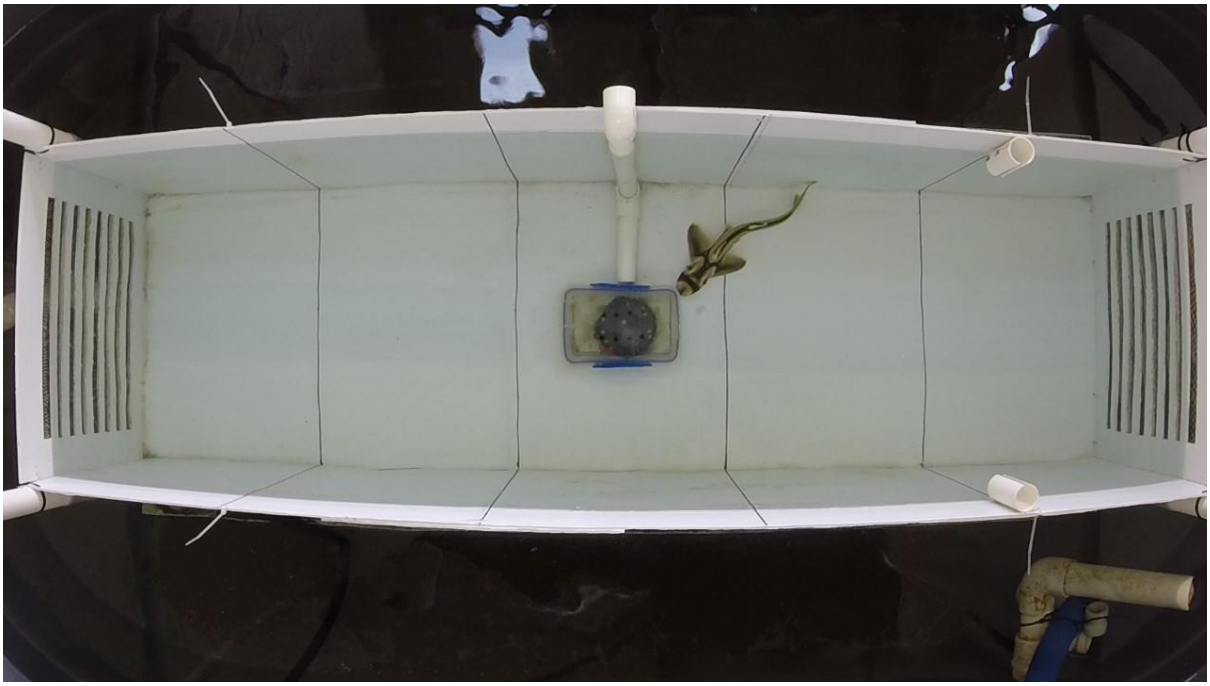
Chapter 2

Juvenile Port Jackson sharks (*Heterodontus portusjacksoni*) habituate to a food-related olfactory cue



2.1 Abstract

Learning is a process that allows animals to develop adaptive behavioural responses to novel situations within an individual's lifetime. The simplest form of learning, habituation, acts a fundamental filter mechanism, which allows animals to ignore irrelevant recurring stimuli, thereby freeing up fitness-related resources, such as time and energy, as well as neural capacity. Although earlier studies have demonstrated that habituation occurs in a variety of taxa ranging from insects to mammals, our knowledge about this process in elasmobranchs is limited. Sharks and rays face an increasingly popular shark-diving industry so it is important to understand how sharks respond to attractants used by these diving operations in the short- and the long-term. Our study investigated whether sharks habituate to the smell of a potential food source, which has been proposed as a more sustainable alternative to actively feeding sharks. We exposed 11 captive juvenile sharks individually to the smell of squid for four minutes, three times per day for 21 days in the absence of a food reward. We found declining responses to the stimulus within and across daily exposure sessions, indicating both short- and long-term habituation. Our findings suggest that sharks learn to avoid wasting time and energy on inaccessible food sources. As a result, using smell instead of feeding sharks during wildlife tourism operations may not be a viable alternative, given the decreasing response observed in our study. Instead, our results suggest that occasionally feeding sharks may be more effective as it maintains shark response while limiting the amount of food consumed by sharks, thereby reducing dependency.

2.2 Introduction

Learning is a process that is based on individual experience and is essential in shaping animal behaviour through developing adaptive responses to novel situations (Dill, 1983, Guttridge et al., 2009, Kawecki, 2010, Pearce and Bouton, 2001). Whilst some forms of learning result in an association between two distinct events (associative learning), others result from a repeated stimulation with a single stimulus (non-associative learning) (Lieberman, 1999). As one of three forms of non-associative learning, habituation has been defined as a declining response to a repeated stimulation, in the absence of any reward or punishment (Rankin et al., 2009, Thompson and Spencer, 1966, Marcus et al., 1988). It is a fundamental and important filter mechanism that allows organisms to ignore irrelevant recurring stimuli and is commonly referred to as the simplest and perhaps most elementary form of learning and behavioural plasticity (Groves and Thompson, 1970, Thompson and Spencer, 1966, Rankin et al., 2009).

Despite being generally thought off as the simplest form of learning, habituation is also considered to be a cognitive building block as it allows for a re-allocation of freed up neuronal resources to be used in more complex cognitive functions (Rankin et al., 2009, Wilson and Linster, 2008, Fabiani et al., 2006, Sinding et al., 2017). It may further assist in optimising an animal’s foraging strategies as part of the optimal foraging theories (Boyd et al., 1997, Haswell et al., 2018). Habituation has been demonstrated in a wide range of phyla, including nematodes (Rankin and Broster, 1992, Hilliard et al., 2005), insects (Das et al., 2011, Haupt and Klemt, 2005, Baracchi et al., 2018), birds (Dong and Clayton, 2009, Mbise et al., 2020, Petrinovich and Peeke, 1973), teleosts (Staven et al., 2019, Randlett et al., 2019, Figler, 1972, Fernandes-de-Castilho et al., 2008), and mammals (Kirmani et al., 2010, Uchida et al., 2019, Bolivar, 2009). In teleosts, habituation to novelty was tested in electric fish (*Gnathonemus petersii*) and zebrafish (*Danio rerio*) using brief novel stimuli of four sensory modalities (acoustic, visual, electrical, electrolocation) and the novel tank test respectively (Post and von der Emde, 1999, Wong et al., 2010). Short- and long-term habituation were successfully demonstrated in both species, but high intensity acoustic stimuli and anxiogenic agents had habituation-impairing effects (Post and von der Emde, 1999, Wong et al., 2010). The zebrafish has subsequently been used as an ecotoxicology model species to further test the influence of certain chemical compounds on the process of habituation (Wong et al., 2010). Habituation can also occur toward ecologically relevant stimuli, such as cues from predators or conspecifics, with a potential impact on individual fitness (Daniel et al., 2019, Fernandes-de-Castilho et al., 2008, Staven et al., 2019). For instance, female Trinidadian guppies (*Poecilia reticulata*) habituated to male colour patterns when repeatedly exposed to a single male specimen. Consequently, females become less attracted to familiar males that may be genetically similar, encouraging outbreeding and ensuring the population's genetic variability (Daniel et al., 2019).

In contrast to the growing body of knowledge on habituation in teleosts (Daniel et al., 2019, Post and von der Emde, 1999, Wong et al., 2010), our knowledge about habituation in elasmobranchs is limited. This is possibly due to the former misconception regarding the learning capacities of elasmobranchs and the former believe that learning within this taxonomic group is of little importance (Northcutt, 2011, Schluessel, 2015). Furthermore, habituation has often referred to as the simplest form of learning kindling little interest within the scientific community (Lieberman, 1999, Thompson, 2009). A final reason may also be the logistical challenges working with large aquatic animals, such as sharks. Early observations on various shark species, including blacktip reef (*Carcharhinus melanopterus*), gray reef (*C. menisorrah*), silky (*C. falciformis*), sharpnose (*Rhizoprionodon* sp.), and nurse sharks (*Ginglymostoma cirratum*)described signs of habituation to underwater sounds (Myrberg et al., 1969, Nelson and Johnson, 1972, Nelson et al., 1969). Small-spotted catsharks (*Scyliorhinus canicular*) habituated to weak electric currents resembling the sharks' natural prey items following the repeated unrewarded exposure to the stimulus (Kimber et al., 2014).

As part of wildlife tourism, one of the fastest growing sectors of the tourism industry, shark feeding is becoming increasingly popular (Newsome et al., 2004, Orams, 2002). Wildlife tourism describes any activities involving the watching and viewing of wild animals (Knight, 2009). It has been associated with numerous benefits, including local economic growth and stability, reduced fishing pressure, and increased conservation efforts (Huveneers et al., 2017, Newsome et al., 2019, Orams, 2002, Apps et al., 2018). However, a number of negative impacts on both focal and non-focal species have also been documented (Green and Giese, 2004, Meyer et al., 2020, Semeniuk and Rothley, 2008, Rizzari et al., 2017). These impacts may include a shift in the movement patterns of the focal species, which may result in an increase in conspecific bites, increased parasite loads and an overall decrease in individual health (Corcoran et al., 2013, Semeniuk and Rothley, 2008). Feeding wildlife may further cause a shift in the diet of smaller non-focal species with yet unknown consequences for the surrounding ecosystem (Meyer et al., 2020, Rizzari et al., 2017). Therefore, some operators do not feed sharks and only use olfactory cues to attract sharks in an effort to promote more sustainable practices (Fitzpatrick et al., 2011). In some areas, such as Hawaii, New Caledonia, and Florida, any activities related to the feeding of sharks are banned. Besides protecting the sharks from harmful effects, these bans were put in place to protect humans from a potentially increased risk of shark bites sometimes associated with shark feeding operations (FFWCC, 2002, Johansen, 2013, Techera, 2012, Burgess, 1998). Similarly, cage-diving operators in South Australia are permitted to use bait to attract white sharks to the boat, but are prohibited to feed sharks intentionally (DEWNR, 2016). The use of olfactory stimuli to attract sharks is less likely to result in potentially harmful associations between the food and humans or boats, since no reward is provided to facilitate any conditioning of the animals. The attraction of the olfactory stimuli could still ensure dependable encounters with the sharks during tourist excursions. Whether the use of olfactory cues alone suffices to reliably attract sharks over long periods remains unknown.

Here, we used juvenile Port Jackson sharks *(Heterodontus portusjacksoni*) as a model to investigate the response of sharks to the smell of squid paired with, without, and partially-paired with food rewards. Each shark was exposed to the stimulus three times per day enabling to test for inter- and intra-session habituation. We hypothesise that the initial difference between the behaviour of the control sharks and the behaviour of the sharks that were exposed to the olfactory stimulus but did not get a food reward would progressively decrease. Therefore, we predicted unrewarded sharks to habituate to the smell of squid over time by progressively 1) taking longer to leave the starting compartment, 2) spending more time resting, 3) taking longer to locate and initiate the investigation of the stimulus, and 4) spending less time investigating the stimulus. In contrast, we predict that partially rewarded sharks will be conditioned to the smell of squid and the bait box by progressively, 1) leaving the starting compartment quicker, 2) spending less time resting, 3) initiating the investigation of the stimulus quicker, and 4) spending more time investigating the stimulus. With respect to these four factors, we further predict unrewarded sharks to exhibit no signs of habituation to the stimulus within a single session (intra-session or short-term habituation), due to the relatively long breaks provided between trials.

2.3 Methods and Materials

Port Jackson sharks are benthic elasmobranchs endemic to the temperate waters of Australia. We chose it as our study species for its small size, hardiness and high abundance along the New South Wales coast. These attributes make it a suitable model species for shark-related research performed under controlled laboratory conditions (Byrnes and Brown, 2016, Powter and Gladstone, 2009, Vila Pouca et al., 2020). We collected 36 Port Jackson shark eggs by hand from rocky reef substrates in Jervis Bay in May 2018 and transported them to Macquarie University where they were housed in 60 L plastic tubs until hatching. Sharks were then moved to the Sydney Institute for Marine Science (SIMS, Mosman, NSW) where they were kept inside six 500 L aquaria for the duration of the experiments. We housed six sharks in each tank and assigned two tanks to each of the four treatment groups (control group later acted as feeding group). All tanks at SIMS were continuously supplied with clean, aerated seawater from Chowder Bay, Sydney, NSW, at ambient temperatures (15.61 ± 0.04°C) and received a natural Sydney photoperiod. We provided the sharks with shelters inside the holding tanks, which consisted of pieces of 100 mm PVC pipe and clay pots. Sharks were fed approximately 6% of their wet body mass three times per week. The food consisted of defrosted, whiteleg shrimp *Litopenaeus vannamei* and Basa fish *Pangasius bocourti*.

For each individual, we measured pre-caudal length to the nearest 1 mm (187 ± 1 mm; mean and SE), body mass to the nearest gram (86 ± 1 g) and determined the sex by noting the presence of claspers in males. These measurements were taken on the day before the start of the acclimation phase. PIT tags were sub-dermally implanted at the base of the first dorsal fin to allow for easy identification two days after the sharks were moved from Macquarie University to the facilities at SIMS. Each shark was initially assigned to one of four treatment groups, the control group (no stimulus), the smell group (olfactory stimulus, but no food reward), the partial feeding group (olfactory stimulus paired with food reward every second day during one of three trials), and the feeding group (olfactory stimulus paired with food reward during every trial). We chose the feeding regime for the partial feeding group to would ensure sufficient repetition of the feeding events throughout the experiment, whilst minimising the reinforcement of the searching and investigative behaviour. We ran two treatment groups simultaneously, starting with the smell and control groups. Once the control and the smell groups finished the last day of trials (day 21), the sharks from the control group were re-assigned to act as the feeding group to reduce the number of individuals used in the study. The feeding group was then trained simultaneously with the partial feeding group for 21 days. We used pseudo-randomisation when initially assigning sharks to the treatment groups to ensure an equal sex and mass distribution (one-way ANOVA for mass: $F_3 = 0.397$, $p = 0.755$).

The olfactory stimulus was provided using ice cubes made from a saltwater solution with squid scent. The solution was created by defrosting frozen squid (*Loligo opalescens* and *Nototodarus sloanii*) in saltwater at a ratio of approximately 1.5 kg of squid per 10 L of water. All ice cubes were made from the same solution to ensure equal amount of scent during each trial. Although squid is not part of the natural diet of juvenile Port Jackson sharks, we used it due to its strong sent, which was well preserved in the ice cubes. We also ran a set of pilot trials using three individuals that were omitted from the main experiments. We tested the sharks' responses to olfactory cues from frozen snails, prawns, fish and squid and we found noticeably stronger responses toward the smell of squid compared to the other three sources. The sharks that were part of the feeding and partial-feeding trials were fed approximately 0.7% of wet body mass equivalent in squid during each trial (total sum of food provided each day came up to approximately 2% of wet body mass equivalent). On feeding days, the partially fed sharks received the remaining 1.3% in fish. The smell and control individuals as well as the partially fed individuals on non-feeding days received approximately 2% of wet body mass in white fish fillets (basa, *Pangasius bocourti*) after the last trial of the day had finished. Sharks were provided a minimum of one hour prior to the feeding to avoid any association between the last trial and the food. The daily experimental order of the sharks was random.

Experiments were carried out in a rectangle arena measuring 150 x 50 x 40 cm (length x width x height) that was built from white corflute sheets. The sheets at the long ends of the arena were fitted with 1 cm wide horizontal slits to allow for a consistent water flow through the arena. The olfactory stimulus (ice cube) was placed inside a plastic container that was placed in the centre of the arena. The water inflow was uni-directional through the arena to allow sharks to locate the source of the stimulus. We divided the length of the arena into five equally sized sectors (30 cm each) with the sector the sharks would start from located at the downstream end of the arena (Figure 2.1). The arena itself was then placed inside a 1000 L tank (180 x 100 x 50 cm). We closed the starting sector with a non-transparent slide door before transferring the shark from the holding tank to the arena.

2.3.1 Acclimation phase

We exposed the sharks to the transfer procedures and the experimental arena on three consecutive days for the purpose of acclimation. On each of the three days, sharks were transferred individually to the experimental arena, using a non-transparent bucket filled with seawater, where they were free to explore the arena for a total of 30 minutes. The sharks were then transferred back to their holding tanks where they were fed approximately 2% of wet body mass equivalent in white fish fillets (basa). We waited for at least one hour following the last shark of the day before feeding them to avoid any association between the food and the arena or the procedures. We also ran a set of pilot trials during the acclimation phase using three individuals that were omitted from the main experiments. We assessed whether sharks would respond to the water disturbances caused when placing the olfactory stimulus (ice cube) inside the stimulus box and the cold water run-off from the ice cube melting. We exposed the three individuals in turn to the same setup used during the experiments placing an odourless ice cube inside the stimulus box. Sharks did not respond to either the placement of the ice cube or cold water run-off during any of the trials we ran across the three-day acclimation period. Therefore, we did not place seawater ice cubes inside the stimulus box for the control group.

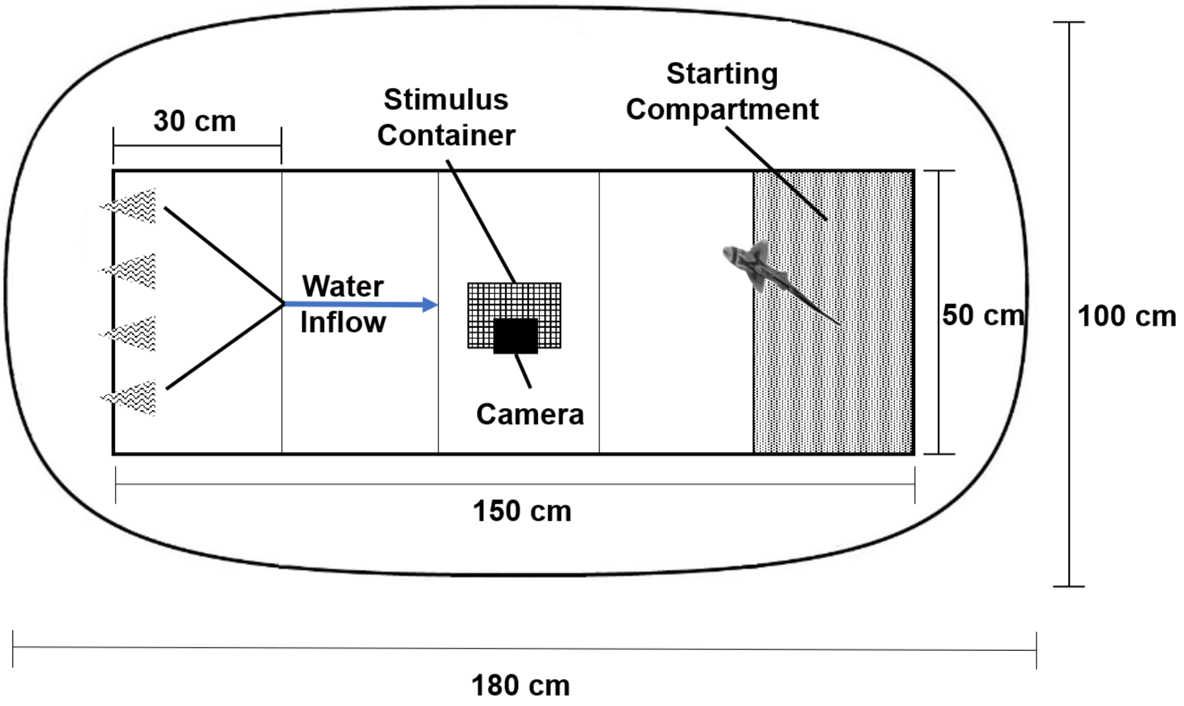


Figure 2.1 Bird-eye view schematics of the experimental arena placed inside the experimental tank. The blue arrow indicates the direction of water flow.

2.3.2 Experimental protocol

Prior to the beginning of the first trial, the subject was provided with a three-minute acclimation period. We then introduced the olfactory stimulus in the case of the smell, partial feeding and feeding groups and opened the starting compartment by removing the sliding door. The sharks of all four treatment groups then had four minutes to freely explore the arena, before being ushered back into the starting sector. During rewarded trials, the food reward was placed inside the stimulus compartment right next to the bait/stimulus box. Of the four minutes total trial duration, the shark was then given approximately 3.5 minutes to locate the reward independently. If a shark did not reach the reward within the allocated time, we attempted to feed it using a pair of aquarium feeder tongues within the remaining 30 seconds of the trial. We recorded the time sharks required to reach the reward and consume it. If a shark did not consume the reward during a trial, we allocated a ceiling value of 240 s. In between trials, a five-minute flushing period followed by a three-minute acclimation period ensured a minimum of 50% of the water volume was exchanged between trials. After the third trial, the shark was removed from the arena and transferred back to the holding tank. We performed a 100% water exchange between individuals to provide baseline background conditions for each shark. The four-minute trials were filmed with GoPro Hero 3 cameras from a birds-eye perspective.

2.3.3 Video analysis

We used the Behavioural Observation Research Interactive Software (BORIS v. 7.4.11) to view and code the video recorded trials (Friard and Gamba, 2016). We recorded shark behaviour using an ethogram with two pre-defined behavioural categories: Resting = the shark was sitting motionless of the bottom; and Investigating = the shark was investigating, i.e. showing a distinct interest in the box holding the squid ice cubes placed in the centre of the arena by putting its snout up against the box. Behaviours that did not fall into either of these two categories were difficult to clearly identify from a birds-eye perspective and were therefore omitted from further analyses. We also determined the latencies to leave the starting compartment and to initiate investigation of the stimulus box (Figure 2.1). Moving from one section to the next was defined as the point in time at which an individual’s head had fully crossed the line between sections – i.e., pectoral fin origin had just passed the line. All times and latencies were recorded in seconds. We calculated the percentage of time sharks engaged in each of the recorded behaviours by dividing the time engaged in a behaviour by the total duration of the respective trials (240.46 ± 0.007; mean ± SE).

2.3.4 Data analysis

To analyse the data, we performed Linear Mixed Model in the Bayesian framework using the package MCMCglmm (Hadfield, 2010) in R version 4.0.0 (R-Core-Team, 2020). We preferred this to the more common frequentist approach as Bayesian linear models reveal joint probabilities of combinations of parameter values without a type I Error inflation and fit the data structure without the requirement to make any approximation assumptions. We ran four independent models assessing: 1) the latency sharks took to leave the starting section of the arena on the floor; 2) the percentage of time sharks spent resting; 3) the latency sharks took to locate and initiate the investigation of the stimulus box; and 4) the percentage of time sharks spent investigating the stimulus box. We ran independent models for the four response variables due to the relatively small dataset which did not allow for a co-variate analysis using the Bayesian framework. The predictor variables included the three-way interaction between days, trial number and treatment group, as well as weight and sex. The model also included any possible two-way interaction and all single factors. This provided an estimation of the effect of repeated stimulation on the behavioural responses of the sharks within and among days. It also allowed for the estimation of the effects of continuous and partial-feeding regimes on the responses to the stimulus and to estimate the effects of size and sex. Random factors included Shark ID (intercept) with a random slope on Days. This was done to account for individual variability across days. We used a Gaussian error distribution and an inverse Wishart prior with a degree of belief parameter (nu) of 0.002. We ran the models with 100,000 iterations, a burn in of 20,000 iterations and a thin term of 20, resulting in non-autocorrelated Monte Carlo Markov Chains with a sample size of 4,000 (Appendix R-Code A2.1). We ensured that the chains were genuinely representative samples from the posterior distribution and that the numerical estimates were accurate by examining the trace plots and the effective sample sizes (Appendix Figure A2.1). To further test the model fit, we did a visual inspection of the residuals.

2.4 Results

Out of the 12 sharks used in each of the four treatment groups, we had to remove one shark from the smell group from the analyses. This individual repeatedly swallowed air and began to float at the water surface throughout the course of the experiment, which had a noticeable impact on its behaviour during approximately 30% of the trials. For the remaining sharks, we viewed and scored approximately 12,000 minutes of video footage.

2.4.1 General responsiveness and activity

Members of the smell group exhibited a decrease in responsiveness and overall activity over time. They took progressively longer to leave the starting compartment and spent more time resting on the floor of the experimental arena over the course of the experiment (Figure 2.2, Table 2.1). Members of the control group took longer to leave the starting compartment and spent more time resting on the floor at the beginning of the experiment, compared to the smell and feeding group. In contrast, members of the partial feeding group and the feeding group took progressively less time to leave the starting compartment. However, members of the partial feeding group exhibited a lower responsiveness during the first day of experiments compared to the smell and feeding groups (Figure 2.2). Feeding group sharks also spent progressively less time resting, whereas the resting period of the partial feeding group increased throughout the experiment, at a rate significantly slower compared to the rate of change shown for the smell group (Figure 2.2 b, Table 2.1). The latency to leave the starting compartment and the time sharks spent resting further differed between daily trials (Table 2.1) with sharks from the feeding group leaving the starting section sooner during the first trial of the day compared to trials two and three for the first three days (Figure 2.3 d). Members of the feeding and the smell group also spent less time resting during the first trial of the day for approximately the first 6 days compared to trials two and three. Unlike the smell group, the feeding group continued to spend less time resting during the first trial of the day on most of the remaining experimental days (Figure 2.4 b, d).

Table 2.1 Estimated effect means from the posterior distribution (post. mean) of 1) the latency to leave the starting compartment, and 2) the percentage of time spent resting, and their 95% confidence intervals (lower-95% CI and upper-95% CI), effective sample sizes and the individual mean Type I error estimate (pMCMC). The abbreviation 'tg' refers to the treatment groups, the factor 'Trials' consists of the three daily trials. The baseline level was the Control group.

Effect	**post. mean**	**lower-95% CI**	**upper-95% CI**	**Effective sample**	**pMCMC**
Latency to leave starting compartment					
(Intercept)	73.90	43.87	102.34	4000	<0.0001
Day	4.68	3.06	6.52	3707	<0.0001
tg (Feeding)	-22.43	-57.65	14.37	4000	0.22
tg (Partial Feed)	6.85	-33.39	43.96	4000	0.73
tg (Smell)	-44.54	-87.30	-8-56	4000	0.032
Trials	-14.48	-24.48	-5.15	4000	0.0050
Weight	8.12	-3.72	20.91	4000	0.19
Sex	-4.16	-27.41	19.00	4000	0.73
Day * tg (Feeding)	-6.93	-9.45	-4.55	4000	<0.0001
Day * tg (Partial Feed)	-6.21	-8.67	-3.86	4250	<0.0001
Day * tg (Smell)	-3.27	-5.57	-0.57	4000	0.0075
Day * Trials	-0.16	-0.94	0.68	4000	0.68
tg (Feeding) * Trials	27.32	13.74	40.99	3617	<0.0001
tg (Partial Feed) * Trials	19.11	6.24	33.26	4000	0.0055
tg (Smell) * Trials	27.06	12.72	40.68	4000	<0.0001
Day * tg (Feeding) * Trials	-0.62	-1.78	0.52	3526	0.29
Day * tg (Partial Feed) * Trials	0.12	-1.03	1.32	4000	0.82
Day * tg (Smell) * Trials	-1.18	-2.34	0.018	4000	0.053
Percentage of time spent resting					
(Intercept)	69.10	59.54	79.17	4000	<0.0001
Day	1.02	0.58	1.45	4000	<0.0001
tg (Feeding)	-42.14	-53.28	-29.97	4000	<0.0001
tg (Partial Feed)	-18.59	-31.21	-4.46	4000	0.0090
tg (Smell)	-49.90	-64.04	-37.11	4000	<0.0001

Trials	-4.95	-7.87	-2.06	4000	0.0010
Weight	3.58	-0.58	7.55	4000	0.084
Sex	-0.58	-9.05	7.03	4000	0.89
Day * tg (Feeding)	-1.42	-2.05	-0.84	4000	<0.0001
Day * tg (Partial Feed)	-0.60	-1.26	-0.011	4000	0.059
Day * tg (Smell)	1.11	0.45	1.71	4000	0.0005
Day * Trials	0.082	-0.17	0.34	4513	0.53
tg (Feeding) * Trials	14.75	10.53	18.82	4000	<0.0001
tg (Partial Feed) * Trials	11.69	7.43	15.69	4000	<0.0001
tg (Smell) * Trials	14.67	10.33	18.83	4000	<0.0001
Day * tg (Feeding) * Trials	-0.38	-0.73	-0.023	4362	0.036
Day * tg (Partial Feed) * Trials	-0.12	-0.48	0.25	4000	0.50
Day * tg (Smell) * Trials	-0.84	-1.20	-0.48	4000	<0.0001

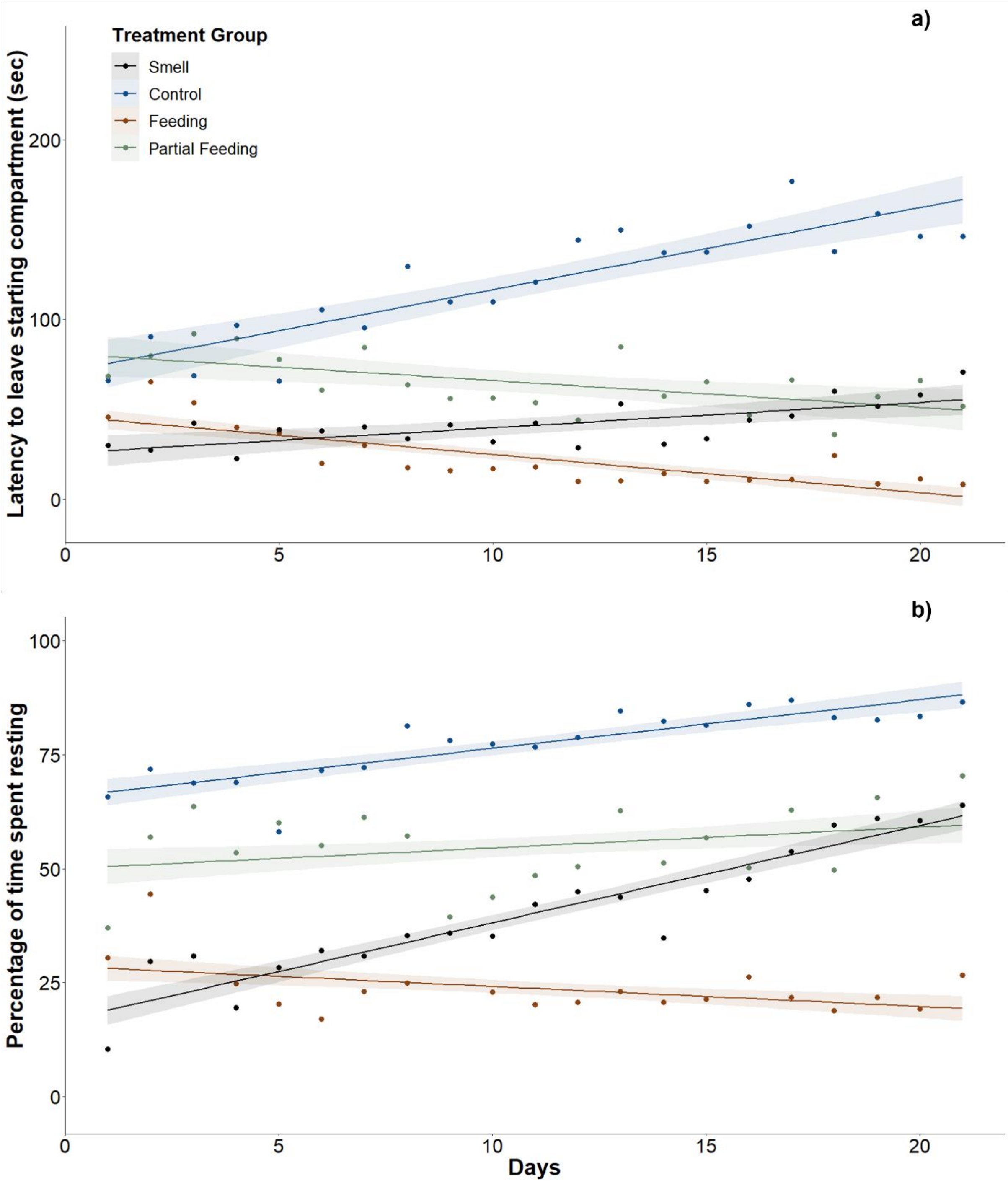


Figure 2.2 Linear plots with 95% confidence interval illustrating the daily trends for a) the latency sharks took to leave the starting compartment and b) the percentage of time sharks spent resting on the arena floor. Each dot represents the daily mean of the respective treatment group.

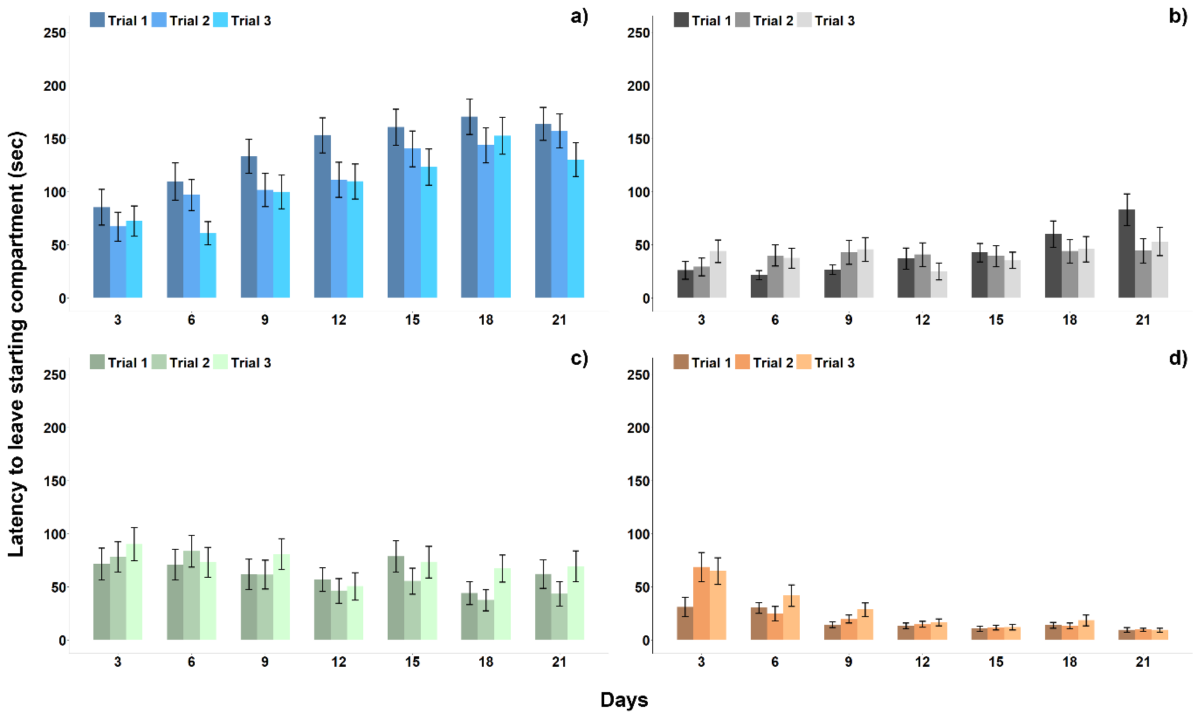


Figure 2.3 Bar graph ± SE illustrating the latency sharks took to leave the starting compartment in a) the control group, b) the smell group, c) the partial feeding group, and d) the feeding group. Each bar represents the three-day mean for one of the three daily trials.

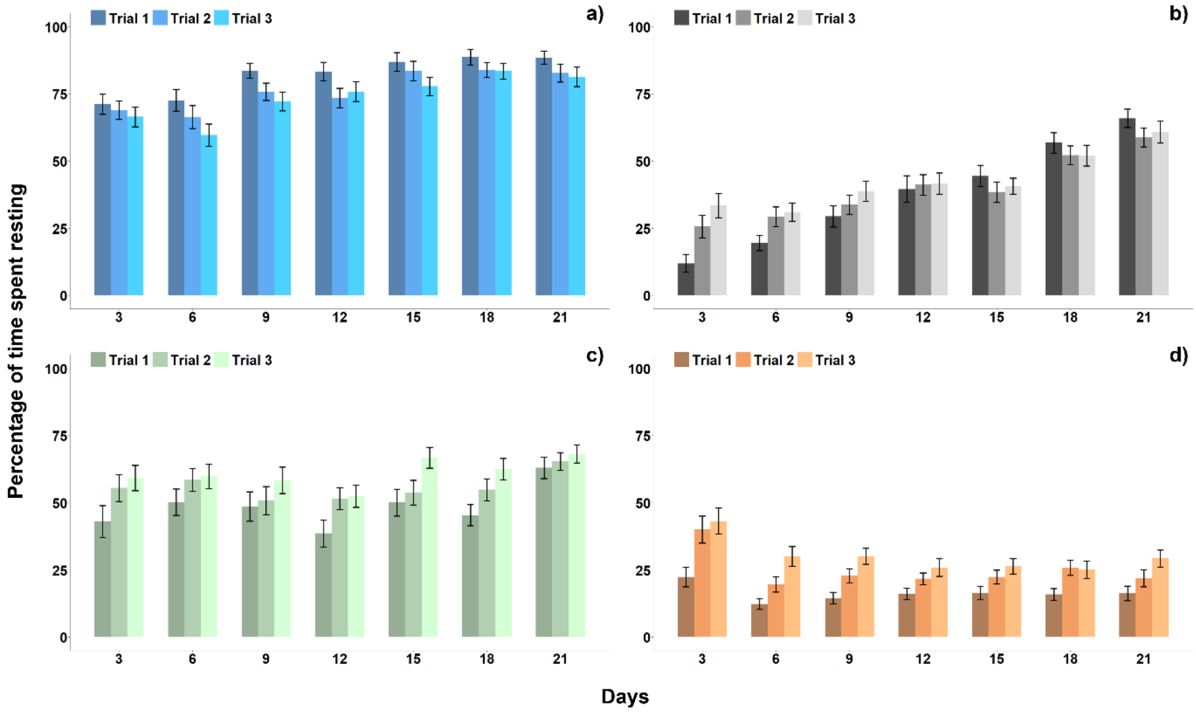


Figure 2.4 Bar graph ± SE illustrating the percentage of time sharks spent resting in a) the control group, b) the smell group, c) the partial feeding group, and d) the feeding group. Each bar represents the three-day mean for one of the three daily trials.

2.4.2 Stimulus-specific response

The direct response to the stimulus, measured as the latency sharks took to start investigating the bait/stimulus box and the percentage of time sharks spent investigating the box, significantly decreased over time in members of the smell group (Figure 2.5, Table 2.2). In contrast, the latency to start investigating the bait box progressively decreased in the feeding and the partial feeding groups. However, the percentage of time sharks spent investigating the box remained largely unchanged in both groups (Figure 2.5 b, Table 2.2). Members of the smell group took progressively longer to start investigating the bait box and spent significantly less time doing so over the course of the experiment (Figure 2.6, Table 2.2). Looking toward intra-session differences, the members of the smell group showed some signs of intra-session habituation with regards to the latency to start investigating the bait box during the first three days of experiments (Figure 2.6 b). Both behavioural responses decreased significantly between trials in the feeding and the partial feeding groups (Figure 2.6 c, d, Table 2.2). However, sharks from the feeding group became progressively more consistent with regards to the latency they took to start investigating the bait box (Figure 2.6 d). The differences among trials were only significant during the first nine days. This is shown by the three-way interaction Day * tg (feeding) * Trials (Table 2.2). In contrast to the smell, partial feeding and feeding groups, members of the control group rarely investigated the bait box at all (Figure 2.5).

Table 2.2 Estimated effect means from the posterior distribution (post. mean) of 1) the latency to start investigating the bait/stimulus box, and 2) the percentage of time spent investigating the bait/stimulus box, and their 95% confidence intervals (lower-95% CI and upper-95% CI), effective sample sizes and the individual mean Type I error estimate (pMCMC). The abbreviation 'tg' refers to the treatment groups, the factor 'Trials' consists of the three daily trials. The baseline level was the Control group.

Effect	**post. mean**	**lower-95% CI**	**upper-95% CI**	**effective sample**	**pMCMC**
Latency to start investigating bait/stimulus box					
(Intercept)	221.99	197.00	244.89	3413	<0.0001
Day	0.68	-0.77	1.98	2989	0.34
tg (Feeding)	-103.10	-134.43	-73.94	4000	<0.0001
tg (Partial Feed)	-43.92	-75.41	-11.18	4377	0.010
tg (Smell)	-102.19	-134.06	-68.11	4000	<0.0001
Trials	6.31	-4.26	17.56	4187	0.25
Weight	2.17	-6.90	11.50	4000	0.64
Sex	-1.37	-18.71	17.53	4000	0.89
Day * tg (Feeding)	-5.19	-6.98	-3.16	4000	<0.0001
Day * tg (Partial Feed)	-2.01	-4.07	-0.13	3059	0.048
Day * tg (Smell)	3.76	1.74	5.76	4000	0.0005
Day * Trials	-0.40	-1.30	0.61	4000	0.39
tg (Feeding) * Trials	26.08	10.01	41.26	4000	0.0010
tg (Partial Feed) * Trials	19.71	4.19	34.96	4219	0.017
tg (Smell) * Trials	12.69	-4.14	27.39	5070	0.12
Day * tg (Feeding) * Trials	-1.47	-2.90	-0.22	3751	0.043
Day * tg (Partial Feed) * Trials	-0.064	-1.39	1.21	4189	0.93
Day * tg (Smell) * Trials	-0.15	-1.49	1.24	4411	0.83
Percentage of time spent investigating bait/stimulus box					
(Intercept)	-0.36	-3.15	2.63	3824	0.81
Day	-0.0098	-0.18	0.17	4000	0.91
tg (Feeding)	22.14	18.71	26.12	4000	<0.0001
tg (Partial Feed)	6.66	2.92	10.26	4000	0.0010
tg (Smell)	13.94	10.06	17.62	4394	<0.0001

Trials	-0.11	-1.89	1.87	4000	0.90
Weight	-0.11	-1.30	1.15	3642	0.84
Sex	1.12	-1.31	3.63	4000	0.37
Day * tg (Feeding)	0.17	-0.079	0.42	4000	0.15
Day * tg (Partial Feed)	-0.0011	-0.24	0.26	4000	1.00
Day * tg (Smell)	-0.63	-0.89	-0.38	4000	<0.0001
Day * Trials	0.0083	-0.16	0.16	4000	0.91
tg (Feeding) * Trials	-7.23	-9.79	-4.44	4000	<0.0001
tg (Partial Feed) * Trials	-4.30	-6.71	-1.41	4534	0.0015
tg (Smell) * Trials	-2.87	-5.65	-0.24	4000	0.41
Day * tg (Feeding) * Trials	0.19	-0.037	0.41	4000	0.099
Day * tg (Partial Feed) * Trials	0.15	-0.073	0.37	4409	0.20
Day * tg (Smell) * Trials	0.14	-0.094	0.38	4000	0.23

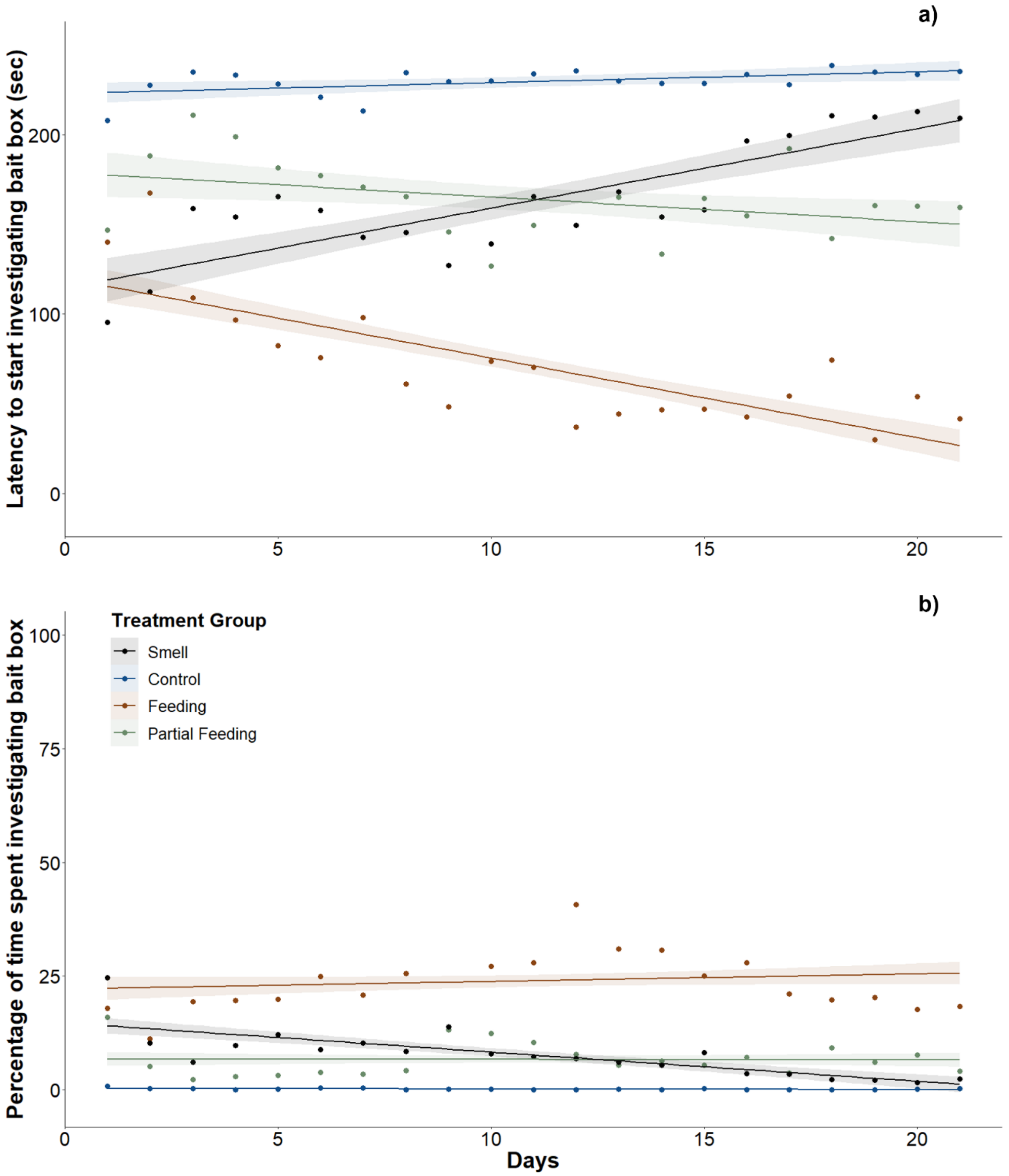


Figure 2.5 Linear plots with 95% confidence interval illustrating the daily trends for a) the latency sharks took to start investigating the bait/stimulus box and b) the percentage of time sharks spent investigating the bait/stimulus box. Each dot represents the daily mean of the respective treatment group.

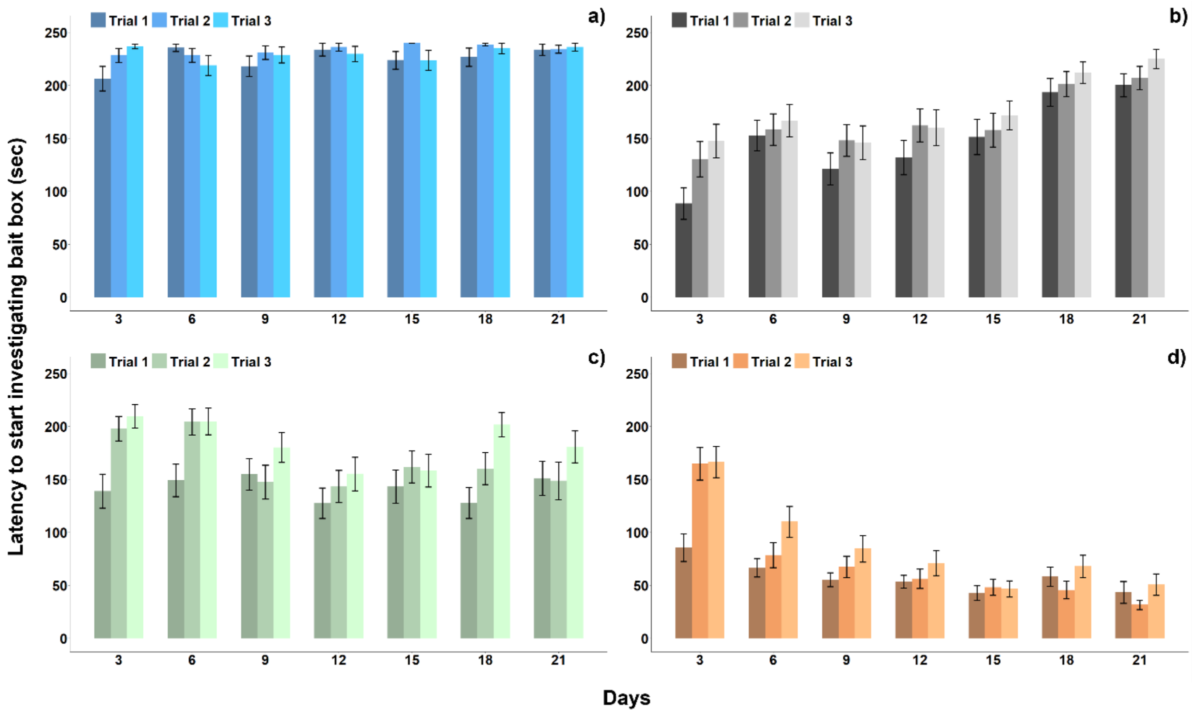


Figure 2.6 Bar graph ± SE illustrating the latency sharks took to start investigating the bait box in a) the control group, b) the smell group, c) the partial feeding group, and d) the feeding group. Each bar represents the three-day mean for one of the three daily trials.

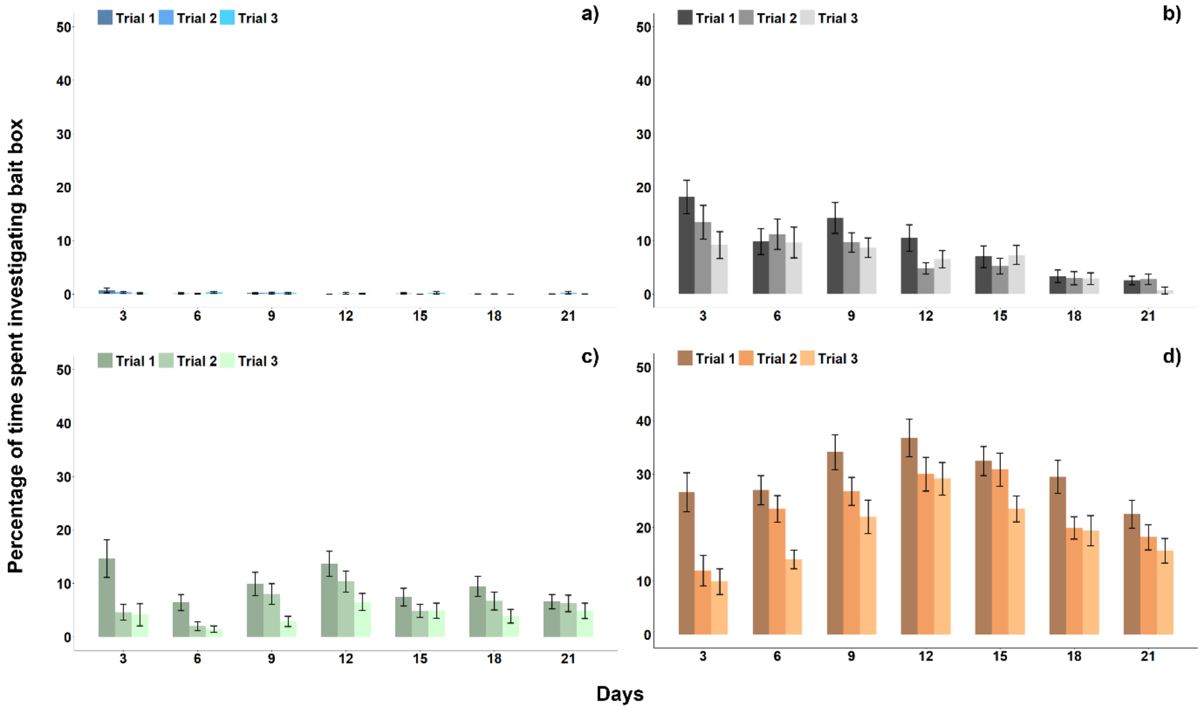


Figure 2.7 Bar graph ± SE illustrating the percentage of time sharks spent investigating the bait box in a) the control group, b) the smell group, c) the partial feeding group, and d) the feeding group. Each bar represents the three-day mean for one of the three daily trials.

2.5 Discussion

We investigated habituation in juvenile Port Jackson sharks to a food-related olfactory cue in a controlled environment. Each shark was subjected to the stimulus three times per day for 21 consecutive days to assess intra- and inter-session habituation. We found clear signs of long-term habituation, with juvenile Port Jackson sharks exposed to the smell only (i.e. no food reward) showing increasingly less interest in locating and investigating the source of the smell while becoming less active and responsive. In contrast, sharks from the partial feeding group neither habituated nor got conditioned to the smell of squid to the extent of the feeding group over the course of the experiment. Whilst slightly increasing their percentage of time spent resting (Figure 2.2 b), sharks tended to leave the starting compartment quicker and start to investigate the bait box sooner during the last days of experiments compared to the beginning (Figure 2.2 a; Figure 2.5 a). We also found some signs of intra-session or short-term habituation in the smell group. The latency to investigate the bait box increased across the trials within a session during the first days of experiments (Figure 2.6 b), as did the percentage of time sharks spent resting (Figure 2.4 b). However, there was no clear pattern that such short-term habituation was affected by the treatment.

The behavioural response observed was also affected by the frequency of feeding, suggesting that the level of response to a food-related olfactory stimulus depended on the rate of successful foraging. A nil pay-off results in habituation and, eventually, in a complete disregard of the stimulus, whereas an approximately 17% success rate (rewarded in 1 out of 6 trials) results in a mixed behavioural plasticity over the course of the experiment. Sharks of the partial feeding group exhibited similar rates of changes with respect to the percentage of time resting and investigating the bait box compared to the control group. However, given the contradictory results on the latency to leave the starting compartment and start investigating the bait box sharks of the partial feeding group exhibited signs of both habituation and conditioning. This suggests that members of this group neither truly habituated nor became conditioned to the stimulus, but instead maintained a relatively consistent level of attraction. In contrast, members of the feeding group clearly became conditioned to the olfactory cue and increased their response to the stimulus with respect to all four behavioural traits tested. Conditioning in animals is wide-spread and has been demonstrated in invertebrates (Menzel et al., 2006, Perry et al., 2013, Vallortigara, 2020), amphibians (Amiel et al., 2011, Bisazza et al., 1998), reptiles (Wilkinson and Huber, 2012, Amiel et al., 2011, Bisazza et al., 1998), teleosts (Brown, 2015, Brown, 2012, Bshary, 2011, Bshary et al., 2002), birds (Bailey et al., 2014, Emery and Clayton, 2009, Marino, 2017, Sol et al., 2005), mammals (Clark, 2013, Deecke, 2006, Hart et al., 2008, Emery and Clayton, 2009) and sharks (Guttridge et al., 2009, Schluessel, 2015). For example, grey bamboo sharks (Chiloscyllium griseum) successfully categorised images of fishes and associated them with a food reward, irrespective of the size, scale, colour or shape of the illustrated fish (Schluessel and Duengen, 2015). The variability in the behavioural response we observed among the different treatment groups likely results from the trade-off between foraging costs and benefits. Such trade-offs may ensure that sharks allocate their resources to potential foraging sites proportionately to the sites' respective payoffs (Charnov, 1976, Fuss et al., 2014a, Watanabe et al., 2014).

Our results indicate that Port Jackson sharks exhibit long-term habituation (i.e. increased latencies to leave the starting sector and to start investigating the bait box, increased time spent resting and decreased time spent searching across experimental days) with some signs of short-term habituation (i.e. increased latency to start investigating the bait box and increased time spent resting across the trials within a day) to the repeated stimulation of a food-associated olfactory cue. Both forms of habituation have been reported in response to an ecologically relevant stimulus. For example, long- and short-term habitation can influence mate choices of Trinidadian guppies (*Poecilia reticulata*) (Daniel et al., 2019). Small-spotted catsharks also exhibit signs of habituation within six to seven sessions (3 – 4 days) when repeatedly exposed to a weak electrical current that resembles their natural prey items (Kimber et al., 2014). The speed at which these catsharks stopped biting the electrodes transmitting the electrical current is faster than the reduction in behavioural response that we observed in Port Jackson sharks, possibly due to stimulus used and inter-species differences (Rankin et al., 2009, Thompson, 2009). Sensory hierarchy in elasmobranch may influences the speed at which sharks habituate to various stimuli. Olfaction is one of the first sense used by elasmobranchs during foraging, whereas electroreception is typically used to detect preys within < 1 m (Hodgson et al., 1978, Kimber et al., 2014, Gardiner et al., 2014, Newton et al., 2019, Meredith and Kajiura, 2010). Across the first six days of our study, members of the smell group exhibiting higher levels of activity during the first trial of the day compared to the subsequent second and third trial. However, similar results of intra-session differences were found in the partial feeding and the feeding groups, and we did not find patterns that would clearly identify a treatment effect. Further research is required to determine the influence of different reward regimes on shark intra-session learning behaviour, and to tease out whether intra-session habituation is influenced by sensory stimulus.

Our findings provide support that the process of foraging is governed by optimal foraging theories and that learning mechanisms play a key role in optimising an animal's foraging strategies. These theories predict that an animal's foraging activities are optimised to maximise net energy gain (Boyd et al., 1997, Haswell et al., 2018). The smell, partial feeding, and feeding groups elicited three different responses, ranging from a reducing (smell group) to an increasing (feeding group) response to the stimulus. Similar behavioural adjustments to varying resource availability have previously been demonstrated in penguins (Watanabe et al., 2014). Adélie penguins (*Pygoscelis adeliae*) adjust their dive times in response to diminishing food availability when preying on mobile and patchily distributed krill (*Euphausia superba* and *E. crystallorophias*) (Watanabe et al., 2014). The behavioural adjustments observed in penguins matched those predicted by the marginal value theorem stating that animals should move from a foraging patch to another once food gain drops below a certain threshold (Charnov, 1976, Watanabe et al., 2014). The decision of an individual to either keep foraging at a certain patch or to move on to the next patch is based on trade-offs between benefits (foraging success) and costs (i.e. energetic cots, risk of predation), and are made based on an individual's experience (i.e. learning) (Watanabe et al., 2014). Habituation may also be linked to optimal foraging theories that apply to escape responses, whereby it is advantageous to adjust antipredator behaviours to match perceived risk levels (Cooper Jr and Frederick, 2007, Ydenberg and Dill, 1986). For instance, habituation allowed hyraxes (*Dendrohyrax spp., Heterohyrax spp., Procavia spp.*) in east Africa to reduce their flight initiation distance, which resulted in decreased costs of unnecessary fleeing, facilitating the allocation of more time and energy to fitness-increasing activities (Mbise et al., 2020). A reduction in flight initiation distance due to habituation towards humans has also been reported in a number of other species, including wild horses and zebras (*Equus caballus ferus and E. quagga*) (Brubaker and Coss, 2015), eastern grey squirrels (*Sciurus carolinensis*) (Engelhardt and Weladji, 2011), skinks (*Emoia impar*) (McGowan et al., 2014), and common blackbirds (*Turdus merula*) (Rodriguez-Prieto et al., 2009). Freeing up resources, such as time, energy, or cognitive capacities is one of the main reasons habituation plays such an important role in learning and has been suggested to be a building block for higher cognitive functions (Fabiani et al., 2006, Rankin et al., 2009, Sinding et al., 2017, Wilson and Linster, 2008). The habituation we observed in Port Jackson sharks to the repeated stimulation with a food-related olfactory cue likely serves the purpose of time and energy conservation. As a benthic elasmobranch species capable of buccal ventilation, Port Jackson sharks tend to spend a significant amount of time resting on the seafloor, conserving energy (Kadar et al., 2019, Kelly et al., 2020a, Kelly et al., 2020b, Powter and Gladstone, 2009). Foraging in search of an inaccessible food source such as those hidden in crevices, would be energetically wasteful. Conserving this energy through habituation to this specific olfactory stimulus would be advantageous and free up time and energy that can be used to track down a more beneficial food sources, potentially increasing an individual's fitness (Boyd et al., 1997, Haswell et al., 2018, Dill, 1983).

2.5.1 Implications for wildlife tourism

In an effort to make the shark-diving industry more socially acceptable and to minimise potential associations, operators sometimes use bait to attract sharks without feeding them (Fitzpatrick et al., 2011). Given the relatively fast rate of habituation we observed during this study, the use of olfactory cues to attract sharks might not yield the same consistent level of attraction as feeding sharks. Based on our findings, the frequent use of prey odour might lead to a decrease response from sharks and to sharks becoming less likely to interact with tourist operators. Given the results we obtained from the partially-fed group, operators could feed sharks on some days (rather than every day) and still maintain shark interest over the long term. In our study, the partially-fed group were fed once every six trials, which occurred every other day. It is, however, unknown how frequently shark-diving operators need to feed sharks to avoid habituation and ensure continued behavioural response to bait odour, and the required frequency of feeding is likely to be context- and species-dependent, and difficult to coordinate between independent tour operators. While feeding sharks every day will likely result in a higher level of interest compared to partially-feeding them, it may also condition them to a specific site and time of day, and alter shark behaviour through dependency (Brunnschweiler and Barnett, 2013, Clarke et al., 2011, Huveneers et al., 2013, Huveneers et al., 2018). Daily feedings of the southern stingray (*Dasyatis americana*) at the Cayman Islands, for example, have resulted in rays becoming conditioned to the site and a subsequent change from a solitary to a group-living lifestyle (Corcoran et al., 2013, Semeniuk and Rothley, 2008). Increase in conspecific bite marks and parasite loads, and overall decline in body condition have since been documented at the feeding site (Corcoran et al., 2013, Semeniuk and Rothley, 2008).

The capacity of sharks to become conditioned and associate certain stimuli, locations, or foraging pathways with a food reward has been shown in several studies (Fuss et al., 2014b, Guttridge and Brown, 2013, Vila Pouca and Brown, 2018, Schluessel and Bleckmann, 2012, Schluessel and Ober, 2018). For example, grey bamboo sharks

(*Chiloscyllium griseum*) successfully learnt a fixed turn response applying both directional information, possibly in combination with some form of place learning and landmark cues (Fuss et al., 2014a). Forming such associations appears to be the underlying mechanism driving many of the behavioural alterations reported in species that are frequently subjected to wildlife tourism activities. A common fear of the public is that sharks may also associate humans with food, resulting in an increased risk of dangerous encounters (Burgess, 1998). While there are currently no empirical evidence linking shark tourism to increased shark bite risk, minimising feeding frequency could reduce the potential for conditioning taking place. Our results show that partially-fed sharks neither habituated nor were conditioned, but rather maintain a relatively stable response to the stimulus. The most effective rate of feeding to avoid habituation and conditioning is, however, likely to be species- and context-dependent.

2.6 Conclusion

Juvenile Port Jackson sharks exhibited linear habituation to an olfactory cue. Habituation to an unyielding food stimulus can free up resources, such as time and energy to be invested in other fitness increasing activities, similar to the adaptations in anti-predator responses and foraging behaviours seen in other species (Boyd et al., 1997, Dill, 1983, Haswell et al., 2018, Kirmani et al., 2010, Mbise et al., 2020, Watanabe et al., 2014). Our results suggest that habituation may act as a mechanism driving optimal foraging behaviour, such as the marginal value theorem, enabling individuals to recognise diminishing food availability through short- and long-term habituation. This would incentivise individuals to move to the next foraging patch (Watanabe et al., 2014) and to potentially abandon depleted or inaccessible foraging patches. From an applied perspective, long-term habituation may be problematic for shark-diving operators who only use the smell of food to attract sharks, a practice that is sometimes promoted to be more sustainable or socially acceptable. However, our results suggest that sharks could eventually lose interest and become less attracted to odour if they are not rewarded with food on occasion. Alternatively, we suggest that feeding sharks occasionally could maintain a stable level of response without conditioning sharks.

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Chapter 3

Effects of reward magnitude and training frequency on the learning rates and memory retention of the Port Jackson shark *Heterodontus portusjacksoni*

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3.1 Abstract

The development of adaptive responses to novel situations via learning has been demonstrated in a wide variety of animal taxa. However, knowledge on the learning abilities of one of the oldest extant vertebrate groups, Chondrichthyes, remains limited. With the increasing interest in global wildlife tourism and shark feeding operations, it is important to understand the capacities of these animals to form associations between human activities and food. We used an operant conditioning regime with a simple spatial cognitive task to investigate the effects of reinforcement frequency and reward magnitude on the learning performance and memory retention of Port Jackson sharks (*Heterodontus portusjacksoni*). Twenty-four Port Jackson sharks were assigned one of four treatments differing in reward magnitude and reinforcement frequency (large magnitude – high frequency; large magnitude – low frequency; small magnitude – high frequency; small magnitude – low frequency). The sharks were trained over a 21-day period to compare the number of days it took to learn to pass an assigned door to feed. Sharks trained at a high reinforcement frequency demonstrated faster learning rates and a higher number of passes through the correct door at the end of the trials, while reward magnitude had limited effects on learning rate. This suggests that a reduction in reinforcement frequency during tourism-related feeding operations is likely to be more effective in reducing the risk of sharks making associations with food than limiting the amount of food provided.

3.2 Introduction

Learning has been demonstrated in a wide variety of animal taxa, including invertebrates (e.g. Kawecki, 2010, Schatz et al., 1994, Srinivasan, 2010) and vertebrates (e.g. Fuss et al., 2014a, Gruber and Schneiderman, 1975, Taylor et al., 2010, Brown et al., 2008). The ability to learn enables individuals to develop adaptive responses to changing conditions and novel situations within an individual's lifetime. This is particularly important for animals living in highly fluctuating and complex environments, such as fishes living in some coastal areas (Dill, 1983, Dodson, 1988). The learning abilities in fishes range from complex spatial learning tasks to object discrimination, and recognition of individuals of the same and different species (Brown, 2015, Brown et al., 2008). However, knowledge on the learning capacities of elasmobranchs remains limited. Early studies demonstrated the ability of sharks to learn (Gruber and Schneiderman, 1975), with recent studies showing that elasmobranchs are capable of solving spatial cognitive tasks, recognising and discriminating between objects (Aronson et al., 1967, Fuss et al., 2014b, Graeber and Ebbesson, 1972), and use different orientation strategies and spatial memory systems to navigate during long- and short-distance migrations (Edrén and Gruber, 2005, Meyer et al., 2010, O'Gower, 1995, Papastamatiou et al., 2011, Schluessel and Bleckmann, 2005, Schluessel and Bleckmann, 2012). Furthermore, sharks can associate artificial sounds with food rewards (Vila Pouca and Brown, 2018), discriminate different quantities (Vila Pouca et al., 2019), and engage in social learning (Guttridge et al., 2013, Vila Pouca et al., 2020). Previous studies have shown that learning rate can be influenced by reinforcement frequency, i.e. the number of rewarded trials per training session (Kerpelman and Himmelfarb, 1971, Lauer and Estes, 1955, van den Akker et al., 2014) and reward magnitude, i.e. the size of the reward per trial (Gonzalez et al., 1974, Muzio et al., 1992, Neuringer, 1967). However, despite the growing body of knowledge on the cognitive abilities of elasmobranchs, the factors that influence the rate of learning in this taxonomic group is yet to be investigated.

Wildlife tourism is considered one of the fastest growing sectors of the tourism industry (Scheyvens, 1999), generating billions of dollars annually globally (Corcoran et al., 2013). Many of the targeted species, including large marine predators are difficult to observe due to their shy and elusive nature (Bres, 1993, Burgess, 1998). These animals are often fed to maximise interactions with humans and produce reliable and good viewing opportunities. This increasingly popular practice is known as provisioning and it has been subject to scrutiny in recent decades (Newsome et al., 2004, Orams, 2002). The deliberate feeding of large predators is suspected to lead to detrimental effects on the target animals, their environments, and humans (Dobson, 2006, Hammerschlag et al., 2012, Newsome and Rodger, 2008). These effects can range from decreased physiological condition to behavioural alterations that could cause cascading effects throughout the marine ecosystem, or increase the risk for humans resulting in injuries due the learnt association between humans and food (Huveneers et al., 2013, Orams, 2002, Gallagher and Huveneers, 2018).

Due to these potential risks, a few regions have banned shark feeding (e.g. New Caledonia, Florida, Hawaii) (FFWCC, 2002, Johansen, 2013, Techera, 2012). A bill was also introduced into the US Congress in 2016 (3099 “Access to Sportfishing Act of 2016”) that would render shark feedings illegal in all United States federal waters (Nelson, 2016). It has yet to be passed by the Senate. Currently, there are a few individual states, e.g. Florida (FFWCC, 2002) and Hawaii (Techera, 2012), that have already banned shark feeding operations in federal waters. In the case of the white shark (*Carcharhorodon carcharhias*) cage-diving industry, the use of bait and berley is strictly regulated in all locations where cage-diving occurs (i.e. USA (California, Farallon Islands), Mexico (Guadalupe Island), Australia (South Australia, Neptune Islands), and New Zealand (Stewart Island) (Bruce, 2015). However, these regulations differ between locations in terms of the amounts of bait and berley that can be used. In California, baiting and the use of berley to attract white shark is prohibited. In South Africa, bait and berley have a maximum daily limit of 25 kg per tour operator whereas in South Australia, operators have a fortnightly limit of 1,000 kg. To further strengthen the incentive to remove the bait before the shark can take it, South Australia also introduced a 15-mins penalty during which no bait or berley can be used when a shark takes the bait. Compliance to regulations and limits is monitored and legislated by the South Australian Department for Environment and Water (DEW) using a logbook system and remote camera placed on one of the islands. While not stipulated in policy documents, limits on the amount of food-based attractant and number of baits used minimise the risk of learning or making an association between humans and food while interacting with cage-diving vessels. While associative learning can be influenced by reward frequency or magnitude in a range of taxa (Kerpelman and Himmelfarb, 1971, Muzio et al., 1992, Neuringer, 1967, van den Akker et al., 2014), it is currently unknown whether the number of baits or size of baits (i.e. amount of food) most affect the rate at which sharks learn or which should be regulated to reduce potential associations being made.

Here, we investigate the effect of reinforcement frequency and reward magnitude on the rate of learning in the benthic Port Jackson shark (*Heterodontus portusjacksoni*). The experiment consisted of a simple operant conditioning regime based on a spatial task in a controlled laboratory environment. We predicted that sharks subjected to higher reinforcement frequencies and larger reward magnitudes would learn the given task at a significantly faster rate than those trained at a low frequency and a small reward.

3.3 Methods

Port Jackson sharks (*H. portusjacksoni*) are benthic elasmobranchs that are endemic to the temperate waters of Australia. Its small size, hardiness and high abundance along the New South Wales coast, makes it a suitable model species for shark-related research performed under controlled laboratory conditions (Byrnes and Brown, 2016, Byrnes et al., 2016a, Powter and Gladstone, 2009). Moreover, the logistical difficulties holding and conducting experiments on species typically targeted by shark-diving tourism in captivity is prohibitive. Twenty-four Port Jackson shark eggs were opportunistically collected from rocky reef substrates by hand in Jervis Bay during a single trip around May 2016. They were transported to the Sydney Institute for Marine Science (SIMS, Mosman, NSW) where they were housed in four 500 L aquaria. Upon hatching sharks were moved into three 1,000 L aquaria where they were kept for the duration of the experiments. All tanks were continuously supplied with clean, aerated seawater from Chowder Bay, Sydney, NSW, at ambient temperatures (23.25 C° ± 2.75 C°) and received a natural Sydney photoperiod. Shelters, consisting of pieces of 100 mm PVC pipe and clay pots, were provided in each holding tank. Under the initial husbandry conditions, *H. portusjacksoni* were fed approximately 6 % of their body mass in food three times per week with defrosted squid *Loligo opalescens* and *Nototodarus sloanii*, whiteleg shrimp *Litopenaeus vannamei*, and Basa fish *Pangasius bocourti*.

The total length of each individual was measured to the nearest 5 mm (31.79 ± 3.46 cm; mean ± SD) and the body mass was recorded to the nearest gram using a digital balance (174.25 ± 51.21 g; mean ± SD). Sex was determined by noting the presence of claspers in males. Each shark was sub-dermally implanted with a PIT tag at the base of the first dorsal fin to allow for easy identification. Sharks were assigned to one of four treatment groups, High Frequency – Large Magnitude, High Frequency – Small Magnitude, Low Frequency – Large Magnitude, and Low Frequency – Small Magnitude. We used pseudo-randomisation when assigning the sharks to the treatment groups to ensure an equal sex and mass distribution (one-way ANOVA for mass: $F_3 = 0.288$, $p = 0.834$). The frequency of the treatment groups represents the number of trials a shark received per day (high frequency = 6, low frequency = 3) and the magnitude represents the size of the reward each shark received upon completion of a trial (large = 0.16 % and small = 0.08 % of wet body weight equivalent). The reward magnitudes were small enough to ensure a strong feeding motivation during all trials. To further ensure equal feeding motivation across all treatments, each shark was fed a total of 2% wet body mass equivalent per day (Sims, 1996). This included the rewards fed during the trials and the remaining 1–1.5% body mass depending on the treatment, which were fed after the completion of the daily training.

Experiments were carried out in an oval experimental arena measuring 180 x 100 cm with a water depth of 40 cm. The length of the arena was divided into two equally sized areas using a partition. Two equally sized doors (22 x 10 cm; width x height) were cut out of the wall, and two sheets of clear Perspex were used as guillotine doors to close the openings. Around the door frames, 5-cm wide stripes of laminated paper showing two different patterns (black on the left-side door and black/white squares on the right-side door) were glued to the wall to assist sharks in distinguishing the two doors (Figure 3.1). A partition made of black mesh was used to close off the starting area. On the opposite side of the tank, a black disc with a single white stripe in the middle was located behind the reward area to serve as a visual landmark to assist sharks in locating the food reward. To account for a potential side bias of the sharks they were assigned to either the right-hand or the left-hand door. This assignment was done using pseudo-randomisation to ensure that within each of the four treatment groups three sharks were trained to the left and three sharks were trained to the right-hand door. Water inflow was provided from the side that held the starting compartment. This prevented the olfactory cues from being carried downstream toward the sharks inside the starting compartment, eliminating the possibility of sharks locating the food reward by smell rather than learning the association with the door.

3.3.1 Acclimation phase

Sharks were provided with a period of three days to acclimate to the experimental arena and the transfer between the holding and experimental tank. On each of the three acclimation days, sharks were transferred individually to the experimental basin, using a bucket filled with seawater, where they were free to explore the arena with both doors open for 15 min. The sharks were then fed the equivalent of 2% of their wet body mass in small pieces of squid using tongs. After a total of 30 min the sharks were transferred back to their holding tanks.

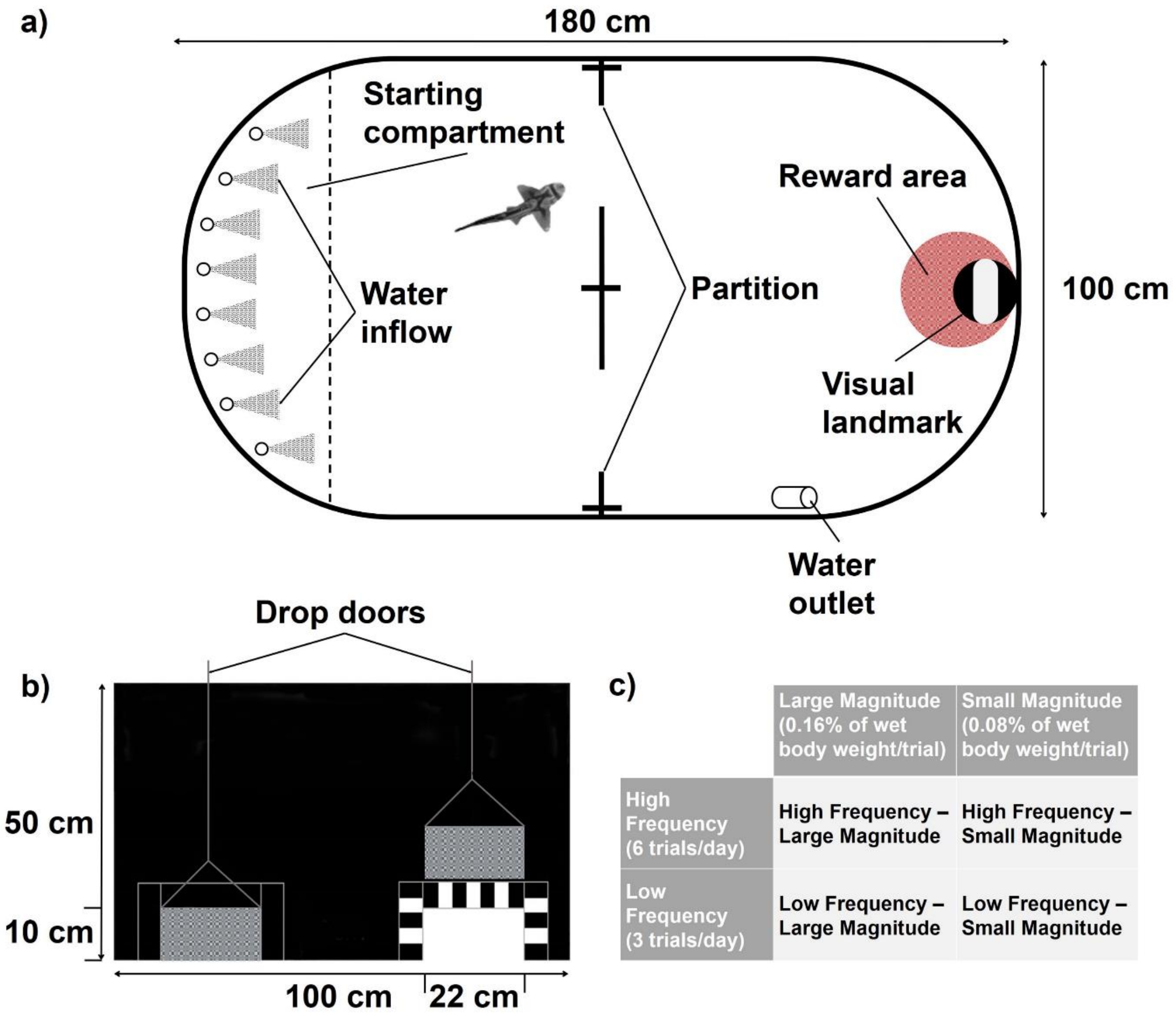
3.3.2 Pre-Training phase

Pre-training trials were run on days one to 10. Training occurred on seven days a week and consisted of a single training session per day. The number of trials per day depended on the treatment group a shark was assigned to. Low-frequency training consisted of three trials per day and high-frequency training of six trials per day. Prior to the first trial sharks were provided with five minutes of acclimation with both doors closed. Sharks were then gently ushered back to the starting area and provided with 30–60 s rest before the first trial commenced. At the start of the first trial, the treatment door was opened whilst the control door was kept closed. The partition closing off the starting area was then removed, and the food was introduced on the opposite side of the door. The location of the food reward was moved further away from the door each day during the pre-training phase. At the end of the pre-training phase the reward location had reached the opposite end of the tank (Figure 3.1) where it remained throughout the training phase.

Following the removal of the start partition, sharks were provided 90 s to pass through the door before the trial was terminated and recorded as unsuccessful. When sharks passed the treatment door within 90 s, they were given another 30 s to consume the food reward. If the shark did not attempt to consume the reward within 30 s, the trial was terminated and recorded as a correct pass. The time limit of 30 s was used to ensure that sharks could make a link between the food reward and passing the correct door. At the end of a trial, the shark was gently ushered back to the starting area and provided 30–60 s of rest before the start of the next trial. Following the last trial, sharks were given three to six minutes of rest with both doors closed before being ushered back to the starting area where they were fed any left-over rewards and the remainder of the 2% wet body mass equivalent in food.

3.3.3 Training phase

The training phase occurred from day 10 to 21. The treatment protocol remained the same but both doors were opened during the trials and the food reward was provided at the designated reward location (Figure 3.1a). The number of daily trials depended on a shark's treatment group (Low Frequency = 3 trials; High Frequency = 6 trials). An individual was considered to have learnt the task successfully once it passed through the treatment door within 90 seconds during 9 out of 12 consecutive trials (0.75 success rate). Passing through the wrong door resulted in the termination of the trial, which was counted as a mistrial.



	Large Magnitude (0.16% of wet body weight/trial)	Small Magnitude (0.08% of wet body weight/trial)
High Frequency (6 trials/day)	High Frequency – Large Magnitude	High Frequency – Small Magnitude
Low Frequency (3 trials/day)	Low Frequency – Large Magnitude	Low Frequency – Small Magnitude

Figure 3.1 Schematic illustration of a) a birds-eye view of the tank, and b) a frontal view of the partitioning with the left-hand door displayed as being closed and the right-hand door being open, and c) a table summarising the four different treatment groups (large reward groups received 0.16% and small reward groups received 0.08% of wet body mass per trial; high frequency groups received 6 and low frequency groups received 3 trials per day).

3.3.4 Data analyses

All trials were video recorded and trial statistics were collected by one observer using video playback on VLC media player. It was not possible to record data blind because our individuals have uniquely identifying markings. The response variables included: (i) pass rate, (ii) approach index, (iii) latency to pass the correct door, and (iv) success rate (Table 3.1). In cases when sharks did not pass any of the doors during the 90 s provided, we assigned a ceiling score of 270 seconds to the latency to pass variable, which was three times the maximum trial time. The pre-training phase was omitted from the learning analyses.

Table 3.1 List of response variables used in the learning task and the corresponding distribution used for the GLMM.

Variable	Definition	Distribution
Pass rate	Proportion of trials sharks passed through either door within a session. Pass rate ranged between 0 (never passed a door) and 1 (passed a door on all trials). A shark was considered to have passed a door when its head and pectoral fins had crossed the door.	binomial
Approach index	Percentage of approaches made toward the correct door ([number of correct approaches/total number of approaches] x 100). An approach was defined as a direct swim toward the door within one body length of the door.	Gaussian
Latency to pass	Time taken for sharks to pass the correct door.	negative binomial
Success rate	Proportion of trials sharks passed through the correct door within a session. Success rate ranged between 0 (never passed the correct door) and 1 (passed the correct door on all trials).	binomial

For each of the four response variables, Generalised Linear Mixed Models (GLMMs) were used to test the effects of reinforcement frequency and reward magnitude on the learning performance of juvenile Port Jackson sharks. Explanatory variables included *reinforcement frequency*, *reward magnitude*, *experimental day*, *treatment side* (left or right), and the interaction between the *reinforcement frequency*, *reward magnitude,* and *experimental day*. Given the repeated measurements of individuals, individual sharks were included in the model as the random effect nested within holding tank. The error structure of GLMM corrects for non-independence of statistical units due to shared temporal structure and permits the random effect variance explained at different levels of clustering to be decomposed. We determined the most appropriate statistical family and error distribution for each analysis by examining the distribution of the response variables and visually inspecting the residuals for the saturated models in accordance with Zuur et al. (2010) (Table 3.1). We ran all models for all possible combinations of factors, and compared their relative probability using Akaike's information criterion corrected for small sample size (AIC_c) (Burnham and Anderson, 2002). To identify the most influential drivers of shark learning behaviour, a dredge function (R package MuMIn; Barton 2013) was used to identify more-parsimonious nested models according to the AIC_c. A 'confidence set' of models with ΔAIC < 2 were considered equivalent and included in model averaging (Burnham et al., 2011); from which the Relative Variable Importance values (RVI; calculated from the sum of AIC weights of models within the confidence set in which the parameter of interest appears) were used to identify important variables. All analyses were performed in R v.4.0.0 (R-Core-Team, 2020) using the package glmmTMB (Brooks et al., 2017). Graphs were created in R v.4.0.0 (R-Core-Team, 2020) using the packages ggplot2 (Wickham, 2016) and plotly (Sievert et al., 2017).

3.4 Results

The mean number of sessions required to reach the learning criterion (LC) ranged from 13.33 ± 0.88 in the Low Frequency – Small Magnitude, and 13.75 ± 1.44 in the High Frequency – Large Magnitude group to 16.40 ± 0.75 in the Low Frequency – Large Magnitude, and 17.6 ± 0.81 in the High Frequency – Small Magnitude group (Figure 3.2). Neither frequency (ANOVA, F = 0.149, $p = 0.706$) nor magnitude (ANOVA, F = 0.350, $p = 0.564$) had a significant effect on the number of training sessions needed to reach LC. However, not all tested individuals reached the learning criteria within the provided 21 days of training (Figure 3.2). Though the mean number of sessions required to reach the LC was highest in the High Frequency – Small Magnitude group (17.6 ± 0.81) it also had the highest number of sharks to successfully reach the LC (5 out of 6) together with the Low Frequency – Large Magnitude group (Figure 3.2). The performance of Port Jackson sharks with respect to the behavioural traits we investigated was significantly influenced by *reinforcement* *frequency* and *reward magnitude* (Figure 3.3, 3.4). However, reinforcement frequency had a stronger effect on the learning rate of sharks (Figure 3.3, 3.4).

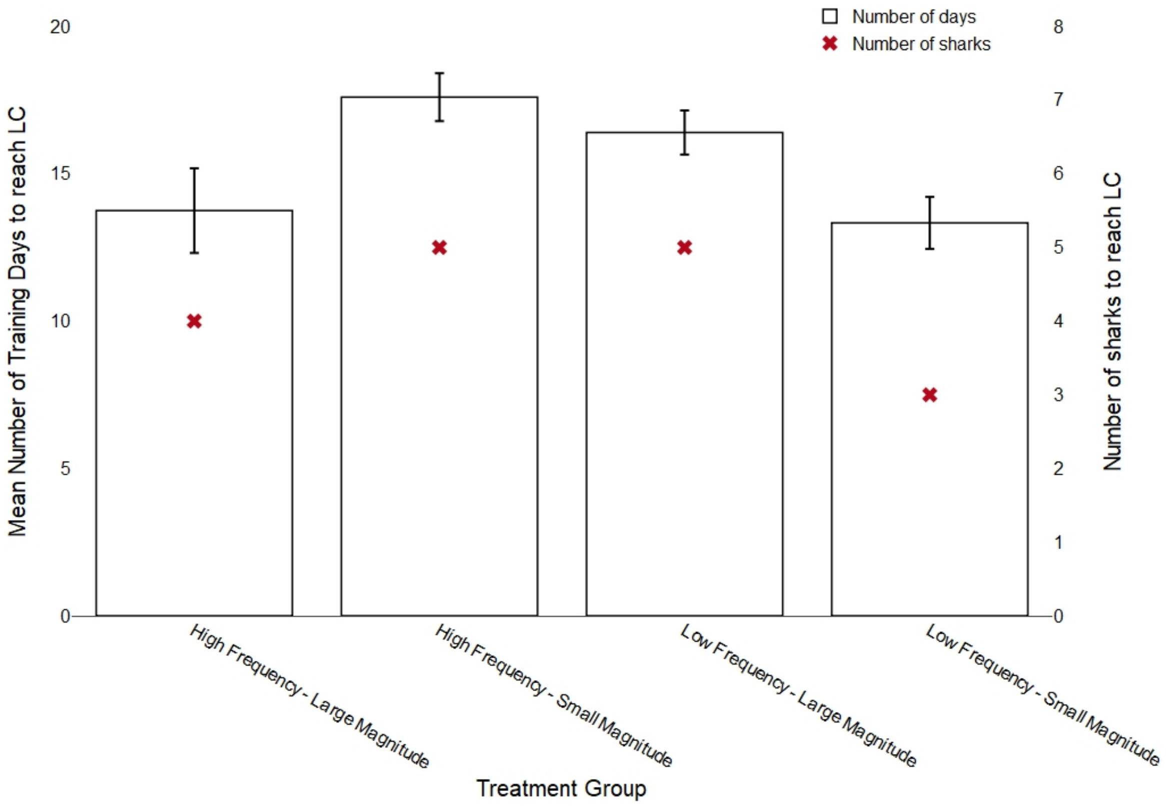


Figure 3.2 The mean number of training days (bars) ± standard error it took sharks to reach the learning criteria (LC). Red crosses represent the number of sharks that reached LC within the 21 days of the experiment. High Frequency – Small Reward sharks took the longest to reach LC but had the most individuals reaching LC within 21 days, together with the Low Frequency – Large Reward sharks.

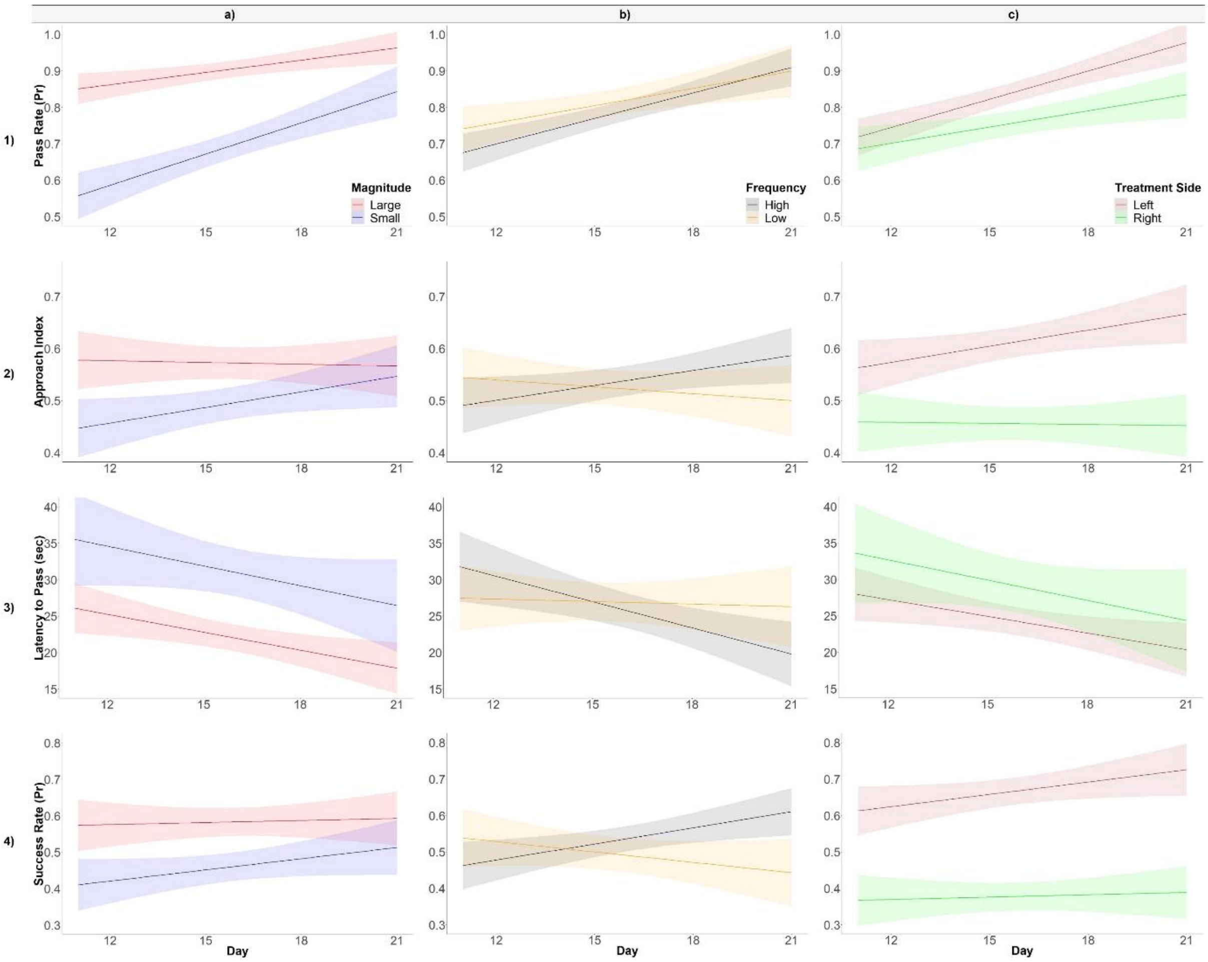


Figure 3.3 Effects of reward magnitude (left), reinforcement frequency (centre), and treatment side (right) on 1) pass rate, 2) approach index, 3) latency to pass the correct door, and 4) success rate through time with 95% confidence bands. High Frequency sharks learnt at a faster rate with regards to the approach index and the success rate. Reward Magnitude only affected the approach index with Small Reward sharks learning at a faster rate.

3.4.1 Pass Rate

When looking at the proportion of trials that resulted in sharks passing either door, the RVI scores for the variables included in the model averaging ranged from 0.22 (day*magnitude) to 1.00 (day, magnitude, Table 3.2). Pass rate increased throughout the experiment by up to ~25% during the 10-day training period (Figure 3.3). Individuals that received large rewards displayed a consistently higher pass rate throughout the experiment than individuals that received small rewards ($p = 0.0084$) (Figure 3.3, Appendix Table A3.1, A3.2). However, the interaction between the *reward magnitude* and the *experimental day* had no significant effect on the pass rate (*p* = 0.46). The lack of any frequency effect and the non-significant effect of the magnitude*day interaction indicates that neither frequency nor magnitude had a significant impact on the learning rate of sharks with respect to the pass rate. Furthermore, sharks trained to pass the left door performed slightly better than those trained to pass through the right door (*p* = 0.10) (Figure 3.3).

3.4.2 Approach Index

When testing the effects of *reward magnitude* and *reinforcement frequency* on the approach index, the RVI scores range from 0.09 (frequency*magnitude) to 1.00 (side, Table 3.2). While sharks that received a small reward increased in performance, individuals trained with a large reward had a relatively consistent approach index throughout the experiment (*p* = 0.064) (Figure 3.3). In contrast, individuals trained at a high reinforcement frequency demonstrated an increasingly higher approach index; whereas the approach index decreased in sharks trained less frequently (*p* = 0.047) (Figure 3.3, Appendix Table A3.3, A3.4). *Treatment side* also had a significant impact, with individuals trained to pass through the left door performing better than those trained to the right door (*p* = 0.0015) (Figure 3.3, Table 3.2, Appendix Table A3.3, A3.4).

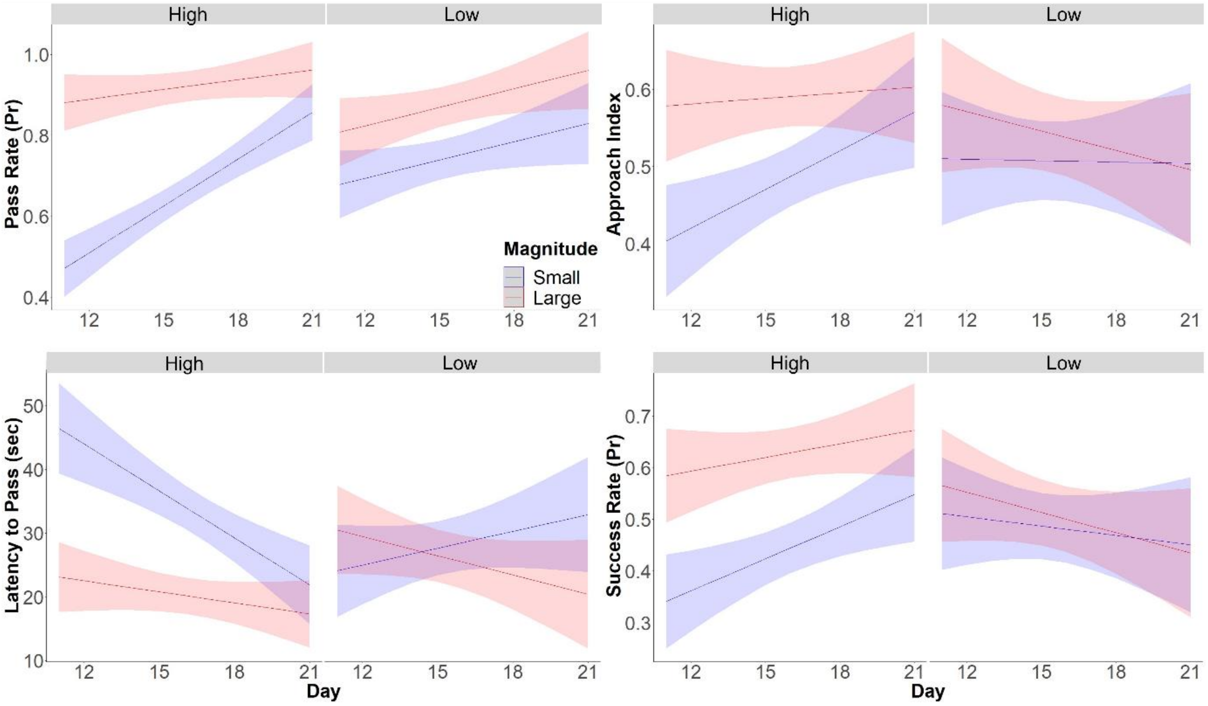


Figure 3.4 Interaction effect between the reinforcement frequency (left: high, right: low), the reward magnitude (blue: small, red: large), and the experimental day on a) pass rate, b) approach index, c) latency to pass the correct door, and d) success rate with 95% confidence bands. Reinforcement Frequency and Reward Magnitude had a significant interaction effect on the latency to pass with Large Reward sharks learning significantly faster when subjected to a Low Reinforcement Frequency.

3.4.3 Latency to pass correct door

The latency to pass through the correct door was significantly affected by the three-way interaction between the *reinforcement frequency*, the *reward magnitude* and the *experimental day* (*p* = 0.0030) (Table 3.2, Appendix Table A3.5, A3.6). When trained at a high reinforcement frequency, latency to pass the correct door decreased more rapidly in individuals that received small rewards than those receiving large rewards (*p* = 0.0030) (Figure 3.4). In contrast, when trained at a low reinforcement frequency, sharks that received small rewards took progressively longer to pass the correct door, whereas sharks that received large rewards passed the correct door faster over time (*p* = 0.0030) (Figure 3.4).

Table 3.2 Table listing the Relative Importance Values for all explanatory variables. Values marked with * indicate that they were not included in the top-ranking models ($\Delta AIC_c < 2.0$) and as a result were not part of the model averaging.

	Pass Rate	**Approach Index**	**Latency to Pass**	**Success Rate**
Day	1.00	0.85	1.00	1.00
Magnitude	1.00	0.78	1.00	0.84
Side	0.68	1.00	0.38	1.00
Frequency	0*	0.50	1.00	1.00
Day*Magn	0.22	0.51	1.00	0.24
Day*Freq	0*	0.43	1.00	1.00
Freq*Magn	0*	0.09	1.00	0.21
Day*Freq*Magn	0*	0*	1.00	0*

3.4.4 Success Rate

The RVIs of the variables included in the top-ranked models (ΔAICc<2, Appendix Table A3.7, A3.8) ranged 0.21 (frequency*magnitude) to 1.00 (day*frequency, side, Table 3.2). Sharks trained at a high frequency performed increasingly better; whereas the proportion of correct passes decreased over time in sharks that were trained at a low frequency ($p = 0.0078$) (Figure 3.3). *Reward magnitude* had no significant effect on the success rate of the sharks ($p = 0.14$). However, sharks trained to the left door performed significantly better than those trained to the right door ($p < 0.001$) (Figure 3.3, Table 3.2, Appendix Table A3.7, A3.8).

3.5 Discussion

Our study revealed that reinforcement frequency affects the rate at which Port Jackson sharks learnt aspects of a spatial cognitive task in an operant conditioning regime. The effect of reinforcement frequency varied depending on the approach index, latency to pass, and success rate, but not the pass rate. In contrast, reward magnitude had little effect on the rate at which sharks improved with regards to the four behavioural traits assayed. Still, the overall performance was found to be consistently higher in sharks trained with a large reward. The results were influenced by lateralisation, with individuals trained to pass the left door performing better than those trained to pass the right door. Future studies should assess side preferences prior to the beginning of the experiments and then train individuals to the opposite side.

Port Jackson sharks learnt the spatial cognitive task in this study within 13-18 sessions (or 40-106 trials) depending on the treatment group. This is similar to the 15 sessions taken by grey bamboo sharks (*C. griseum*) and coral cat sharks (*Atelomycterus. marmoratus*) to learn to locate a fixed goal position in a diamond shaped maze from two different starting positions (Fuss et al., 2014a). Grey bamboo sharks and Port Jackson sharks also took a similar amount of time (18 sessions) to learn other cognitive tasks such as visual discrimination and association of two time-separated events respectively (Fuss et al., 2014b, Guttridge and Brown, 2013). Besides sharks, learning has also been investigated in other elasmobranchs. The ocellate river stingray (*Potamotrygon motoro*) for instance, learnt a spatial cognitive task within 17 session at a frequency of ten trials per session (Schluessel and Ober, 2018) and the yellow stingray (*Urobatis jamaicensis*) learnt to discriminate magnetic stimuli within 13 session with four trials per session (Newton and Kajiura, 2017). This suggests that, when trained daily, sharks and rays can learn a range of spatial and visual tasks relatively quickly. In comparison, teleost fish appear to outperform elasmobranchs on similar tasks. For instance, when trained at a frequency of 25 trials per day, goldfish (*Carassius auratus*) achieve a learning criterion of 80% successful trials when subjected to a similar spatial cognitive task within 3–4 days (Portavella and Vargas, 2005). Three spined sicklebacks (*Gasterosteus aculeatus*) required 5–10 trials to reach the learning criterion (three successful consecutive trials) when trained at lower frequencies (one trial every 36 to 48 hours) (Girvan and Braithwaite, 1998). However, Fuss et al. (2018) recently carried out an object discrimination experiment comparing the cognitive abilities of juvenile grey bamboo sharks (*Chiloscyllium griseum*) and Malawi cichlids (*Pseudotropheus zebra*). They demonstrated that the sharks reached the LC within 15 sessions whereas the cichlids required 32 sessions on average. Furthermore, sharks were able to apply previously gained knowledge to novel stimuli and use abstract relational concepts to categorise these novel objects. In contrast, cichlids failed to apply both concepts simultaneously.

3.5.1 Reinforcement frequency

Our findings show that sharks learnt faster when trained at a higher reinforcement frequency with regards to the approach index and the success rate. However, the pass rate, hence, the general association between passing the partition and receiving a food reward was unaffected by the reinforcement frequency. Low reinforcement frequency also increased latency to pass when combined to small reward size. Our findings are supported by previous studies showing positive correlations between reinforcement frequency and performance during the acquisition of a task or behaviour (Devan et al., 2003, Gonzalez and Bitterman, 1967, Muzio et al., 1992). Many of these studies, however, compared continuous (every trial is rewarded) to partial (only a certain percentage of trials is rewarded) reinforcement (Bouton et al., 2014, Kerpelman and Himmelfarb, 1971, Muzio et al., 1992) and tested the active role of inhibition or frustration in addition to the passive role of nonreinforcement (Amsel, 1958, Amsel, 1962). Nonetheless, our findings show a positive correlation between reinforcement frequency and learning rates similar to previous studies on Argentine common toads (*Bufo arenarum*) and Fancy rats (*Rattus norvegicus domestica*) that were subjected to spatial cognitive tasks (Devan et al., 2003, Muzio et al., 1992), and goldfish (*Carassius auratus*) that were trained to press a target to gain access to a food reward (Gonzalez and Bitterman, 1967).

3.5.2 Reward magnitude

The elevated performance (i.e. higher number of correct passes) in individuals that received a large reward was evident from the beginning of the experiments, suggesting that it might be due to individual variability rather than the effect of reward magnitude. Our findings showing that learning rate was not affected by reward magnitude contradict previous studies. For example, goldfish (*Carassius auratus*) and Argentine common toads (*Rhinella arenarum*) exhibited a positive correlation between reward magnitude and the rate at which they learnt a given task (Gonzalez et al., 1972, Muzio et al., 1992).

The lack of correlation observed in our study could be due to the size of the food rewards. Although the amount of food doubled between the low and high reward magnitude, the sizes of the rewards were small due to the number of trials per day and the need to maintain a consistent feeding motivation to the food stimulus during all trials. Therefore, we limited the amount of food provided during the trials to a total of 1% wet body weight equivalent. With the juvenile Port Jackson sharks only weighing ~175 g, the actual difference in the reward magnitude was ~0.2 g per trial, which may have been too small for sharks to learn at a different pace.

3.5.3 Treatment side and lateralisation

Brain lateralisation has recently been demonstrated in both juvenile and adult Port Jackson sharks (Byrnes et al., 2016a, Byrnes et al., 2016b, Vila Pouca et al., 2019, Vila Pouca et al., 2018), and it could explain the significant effects of treatment side we observed during our study. While the ecological benefit of lateralisation has not been demonstrated in elasmobranchs, cerebral and behavioural lateralisation are widespread and have been suggested to offer many selective advantages. For example, strongly lateralised fish perform simultaneous tasks more efficiently than non-lateralised individuals (Dadda and Bisazza, 2006). Brain lateralisation was further found to enhance predator recognition learning (Chivers et al., 2017), and spatial reorientation (Sovrano et al., 2005). An increased performance during these types of learning will likely increase individual survival, especially in predator-rich environments (Ferrari et al., 2015, Stier et al., 2013). Since we could not test the laterality strength of the Port Jackson sharks tested, it was not possible to determine if performance was affected by the shark's lateralisation, other than noticing the strong bias towards the left door.

3.5.4 Implications to wildlife tourism

With the increasing popularity of shark-diving tourism, we are in need of management strategies that will ensure the sustainability of the industry (Gallagher and Huveneers, 2018). The use of bait to attract sharks is a popular method to ensure reliable encounters with these elusive predators (Clua and Séret, 2010). However, these practices can affect the behaviour of the targeted species (Huveneers et al., 2013), putting them at risk of getting caught in an ecological trap (Schlaepfer et al., 2002). Ecological traps occur when the habitat choices of organisms are negatively affected by cues that would normally be associated with habitat quality (Schlaepfer et al., 2002). For instance, the artificial feedings of southern stingrays (*Dasyatis americana*) off the Cayman Islands, Bahamas has caused these animals to change from a solitary to a group-living lifestyle (Corcoran et al., 2013, Semeniuk and Rothley, 2008). Consequently, they suffer from reduced health conditions, increased parasite loads, and an increase in conspecific bite marks. These detrimental effects are due to the changes in residential behaviour caused by the poor habitat choices (Corcoran et al., 2013, Semeniuk and Rothley, 2008). The white shark cage-diving industry in South Australia also affects the behaviour and diet of non-target species, e.g. smooth stingray (*Bathytoshia brevicaudata*), trevally (*Pseudocaranx spp.*), yellowtail kingfish (*Seriola lalandi*) (Meyer et al., 2019, Rizzari et al., 2017).

Shark feeding operations may also cause an increased level of aggression toward conspecifics, other species of sharks, and humans (Burgess, 1998, Gallagher and Huveneers, 2018). An example can be found off Bimini, Bahamas where great hammerhead sharks (*Sphyrna mokarran*) have been provisioned since 2013. Tourism operators were observed dumping bait at the dive sites in between tours to keep the sharks nearby. During the second season of provisioning, bull shark (*Carcharhinus leucas*) had already overrun many of the dive sites exhibiting some aggression toward other shark species and humans (Gallagher and Huveneers, 2018). Aside from the negative effects on the surrounding ecosystem and the safety of the divers, the presence of the bull sharks also impacted the industry itself, since they drove off the great hammerhead sharks (Gallagher and Huveneers, 2018). The increased aggression toward humans is a major public concern. People are afraid that sharks will learn to associate food with humans when being fed for tourism purposes (Burgess, 1998). Our results suggest that a reduction in the frequency of exposure to these operations would contribute towards avoiding behavioural alterations in targeted and non-targeted elasmobranch species.

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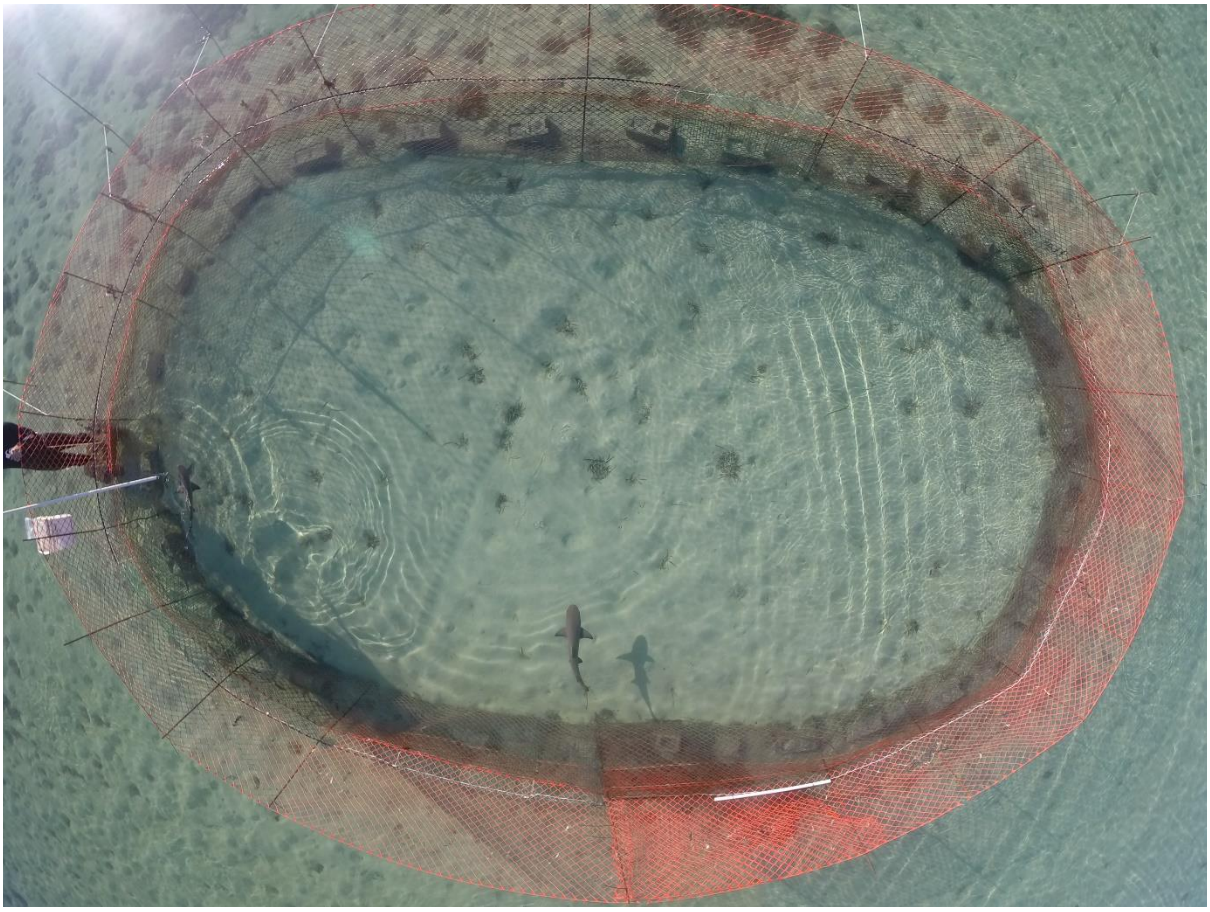
Chapter 4

No evidence for time-place learning in juvenile lemon sharks (*Negaprion brevirostris*)



4.1 Abstract

Endogenous circadian clocks influence a variety of animal behaviour, including the timing of daily foraging events, and is among the most prominent factors dictating an animal’s daily activity patterns. Learning about consistent spatio-temporal patterns in food availability can be advantageous, as it may increase foraging efficiency. Time-place learning abilities have been demonstrated in teleosts but has yet to be studied in elasmobranchs. Understanding time-place learning in sharks will help us understand how anthropogenic factors, including wildlife tourism, may affect shark behaviour. Here, we investigated the ability of sharks to learn time-place associations in a semi-controlled environment. We trained juvenile lemon sharks (*Negaprion brevirostris*) to feed at two different locations at two distinct times of day inside 45 m2 enclosures for 41 days and monitored their learning along with untrained, control individuals. We used time-laps recordings taking an image every five seconds to assess a shark’s position relative to the upcoming feeding location. Based on the mean distance to said location and the time sharks spent within three body lengths of it, we concluded that they were unable to learn the association. Sharks inside the treatment pens displayed distribution patterns similar to those of the control sharks throughout the course of the experiment. The position of the sharks inside the holding pens instead appeared to be significantly affected by the tidal cycle. Such an effect might be linked to the juvenile lemon sharks’ natural tendency to seek protection from large predators inside shallow mangrove inlets during high tides. We suggest that juvenile lemon sharks were unable to learn the association due to the relatively small enclosure size in combination with a strong dependency on the tidal cycles.

4.2 Introduction

Endogenous circadian clocks form the basis of an animal’s ability to recognise and store spatio-temporal information of biologically relevant events (Gómez-Laplaza and Morgan, 2005), which is key to the survival and reproductive success of most animals (Van der Zee et al., 2008). In most animals, these clocks dictate many temporal behavioural aspects, such as the wake and sleep onset, daily timing of foraging, and reproductive activities (Reebs, 1996, Van der Zee et al., 2008). Among the most prominent factors that dictate the organisation of an animal’s daily activity patterns is the availability of and search for food (Gómez-Laplaza and Morgan, 2005). When a food source occurs in a repeatable spatio-temporal pattern, it is advantageous to learn this pattern to increase foraging efficiency. To learn the association between a time of day and a location requires the endogenous clock to maintain a continuous influence on an individual’s behaviour throughout the day (Reebs, 1996). The ability to link the location of an event or stimulus with a time of day is known as time-place learning (Biebach et al., 1991, Schatz et al., 1999) and studies on this subject in wild fishes are relatively scarce.

Time-place learning has been demonstrated in a range of taxonomic groups in captive settings, including invertebrates (Schatz et al., 1999), birds (Biebach et al., 1989, Shettleworth and Plowright, 1992), mammals (Van der Zee et al., 2008), and some mixed results for fishes (Gómez-Laplaza and Morgan, 2005, Reebs, 1996). Cichlid angelfish (*Pterophyllum scalare*) were able to associate the time and place of daily food sources when fed twice per day in two different locations (Gómez-Laplaza and Morgan, 2005). Similarly, zebrafish (*Danio rerio*) were able to learn time-place associations based on social reinforcement, using two locations and times of day (de Almeida Moura and Luchiari, 2016). The fish learnt the location and time at which a group of conspecifics (same size and age) was introduced to the aquarium and even started to exhibit anticipatory behaviours prior to the introduction of the stimulus (de Almeida Moura and Luchiari, 2016). Conversely, when kept under constant light and dark conditions, zebrafish were unable to learn time-place associations, indicating the importance of environmental factors that can be used to synchronise an animal's endogenous circadian clock (de Almeida Moura et al., 2017). Convict cichlids (*Cichliasoma nigrofasciatum*) were also unable to learn spatio-temporal associations in relation to a food reward within 30-consecutive days of training (Reebs, 1993). The ability to learn such spatio-temporal associations also appears to vary with stimulus. For example, common galaxias (*Galaxias maculatus)* learnt the association between a place and a time to receive food within 14 days, but failed to learn to avoid a specific place at a specific time in the presence of predation risk (Reebs, 1999). These results suggest that selection for learning may occur if it is ecologically relevant given that food patches may be more spatio-temporally predictable than predators. While there is still much to discover about time-place learning in fishes, knowledge on this ability in cartilaginous fishes (e.g. sharks and rays) is entirely lacking. Investigating the ability of sharks to learn regular time-place associations with respect to the availability of a food source will help us better understand their diurnal movement patterns and behavioural strategies.

Sharks and rays are capable of learning a wide variety of cognitive tasks at rates similar to those of birds and mammals (Guttridge et al., 2009b). These tasks include spatial learning (Edrén and Gruber, 2005, Fuss et al., 2014b), object discrimination (Fuss et al., 2014c, Schluessel et al., 2012, Schluessel et al., 2014), perception of illusory contours (Fuss et al., 2014a), long-term memory retention (Fuss and Schluessel, 2015, Guttridge and Brown, 2013), social learning (Guttridge et al., 2013, Vila Pouca et al., 2020), and tool use (Kuba et al., 2010). Sharks have also been found to align their long-distance migrations with seasonal peaks in resource availabilities (Hammerschlag et al., 2016, Meyer et al., 2010, Sims et al., 2003, Sims et al., 2005). For example, tiger sharks can travel thousands of kilometres to prey on seasonally abundant fledgling Albatross (*Phoebastria* spp.) at remote Hawaiian atolls (Meyer et al., 2010). Tiger sharks also visit a popular green turtle (*Chelonia mydas*) breeding spot at Raine Island, Australia during the nesting season to scavenge on the abundant carcasses (Hammerschlag et al., 2016). While there have been relatively few studies of shark cognition, results to date suggest capacities similar to terrestrial animals.

These cognitive capacities could be the underlying factors leading to detrimental effects associated with wildlife tourism activities (Semeniuk, 2020, Semeniuk and Rothley, 2008). Marine predators, such as sharks, are often difficult to observe due to their shy and elusive nature (Bres, 1993, Burgess, 1998). The feeding of sharks has, therefore, become a common practice to maximise shark-human interactions and produce reliable and good viewing opportunities (Newsome et al., 2004, Orams, 2002). It has been argued that such shark feeding operations have detrimental effects on the targeted animals, their environment, and possibly on humans (Dobson, 2006, Hammerschlag et al., 2012, Newsome and Rodger, 2008, Burgess, 1998). These effects can range from decreased physiological condition to behavioural alterations that could cause cascading effects throughout the local ecosystem (Brunnschweiler et al., 2018, Brunnschweiler and Baensch, 2011, Burgin and Hardiman, 2015, Corcoran et al., 2013, Semeniuk and Rothley, 2008). Learnt associations between humans and food or between certain locations and food may further result in an increased risk in shark-human encounters that result in injuries or death (Orams, 2002, Gallagher and Huveneers, 2018). These negative impacts can undermine the socio-economic benefits, improved education, and increased conservation resulting from wildlife tourism experiences (Apps et al., 2018, Huveneers et al., 2018). With a net gross income of approximately $165 billion dollars annually worldwide (Corcoran et al., 2013), wildlife tourism is considered one of the fastest growing sectors of the tourism industry (Scheyvens, 1999) and offers a great opportunity to provide *in situ* education to participants and increase public awareness and assist conservation efforts (Apps et al., 2016).

Learning time-place associations with respect to the availability of a food source could put sharks at risk of getting caught in an ecological trap when frequently subjected to feeding operations (Semeniuk and Rothley, 2008, Semeniuk, 2020). An ecological trap refers to scenarios in which rapid environmental change leads organisms to prefer to settle in poor-quality habitats (Schlaepfer et al., 2002). These rapid environmental changes may include the regular introduction of an artificial food source for wildlife tourism purposes. Such an ecological trap has been described by Semeniuk and Rothley (2008) in the southern stingray (*Dasyatis americana*) around the Cayman Islands. These rays, which are normally solitary by nature, were found to live and forage in much higher densities than expected due to food provisioning by tourists. These increased population densities resulted in higher numbers of conspecific bite marks and ectoparasite loads (Semeniuk and Rothley, 2008). Focal animals further suffered from lower body condition, resulting in an elevated risk of being injured by a boat or taken by a predator (Semeniuk and Rothley, 2008). Pini-Fitzsimmons et al. (2018) provided further evidence for the detrimental impacts of regular provisioning on the behaviour of another ray species, the short-tail stingray (*Bathytoshia brevicaudata*). Their findings demonstrated increased densities of individuals around the feeding site and indicated anticipatory behaviour in the afternoon, during times when feedings occurred most often. Such anticipatory behaviour suggests that some form of time-place learning has occurred.

Our study investigated whether elasmobranchs are capable of forming time-place associations, using juvenile lemon sharks (*Negaprion brevirostris*) as a model species. Lemon sharks were chosen as our study species due to their relatively high abundance around the islands of Bimini (Kessel et al., 2016), as well as their relatively small size and hardiness in captivity. They are also among the most extensively studied species of elasmobranchs, allowing for an optimisation of experimental design in captive and semi-captive conditions (Dhellemmes et al., 2020b, Dhellemmes et al., 2020a, Finger et al., 2018, Finger, 2019, Gruber and Schneiderman, 1975, Guttridge et al., 2009a, Guttridge et al., 2013, Kessel et al., 2016). Furthermore, lemon sharks are a common focal species in shark feeding operations, which makes the results of this study more applicable to wildlife tourism activities. Here, we recorded the behaviour of sharks being fed at scheduled times and locations within a confined semi-captive environment consisting of a 45 m^2 arena. Given the evidence of the strong spatial cognitive abilities in sharks and rays (Edrén and Gruber, 2005, Fuss et al., 2014b, Schluessel and Bleckmann, 2012, Heinrich et al., 2020), we hypothesised that juvenile lemon sharks would start to pre-empt feeding times and locations as the experiment progressed. We expected sharks to remain close to the upcoming feeding location prior to the beginning of the feedings during the last day of experiments compared to the first. We further expected to see a significant difference in spatial distributions between the treatment and the control pens based on Kernel Density Estimates (KDE) toward the end of the experiment. Overall, we expect the differences between the treatment and the control group to increase over time, which would signal a stable response in the control sharks and learning in the treatment sharks. The findings from this study will contribute to our limited understanding of elasmobranch cognition and will help advance our theories regarding the behavioural plasticity of sharks in response to novel and changing environmental conditions. They will further contribute to the sustainable management of shark feeding operations by informing operators and management institutions on the susceptibility of sharks to getting caught in ecological traps, based on their capacity to learn time-place associations. If sharks learn an association between a certain time of day and tourism related feeding events, they may eventually start anticipating feeding times, trapping sharks into potentially-harmful changed diurnal behaviours (Semeniuk and Rothley, 2008). Because these associations are based on the time of day, sharks are likely to continue to engage in these harmful behaviours on days the feedings do not occur. Therefore, break-days and short off-seasons would not necessarily help in making these activities more sustainable.

4.3 Methods and Materials

4.3.1 Study Site and Species

This study was conducted at the Bimini Biological Field Station off the southern island of Bimini (25°44'N, 79°16'W), Bahamas. Eight juvenile lemon sharks (*Negaprion brevirostris*, 5 males and 3 females, mean pre-caudal length ± SD = 63.86 ± 6.41 cm) were captured using gillnets and immediately transported to one of the four holding pens, where the sharks would be kept for the remainder of the study (Figure 4.1). Upon arrival at the holding pens, sharks were measured and sexed. Sex was determined by noting the presence of claspers in males and pre-caudal length was measured to the nearest millimetre. Lemon sharks are large-bodied meso-predators with a maximum body length of ~3 m. They generally feed on smaller fish and juvenile sharks (Cortés and Gruber, 1990, Guttridge et al., 2012). Although they are capable of temporal buccal ventilation, they spend most of the time swimming (Bouyoucos et al., 2017b, Bouyoucos et al., 2018).

Sharks were size-matched and kept in pairs in four oval-shaped 45 m^2 pens, built with orange construction site fence (Tenax Sentry) and steel reinforcing bars on a sandbank off the southern coast of the island (Figure 4.1). Sharks were kept in pairs as they are known to regularly interact with conspecifics while using their nursery habitats (Guttridge et al., 2009a, Guttridge et al., 2011a). Sharks were assigned to either the trained group or the control group using pseudo randomisation to ensure an even size and sex ratio between the two treatment groups. The pens were built in line North to South, 5 m apart. This alignment was chosen to avoid mixing of olfactory cues among pens through prevailing tidal currents, which run along an East-West trajectory. Furthermore, placement was chosen to ensure consistent substrate and depth across all experimental pens. A wooden stand (height, 6m) was placed at the North and South ends of the pens, connected with two lines of aircraft cable, spanning a total distance of ~45 m. Across the cables were four equally spaced holders, each containing a video camera (GoPro Hero 3+) that were positioned over the centre of each pen. Each camera was equipped with an additional battery pack (Brunton All Day 2.0 battery) to allow for extensive (10 h) time-lapse recordings.

After capture, sharks were provided with acclimation days before the experiment began, to get them accustomed to the holding pens and to the feeding protocol, which included the use of a feeding pole. During this time, they were fed twice per day at random times and random locations using the feeding pole. Experiments did not commence before all sharks started to feed off the pole (total acclimation/training time: 5 days) to ensure they had settled into the enclosure and introduce them to a novel feeding method. The feeding pole consisted of a 1.5 m PVC pipe with a wooden cloth peg attached at the end. Fish pieces were attached to the pole using the peg. The use of the pole allowed the feeder to closely monitor the feeding, and to control how many pieces of fish each shark in the pen was consuming. However, small bony fishes occasionally entered the enclosures, providing an additional potential food source. It was not possible to record the frequency fishes entered the enclosures, nor whether sharks successfully fed on them.

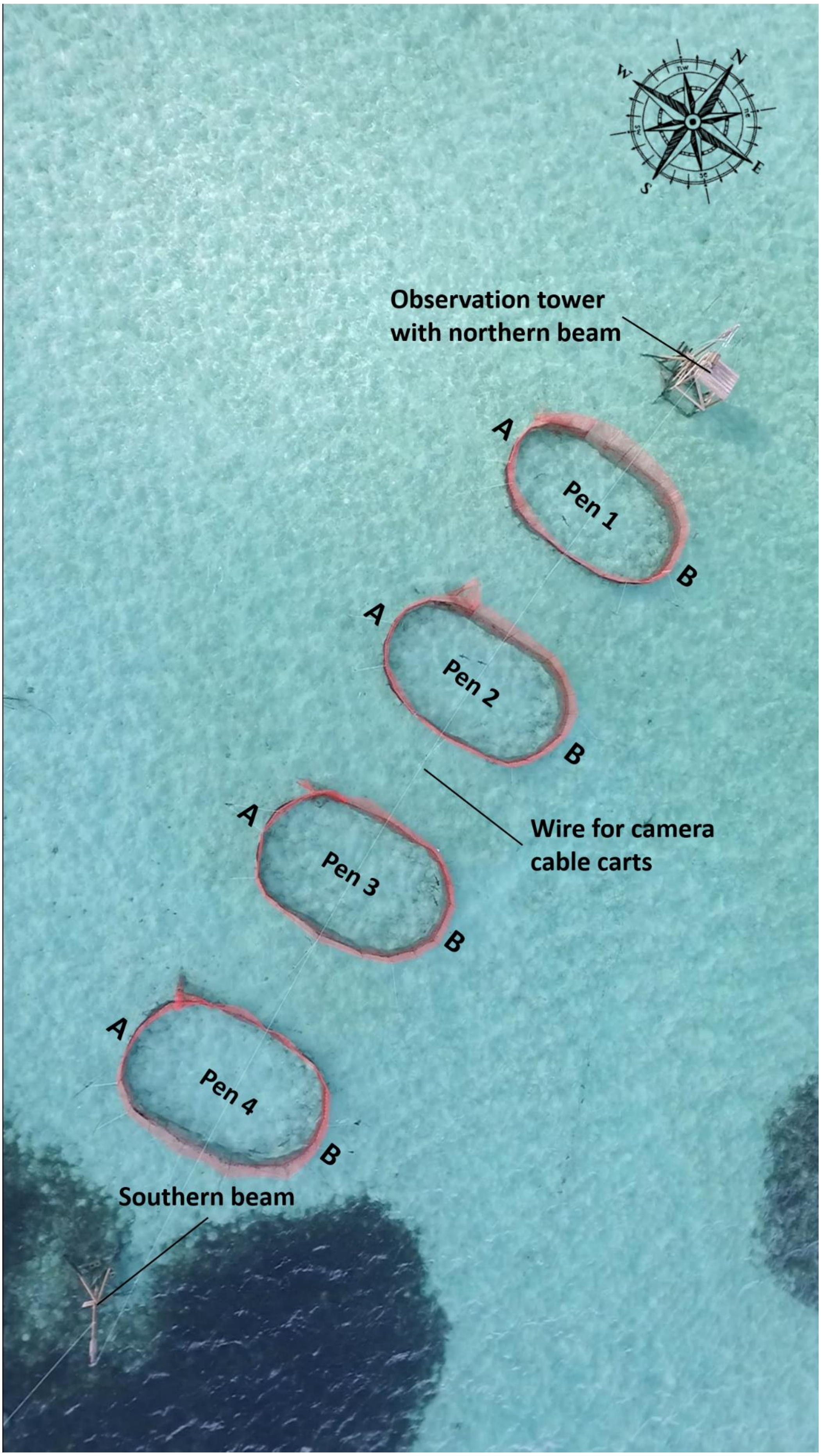

N
W
E
S
nw
ne
sw
se
Observation tower with northern beam
A
Pen 1
B
A
Pen 2
B
Wire for camera cable carts
A
Pen 3
B
A
Pen 4
B
Southern beam

Figure 4.1 Birds-eye view on the pen setup off the southern coast of Bimini, Bahamas. Pens are 5 x 9 m and 5 m apart. Pen 1 and Pen 3 were training pens and were fed on side B in the morning and on side A in the evening. Pen 2 and Pen 4 were the control pens and were fed at random times during the day on either side A or B (chosen randomly).

Sharks in the trained group (pens 1 and 3) were fed at the eastern end of the pen in the morning (09.00 h) and at the western end in the afternoon (17.00 h). Sharks in control pens were fed twice during the day at random times at either the eastern or western end of the pen (chosen randomly). The feeding protocol was the same every time: an observer approached the enclosure at the side of the feeding area with a bucket filled with fish, the feeding pole and a clipboard for data recording. The observer attached a fish piece to the feeding pole and gently tapped the water surface until a shark approached the pole and consumed the food. This was repeated until each shark in the pen had eaten the equivalent of approximately 1% of their wet body weight, which equates to half of their daily ration calculated in the field (Cortés and Gruber, 1990, Guttridge et al., 2013). This meant that each shark reached their intended daily ration by the end of the two daily feedings. We used a mixture of different white fish species (scaled sardine (*Harengula jaguana)*, blue striped grunt (*Haemulon sciurus*), West African ladyfish (*Elops lacerate*)).

Whenever either of the four pens was subjected to a feeding session, the remaining pens received a sham feeding with a maximum 10-min delay. Although this may have caused some confusion for the sharks, we deemed it necessary to ensure that sharks could not simply associate the arrival and presence of the feeding person at their enclosure with the food rewards, rather than actually learning the time-place association. During these sham feeds the normal feeding procedures remained the same, from the arrival of the feeding personnel to the equipment that was used but the food reward was absent at the end of the feeding pole. Sham feeds were conducted for approximately three minutes in the same way feedings were. Throughout the day each pen received up to four sham feedings, depending on the random timing of the control pen feedings. Therefore, sham feeds occurred more often than the actual feeds making it more difficult for the sharks to form an association between the feeding personnel and the food. Any anticipatory behaviour seen in the trained groups can therefore be assumed to be driven by the time of day rather than the arrival or presence of the person feeding the sharks. The cameras, used to record the sharks spatial use of the pens, were setup and the recordings started one hour prior to the test groups' morning feeding, which started at 9 am. They were programmed to record a continuous time-lapse with an image taken every five seconds until one hour after the test groups' evening feeding at 5 pm.

After 28 days of feeding, preliminary data analysis revealed little evidence of time-place learning. As a highly visible landmarks had been successfully used in previous learning tests in lemon sharks (Guttridge et al., 2013), we placed a high contrast target (30 x 80 cm wooden panel with 5 cm wide horizontal black and white stripes) at the Eastern side of each pen to act as landmark for the remaining 13 days of training (Figure 4.2). After a total of 41 days, the experiment was terminated, and the sharks were released back into the wild.

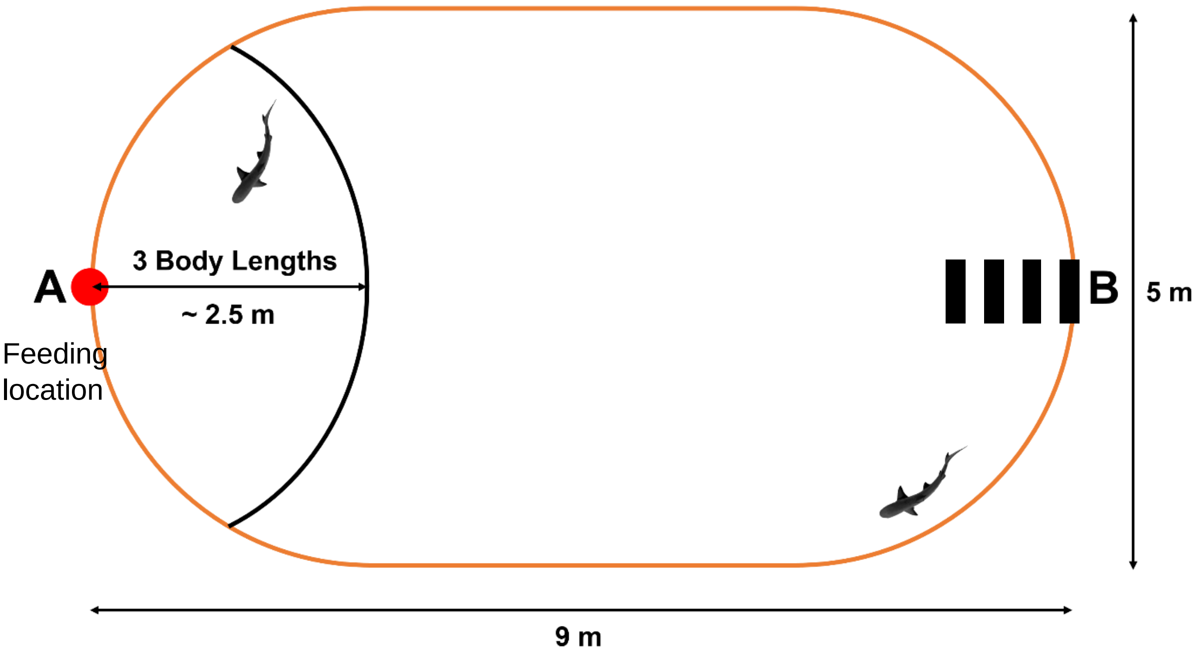
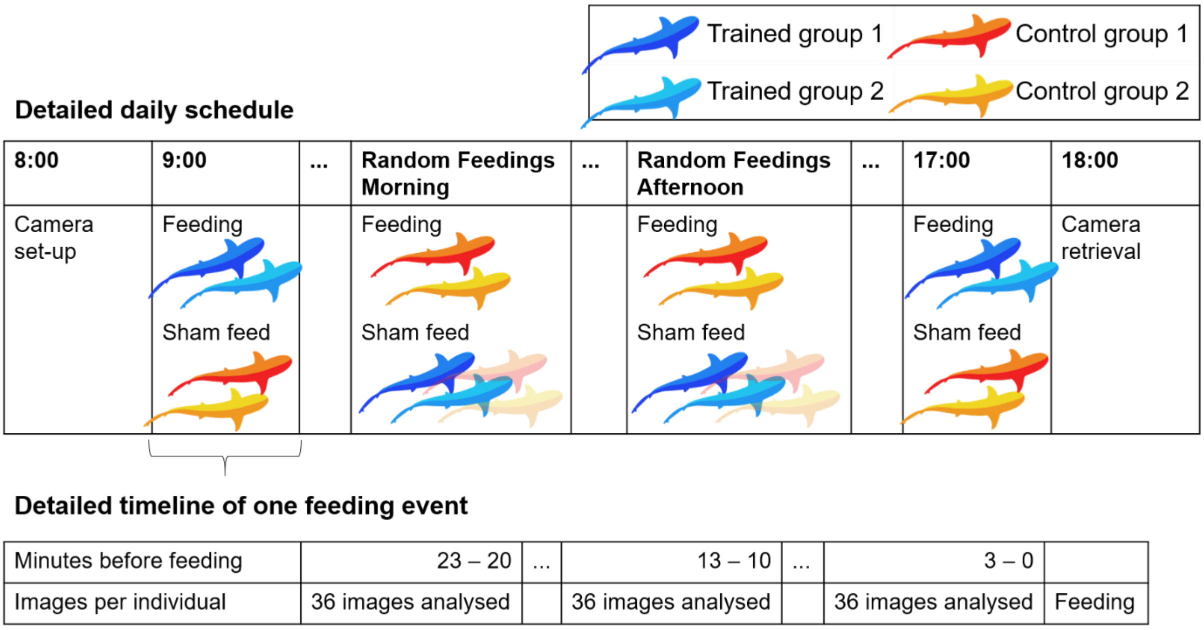


Figure 4.2 Schematics of the holding pens illustrating the shape and dimensions of the pens in approximate relation to the size of the sharks. The black and white target on side B was introduced on day 28 to act as a permanent landmark and assist sharks in making the time-place associations.

4.3.2 Data Analysis

For our assessment of the time-place learning abilities of juvenile lemon sharks, we focused on four feeding events per day, the morning and afternoon feedings of the trained pens and one of the two daily feeding events for each of the control pens, chosen randomly (Figure 4.3). In addition, we included a randomly chosen unfed period each day for all pens to compare spatial behaviours between pens in the absence of any human interference. For each of these events, we investigated three three-minute windows at 23, 13, and three minutes prior to the start of the feedings (Figure 4.3). Each three-minute window consisted of 36 images. We marked the position of each individual in every image using the plugin MTrackJ (Meijering et al., 2012) for the open-source software ImageJ (Schindelin et al., 2012). We then added a reference point to the image marking the respective feeding location. The reference point for the unfed periods was chosen based on the time of day the period fell in. For unfed periods in the morning, we chose the reference point to be consistent with the morning feeding location, the reference point during periods in the afternoon was consistent with the afternoon feeding location. This approach was chosen for the treatment and the control pens. The software was then used to calculate the distance between each individual’s position in an image and the reference point. Based on the obtained information, we focused on two main metrics: 1) the proportion of images that were taken within three body lengths (BL) of the upcoming feeding location ($\frac{\text{number of images taken within 3 BL}}{total\ number\ of\ images}$) , and 2) the mean distance of the sharks from the upcoming feeding location. For the proportion analysis, we assigned a value of one to each image a shark was recorded within three body lengths of the upcoming feeding location and a value of zero to each image a shark was recorded outside this radius. The distance was chosen based on previous behavioural studies using the same species (Guttridge et al., 2009a, Guttridge et al., 2011b). The mean distance from the upcoming feeding location was calculated for each individual shark during each of the three-minute windows. To further assess the spatial distribution inside the holding pens leading up to the feedings, we calculated Kernel Density Estimates (KDE) based on the x and y coordinates we obtained for each image to compare the extent of the sharks' space use inside the pens prior to the feeding events. To do so, we divided the data into five events: Feeding Trained Morning, Feeding Trained Afternoon, Feeding Control (all feedings of the control sharks), No Feeding Trained, and No Feeding Control. We then combined the data into groups of seven days, due to the large data demand of the analysis. For each of these events and each of these time intervals, we estimated the KDE-95 and KDE-50 ranges inside each pen using the R-packages ks (Duong et al., 2020) and GEOmap (Lees, 2018).



Detailed daily schedule

8:00	9:00	...	Random Feedings Morning	...	Random Feedings Afternoon	...	17:00	18:00
Camera set-up	Feeding Sham feed		Feeding Sham feed		Feeding Sham feed		Feeding Sham feed	Camera retrieval

Detailed timeline of one feeding event

Minutes before feeding	23 – 20	…	13 – 10	…	3 – 0	
Images per individual	36 images analysed		36 images analysed		36 images analysed	Feeding

Figure 4.3 Timeline of the daily feeding events illustrating which groups were fed during each event and which groups received a sham feeding. Random Feedings Morning and Random Feedings Afternoon each consisted of two distinct feeding events, one for each control group.

4.3.3 Anticipatory behaviour

To test if sharks started to anticipate the feedings based on the proportion of images taken within three body lengths and the mean distance from the upcoming feeding location we used Generalised Additive Mixed Models (GAMMs) from the R-package mgcv (Wood and Wood, 2015) for each of the three three-minute windows. We used GAMMs instead of a linear approach due to the non-linear nature of the data. Because lemon sharks are known to adjust their behaviour according to the tidal cycle, seeking refuge in shallow water mangrove inlets during high tides (Guttridge et al., 2012), we controlled for tidal effects by including tide in an interaction with events. Explanatory variables included a smooth term on *Day* by *Event* with cubic regression splines, and the parametric interaction term between *Event* and *Tides*. *Event* consisted of the feeding events of the trained sharks (feedings in the morning and feedings in the afternoon) and the control sharks, as well as no feeding events for both trained and control sharks. For the factor *Tides*, we categorised the tidal phases during each of the events into one of four categories: low tide, flood tide, high tide and ebb tide. We included individual sharks nested within pens as a random factor in GAMM models, due to the repeated measurements of individuals. The error structure of generalised mixed models allows for the analysis of non-independent data sets (Zuur et al., 2009). We examined the distribution of the response variables and visually inspected the residuals for the saturated models in accordance with Zuur et al. (2010) to determine the most appropriate statistical family and error distribution for each analysis. For the model assessing the proportion of time spent within three body lengths of the upcoming feeding location, we used a binomial distribution with a logit function. For the mean distance assessment, we used a Gaussian distribution with an identity link function.

4.3.4 Spatial distribution inside the pens

Given the low sample size and the linear nature of the regression, we used GLMMs from the R-package glmmTMB (Brooks et al., 2017) to assess changes in the size of the KDEs in relation to upcoming feeding events. To achieve a better model fit we used a square-root transformation and applied a Gaussian distribution with an identity link function. We used *Event* and *Days* as predictor variables and included an interaction term between them. However, *Days* were combined in groups of five days for this analysis. The factor *Tides* could therefore not be included as it shifted by approximately one hour each day of the 5-day groupings. For the GLMMs, we used the dredge function from the R package MuMIn

(Barton, 2020) to compare all possible combinations of factors and to identify the most parsimonious model based on Akaike's information criterion corrected for small sample size (AIC_c) (Burnham and Anderson, 2002). We determined the Relative Variable Importance values (RVI) for a confidence set of models with $\Delta AIC_c < 2$ (Burnham et al., 2011). These models were included in averaging and the RVI scores for each explanatory factor were calculated based on the models' AIC_c weights.

All analyses were performed in R v.4.0.0 (R-Core-Team, 2020) using the above mentioned packages, We used ggplot2 (Wickham, 2016) to create the graphical display of the results.

4.4 Results

4.4.1 Do trained sharks spend more time within three body length of the feeding location prior to a feeding event?

In the GAMM testing the influence of daily feedings on the time sharks spent within three body lengths of the upcoming feeding location, the smooth term on *Day* by *Event* was significant during all three three-minute windows, indicating that the smoother placed on *Day* by *Event* explained a significant amount of the response we see in the data (Figure 4.4, Table 4.1). The interaction term between *Event* and *Tides* had a significant effect on the proportion of images taken within three body lengths of the feeding location 20 minutes prior to the no feeding events but had no effect during the feeding events of the trained sharks (GAMM: 20 minutes prior: $\beta = -0.247$, $t = -3.582$, $P < 0.001$, Figure 4.4, Appendix Table A4.1). The amplitude of the predictable variation decreased over time during the feeding events of the trained sharks and the no feeding events. Whilst the data of the feeding location mimics a wave-like pattern when fitted with a GAMM, the feeding control event displays a relatively consistent proportion of images taken within three body lengths throughout the experiment. In contrast to the time window 20 minutes prior to the feedings, the interaction term had significant effects on the proportion of images taken, ten and three minutes before the feeding events of the trained sharks (GAMM: 10 minutes prior: $\beta = -0.301$, $t = -4.552$, $P < 0.001$; 3 minutes prior: $\beta = 0.343$, $t = 4.444$, $P < 0.001$, Figure 4.4, Appendix Table A4.1).Ten minutes prior to feeding, trained sharks show a wavelike periodicity in the proportion of images taken within three body lengths of the upcoming feeding location. In contrast, the proportion remained relatively stable before control feedings throughout the experiment. Three minutes prior to the afternoon feedings of the trained sharks, more sharks were within three body lengths of the feeding location during the second half of the experiment. However, overall effect sizes were relatively small (Figure 4.4).

Table 4.1 Approximate significance of the smooth terms, assessing the proportion of images taken within three body lengths of the feeding location at a) 20 minutes, b) 10 minutes, and c) 3 minutes prior to the start of the feedings. edf = estimated degrees of freedom, F = the F-statistic for each individual smooth term, P = the individual smooth term type I error.

	Smooth term	**edf**	***F***	***P***
a)	s(Day) : Feeding Control	12.86	9.85	< 0.001
	s(Day) : Feeding Trained Morning	13.50	25.60	< 0.001
	s(Day) : Feeding Trained Afternoon	12.12	10.01	< 0.001
	s(Day) : No Feeding Control	12.80	11.16	< 0.001
	s(Day) : No Feeding Trained	13.37	15.64	< 0.001
b)	s(Day) : Feeding Control	13.36	8.88	< 0.001
	s(Day) : Feeding Trained Morning	13.96	19.37	< 0.001
	s(Day) : Feeding Trained Afternoon	8.43	13.66	< 0.001
	s(Day) : No Feeding Control	13.33	14.36	< 0.001
	s(Day) : No Feeding Trained	12.42	12.65	< 0.001
c)	s(Day) : Feeding Control	12.71	11.08	< 0.001
	s(Day) : Feeding Trained Morning	12.10	16.28	< 0.001
	s(Day) : Feeding Trained Afternoon	11.61	15.42	< 0.001
	s(Day) : No Feeding Control	13.07	13.52	< 0.001
	s(Day) : No Feeding Trained	12.87	14.60	< 0.001

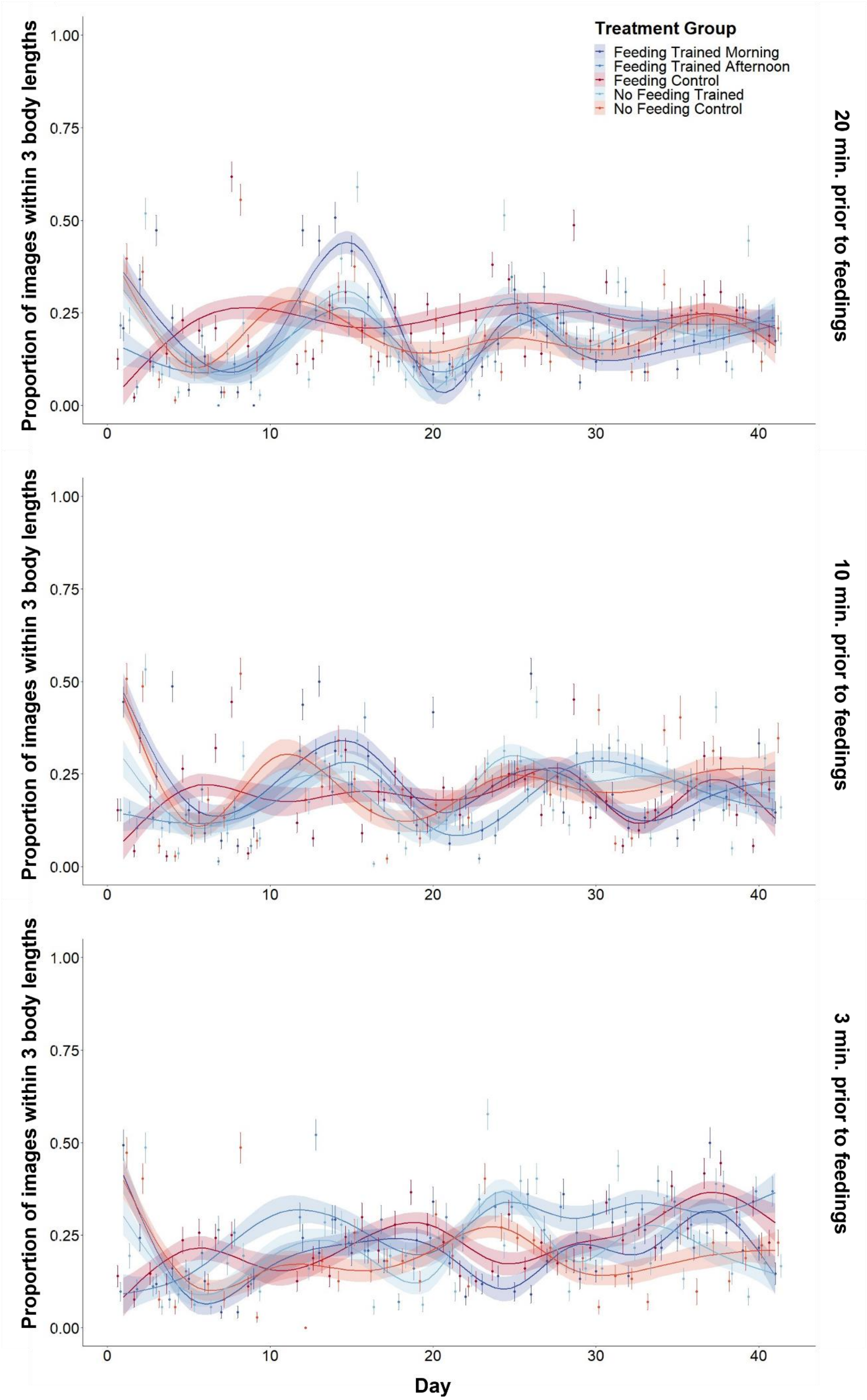

Treatment Group
Feeding Trained Morning
Feeding Trained Afternoon
Feeding Control
No Feeding Trained
No Feeding Control
20 min. prior to feedings
10 min. prior to feedings
3 min. prior to feedings
Proportion of images within 3 body lengths
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Day

Figure 4.4 Proportion of images taken within approximately three body lengths of the upcoming feeding location. Each dot represents the daily mean proportion ± standard error for one of the five event-treatment group combinations: Feeding Trained Morning, Feeding Trained Afternoon, Feeding Control, No Feeding Trained, and No Feeding Control. We added lines of best fit using a GAM smooth with cubic regression splines and 95% confidence bands.

4.4.2 Do trained sharks move closer to the feeding location prior to a feeding event?

In the GAMM testing the influence of daily feedings on the mean distance of the sharks to the upcoming feeding location, the smooth term on *Day* by *Event* was significant during all three three-minute windows, indicating that the smoother placed on *Day* by *Event* explained a significant part of the response we see in the data (Figure 4.5, Table 4.2).

The interaction term between *Event* and *Tides* affected the mean distance from the feeding site 20 minutes prior to the feedings (GAMM: 20 minutes prior: $\beta = 0.208$, $t = 2.734$, $P = 0.00626$). The amplitude of the predictable variation decreased over time during the feeding events of the trained sharks and the sham feeding events. The significant event effects stem from a significantly different pattern during the feeding events of the control sharks. Whilst all other events show a wave-like pattern with decreasing amplitudes, the feeding control event displays a relatively consistent distance from the feeding location throughout the experiment. The interaction term between *Event* and *Tides* also had a significant effect ten and three minutes before feedings (GAMM: 10 minutes prior: $\beta = 0.441$, $t = 6.547$, $P < 0.001$; 3 minutes prior: $\beta = -0.454$, $t = -6.753$, $P < 0.001$, Figure 4.5, Appendix Table A4.2). Three minutes prior to the afternoon feedings, the trained sharks indicate a closer distance during the second half of the experiment. However, overall effect sizes were relatively small (Figure 4.5, Appendix Table A4.2).

Table 4.2 Approximate significance of the smooth terms, assessing the mean distance from the upcoming feeding location at a) 20 minutes, b) 10 minutes, and c) 3 minutes prior to the start of the feedings. edf = estimated degrees of freedom, F = the F-statistic for each individual smooth term, P = the individual smooth term type I error.

	Smooth term	**edf**	***F***	***P***
a)	s(Day) : Feeding Control	14.96	14.31	< 0.001
	s(Day) : Feeding Trained Morning	15.08	35.28	< 0.001
	s(Day) : Feeding Trained Afternoon	14.64	30.61	< 0.001
	s(Day) : No Feeding Control	14.87	30.33	< 0.001
	s(Day) : No Feeding Trained	15.32	53.00	< 0.001
b)	s(Day) : Feeding Control	14.54	19.14	< 0.001
	s(Day) : Feeding Trained Morning	14.79	34.65	< 0.001
	s(Day) : Feeding Trained Afternoon	14.38	23.46	< 0.001
	s(Day) : No Feeding Control	15.25	44.00	< 0.001
	s(Day) : No Feeding Trained	13.74	39.47	< 0.001
c)	s(Day) : Feeding Control	13.64	11.81	< 0.001
	s(Day) : Feeding Trained Morning	12.38	18.23	< 0.001
	s(Day) : Feeding Trained Afternoon	13.01	25.94	< 0.001
	s(Day) : No Feeding Control	13.77	35.41	< 0.001
	s(Day) : No Feeding Trained	13.47	45.83	< 0.001

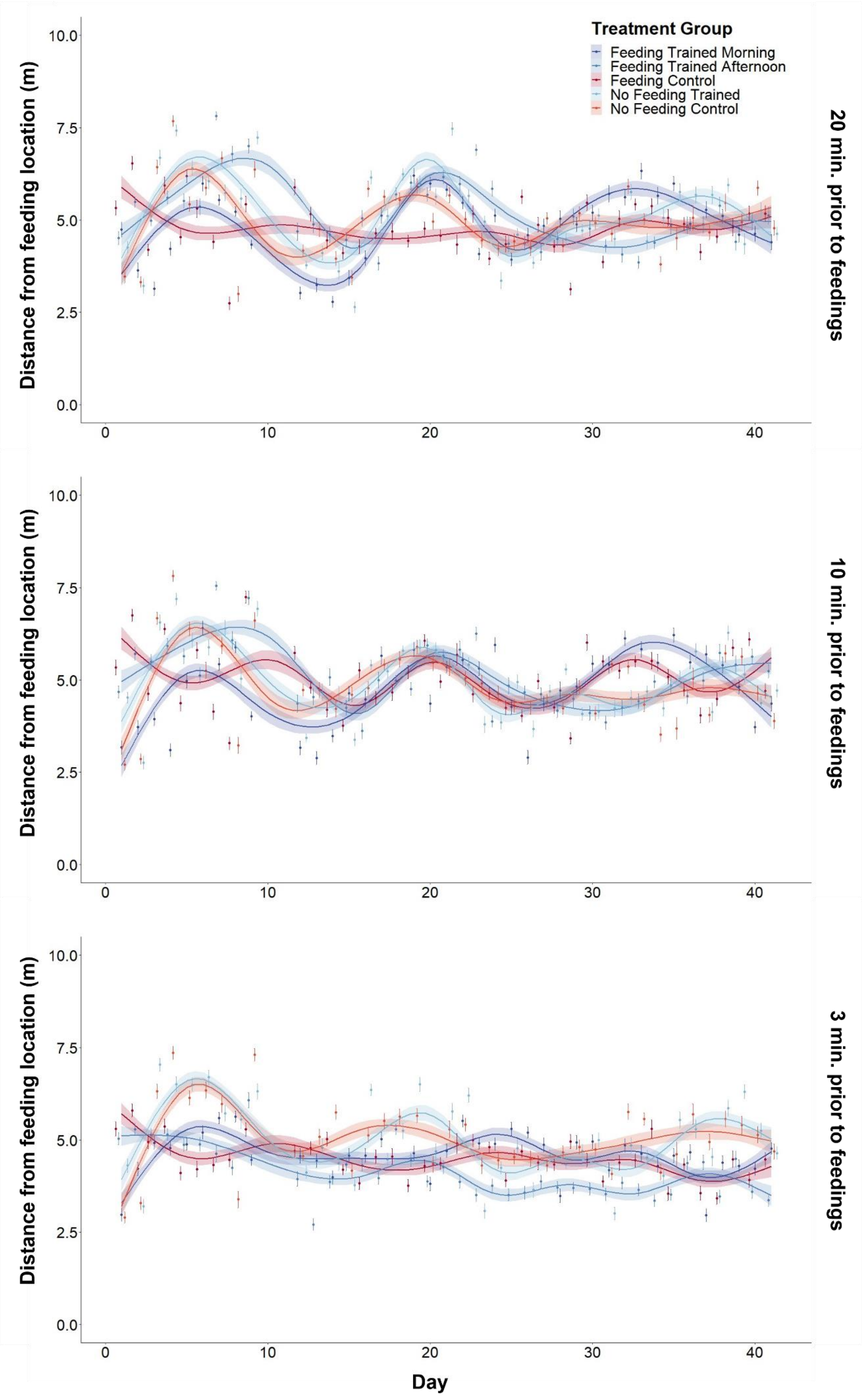

Treatment Group
Feeding Trained Morning
Feeding Trained Afternoon
Feeding Control
No Feeding Trained
No Feeding Control
20 min. prior to feedings
10 min. prior to feedings
3 min. prior to feedings
Distance from feeding location (m)
0.0
2.5
5.0
7.5
10.0
0
10
20
30
40
Day

Figure 4.5 Distance from the upcoming feeding location. Each dot represents the daily mean distance ± standard error for one of five event-treatment group combinations: Feeding Trained Morning, Feeding Trained Afternoon, Feeding Control, No Feeding Trained, and No Feeding Control. We added lines of best fit using a GAM smooth with cubic regression splines with 95% confidence bands.

4.4.3 Distribution throughout the pens

The interaction term between *Day* and *Event* was not included in the model averaging during either of the three three-minute windows when assessing the effects on the KDE-50 and the KDE-95 range. Between the two factors, *Event* had a stronger effect than *Day* (Table 4.3). KDE-50 and KDE-95 were significantly smaller 10 minutes and three minutes before the beginning of the feedings of the trained sharks compared to the control sharks (**GLMM KDE-50:** 10 minutes: Conditional $R^2 = 0.21$; *Event*: $\beta = -0.176$, $z = -2.26$, df = 6, $P = 0.0235$; 3 minutes: Conditional $R^2 = 0.33$; *Event*: $\beta = -0.303$, $z = -3.45$, df = 7, $P = 0.000563$; **GLMM KDE-95:** 10 minutes: Conditional $R^2 = 0.21$; *Event*: $\beta = -0.298$, $z = -2.27$, df = 7, $P = 0.023$; 3 minutes: Conditional $R^2 = 0.41$; *Event*: $\beta = -0.640$, $z = -5.50$, df = 7, $P < 0.0001$; Figure 4.6, Appendix Table A4.3). In addition, KDE-50 of the treatment group changed significantly over time three minutes before the feedings (Conditional $R^2 = 0.33$; *Event*: $\beta = -0.00910$, $z = -3.08$, df = 7, $P = 0.00210$, Appendix Table A4.3).

Table 4.3 Ranking of the best models (ΔAICc<2) assessing the Kernel Density Estimates at a) 20 minutes, b) 10 minutes, and c) 3 minutes prior to the start of the feedings. df = degrees of freedom, AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top ranked model, $wAIC_c$ = model probability. The models listed below were retained for the model averaging. When no model besides the top-ranked one had $\Delta AIC_c<2$, the model summary for the top-ranked model was used. Shark ID nested within Pen was included as a random factor in all models.

		Model	df	AIC_c	ΔAIC_c	$wAIC_c$
KDE-95	**a)**	Event	6	68.34	0.00	0.45
		Null	3	69.29	0.94	0.28
	b)	Event	6	72.64	0.00	0.61
		Day + Event	7	74.45	1.80	0.25
	c)	Day + Event	7	88.33	0.00	0.61
		Event	6	82.36	1.03	0.37
		Model	**df**	**AIC_c**	**ΔAIC_c**	**$wAIC_c$**
KDE-50	**a)**	Event	6	74.46	0.00	0.46
		Day + Event	7	75.16	0.70	0.32
	b)	Event	6	72.51	0.00	0.72
	c)	Day + Event	7	44.25	0.00	0.93

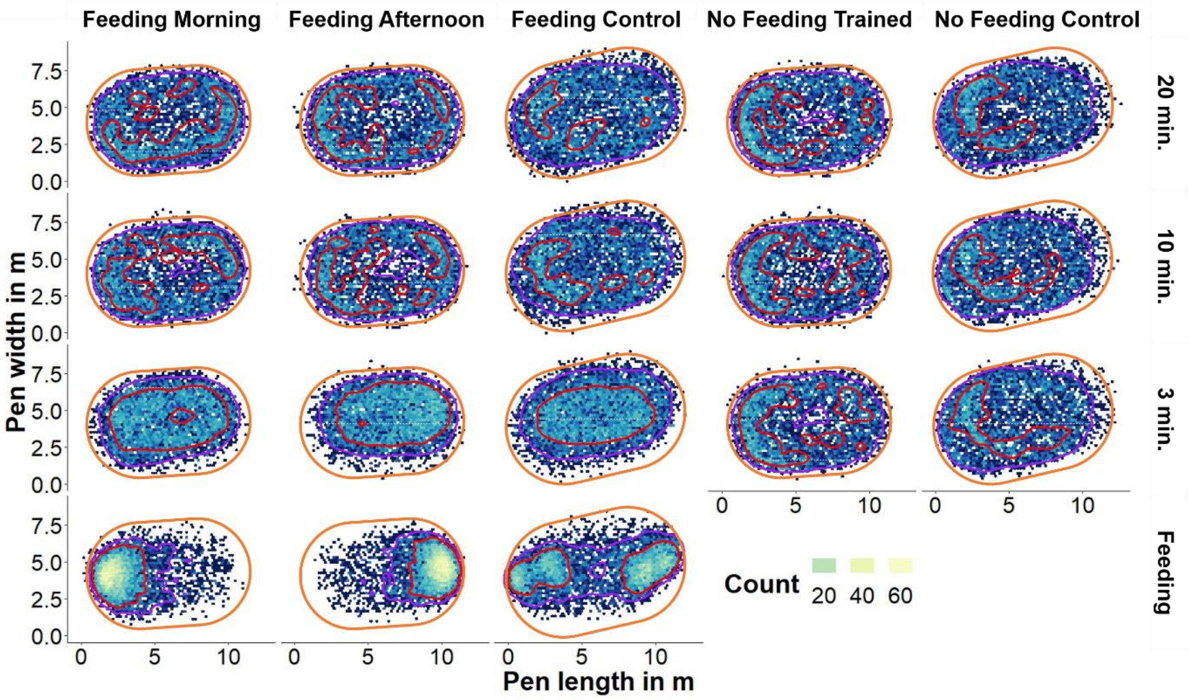


Figure 4.6 Hexagonal density plot illustrating the distribution of the sharks inside the pens 20, 10, and 3 minutes before the feedings and during the feedings (rows). Columns mark the different events with the Feeding morning and Feeding afternoon referring to the two feeding events of the trained sharks. The orange outline marks the shape of the pens, the purple contour marks the KDE-95 (outside) and the red contour marks the KDE-50 (inside) range.

4.5 Discussion

We hypothesised that juvenile lemon sharks were capable of learning time-place associations within a semi-captive environment. However, our results suggest that juvenile lemon sharks were unable to learn this association after 41 days of training, despite the additional introduction of a high contrast landmark on one side of the pens on day 28. There was no evidence for behavioural changes following the introduction of the high contrast landmark. The decrease in activity space, as well as the reduction in mean distance and the increase in the proportion of images taken with three body lengths from the feeding location three minutes prior to feedings shown for trained and control sharks might be due to the arrival of the feeding personnel and the preparations made at the feeding location. Similar results were found during an earlier study on convict cichlids (*Cichlasoma nigrofasciatum*), with the fish failing to learn time-place associations between more than one location and one time (Reebs, 1993). It was suggested that the observed association using a single location and a single time of day was in fact an association between a signal onset (the shutdown of the filtering pump just before the feedings) and the feeding location rather than a time-place association (Reebs, 1993). Therefore, the behavioural changes we observed three minutes prior to the feedings may represent an association between the stimuli produced by arrival of the personnel, i.e. boat engine noise, water movement and visual cues, and the feedings rather than a time-place association, despite our best efforts to mitigate this confounding factor by implementing daily sham feedings. Furthermore, the implemented sham feedings may have caused some confusion in the sharks, potentially making it more difficult for them to successfully identify the real feeding events and learn the time-place associations. Whilst time-place learning has since been demonstrated in multiple species of teleost fish within 21-30 days (Brännäs, 2014, de Almeida Moura and Luchiari, 2016, Gómez-Laplaza and Morgan, 2005), we were unable to demonstrate the same learning capacity in juvenile lemon sharks. A simpler setup with a single location and time may have been easier for juvenile lemon sharks to make the time-place association. However, a single location and time is more likely to be affected by confounding factors, such as the arrival of the feeding personnel (e.g. study on convict cichlids; Reebs, 1993).

Previous studies have shown that the endogenous clock of an animal can be synchronised to the 24-hour day using a variety of abiotic factors, including light-dark cycle and temperature (Kuhlman et al., 2018, Reebs, 2002), cues from conspecific individuals (Rajaratnam and Redman, 1999), or self-regulated molecular processes through gene expressions (Amaral et al., 2014). During our study, juvenile lemon sharks were maintained in a semi-captive environment that was subject to the natural light-dark cycle, and ambient temperatures. Furthermore, the sharks were kept in groups of two due to their known social interactions (Guttridge et al., 2009a, Guttridge et al., 2012). Therefore, these sharks had a multitude of abiotic factors they could use to synchronise their endogenous clocks and identify the correct time and place of the daily feedings (de Almeida Moura et al., 2017, Rajaratnam and Redman, 1999, Kuhlman et al., 2018, Reebs, 2002). This suggests that juvenile lemon sharks were unable to form time-place associations based on their endogenous circadian clock alone, despite the opportunity to use the light-dark cycle, temperature, and social interactions to synchronise it, using cues from older individuals to learn (Guttridge et al., 2013, Vila Pouca et al., 2020). The differences in activity space do not indicate successful time-place learning either given the lack of significant interaction effect between the experimental days and the feeding events. Hence, the differences between trained and control sharks remained relatively stable throughout the experiment, indicating variability between sharks (e.g. personalities (Dhellemmes et al., 2020b, Finger, 2019), rather than a treatment effect. If learning had occurred, we would expect the differences between the groups to increase over time, signalling a stable response in the control sharks and a change in the response of the treatment sharks. Instead, the natural diurnal rhythm of juvenile lemon sharks, which has been suggested to follow the tidal cycle (Guttridge et al., 2012), was resilient to associations based on recurring events. Juvenile lemon sharks around Bimini are known to take refuge in shallow water mangrove inlets during high tides to seek protection from larger predatory sharks, such as subadult and adult lemon sharks (Guttridge et al., 2012). The behavioural dependence of juvenile lemon sharks on the tidal cycle is reflected in the wavelike patterns that can be observed in the scatterplots (Figure 4.4; 4.5). The significant tide effects indicate that these patterns coincide with the recurring tidal cycle. In the pens, sharks were subjected to the natural high tide, which may be associated with an increased risk of predation and the consequent need to seek refuge (Guttridge et al., 2012). Predation risk has previously been demonstrated to curtail the expression of time-place learning based on food availability in the teleost fish, Inanga (*Galaxias maculatus*). Forming a time-place association based on the endogenous circadian clock using other abiotic factors, such as the light-dark cycle to synchronise it, could have detrimental consequences, if juvenile lemon sharks failed to seek refuge during high tides as a result (Guttridge et al., 2012). The decreasing amplitude in the oscillation patterns of the distance data and the proportion of images taken within three body lengths of the feeding location may suggest that this natural rhythm was fading in the semi-captive sharks over the course of the experiment. A prolonged experimental period may have sufficed to eliminate the underlying tidal rhythm and allowed sharks to learn the time-place association we tested for. Subadults or adults may also be more likely to learn time-place associations, as they may be less affected by tides given their reduced need to seek shelter during high tides (Guttridge et al., 2012). Similarly, running this experiment using “tidal time” rather than circadian time may have resulted in successful time-place learning, although this would have posed a significant logistical challenge, as some feeding may have taken place during the night.

The relatively small size of the holding pens (45 m^2; 9 m long) may have affected the expected time-place learning, due to the low benefit and incentive to make a time-place association at such small spatial-scale. The need of juvenile lemon sharks to swim most of the time may have further reduced the incentive to close in on the feeding location prior to the start of the feedings (Bouyoucos et al., 2017a, Bouyoucos et al., 2018). The short 9 m distance may not be biologically relevant to juvenile lemon sharks, given their relatively large space use (Gruber et al., 1988), but recent sociability experiments conducted in a 10 m diameter enclosure found that a pen this size was enough for up to six sharks to choose to swim individually or in groups, suggesting that our pen size is sufficient for sharks not to be forced to use its whole area (Finger et al., 2016). Difficulties with time-place learning due within a confined space have previously been observed in convict cichlids, where these fish could learn to feed at one location, but not at two different locations due to the short distance between the two locations (Reebs, 1993). However, studies using captive-bred teleosts showed successful time-place learning in small aquaria (Gómez-Laplaza and Morgan, 2005, Reebs, 1996). This suggests that captive bred individuals may be more likely to perceive the energetic costs involved travelling within the enclosure to be significant. Bottom dwelling sharks may be better models to investigate general cognitive abilities of elasmobranchs in captivity (Vila Pouca et al., 2019, Schluessel and Bleckmann, 2012, Heinrich et al., 2020), as limitations due to size of the test arena are less likely to arise in sharks that do not swim constantly. However, whilst bottom dwelling sharks may be better suited to investigate general cognitive abilities in sharks, we still need to expand our efforts to include other species with different ecologies to provide sound advice to the wildlife tourism industry and advance our understanding of shark cognition and behaviour. Despite the relatively small space and potentially low incentive to learn the time-place associations, it is unlikely that the sharks were unable to distinguish between the Eastern and the Western end of the pens. The periodicity in the distance data and the proportion of images taken within three body lengths of the feeding location, which was at least partially driven by the diurnal tidal cycles, indicates that the sharks did distinguish between East and West and preferred one side over the other based on the tidal phase.

4.6 Conclusion

We were unable to demonstrate time-place learning in sharks in a semi-captive environment. This lack of time-place learning could limit the effects of provisioning activities on the behaviour and ecology of these sharks. In general, feeding schedules should take the ecology of the focal species into consideration. Species that are less likely to learn circadian based time-place associations due to a strong dependency on other environmental factors, such as the tides, may benefit from being fed at the same time each day. In contrast, species that exhibit diurnal patterns based on the circadian cycle could benefit from a schedule that is linked to factors such as the tides. The lack of time-place learning was potentially due to the spatial restrictions of the experimental setup, not providing enough incentive for sharks to make the association in conjunction with the sharks' strong dependence on the tidal cycles. This study further highlights the difficulties involved when running these types of experiments in an environment where the sharks are exposed to ambient conditions. An animal's ability to learn an association between a time of day and a place will always depend on the risk-benefit trade-off. If the risks posed at said location at that time of day outweigh the benefits, the association will not be learned. A possible follow-up experiment may test for anticipatory behaviours on days the feedings are delayed. These delays should be implemented on pre-determined days throughout the experiment and should be no shorter than 30 min. to account for the shifting sunset and sunrise times. Future studies should also investigate time-place associations across a longer time period, in more controlled conditions (e.g. without the influence of tides), and across different life stages, given the possibility that the trade-off benefit of time-place learning might be greater in older sharks that are less affected by predation risk.

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Chapter 5

Short-term impacts of daily feeding on the residency, distribution and energy expenditure of sharks

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5.1 Abstract

Tourism-related feeding of wildlife can result in detrimental, human-induced changes to the spatial distribution, social behaviour and health of target species. The feeding of sharks as part of shark-viewing activities has become increasingly popular in recent years to ensure reliable and consistent encounters. A common limitation in determining how feeding affects individuals or populations is the lack of baseline data prior to the establishment of a feeding site. Here, we documented the residency, spatial distribution, activity patterns and daily metabolic rates of juvenile lemon sharks, *Negaprion brevirostris*, prior to initiating daily feeding for 27 days to assess the effect of short-term feeding. We implanted acoustic transmitters equipped with accelerometers to record movement and activity in six lemon sharks. Sharks progressively anticipated the feeding events during the 27 days of daily feeding, as shown by a change in activity and increased time spent near the feeding site 1 h prior to feeding events. Shark behaviour did not fully return to baseline levels within the documented 90 days of postfeeding recovery. However, neither spatial distribution outside the refuge nor mean daily activity was affected by feeding. Sharks decreased their metabolic rates over the course of the study, but this was probably due to falling water temperature rather than the effect of feeding. Overall, our study shows that anticipatory behaviour in juvenile lemon sharks can occur within 11 days of daily feeding events, but behavioural changes seem confined to fine-scale movement patterns and may not affect these sharks' daily energy needs. The ability to assess the effects of daily feeding at a site where tourism has not been occurring previously provides new information for operators and managers of wildlife tourism to account for and minimize potentially detrimental effects on the target species.

5.2 Introduction

In recent decades, wildlife tourism has become one of the fastest-growing sectors of the tourism industry (Scheyvens, 1999, Wearing and Neil, 2009), partly due to the increasing desire of people to reconnect with nature (Miller, 2005). Associated with a number of socioeconomic benefits (Huveneers et al., 2017, Orams, 2002, Apps et al., 2018, Newsome et al., 2019), wildlife tourism has been viewed as a tool to assist local economies transitioning from consumptive to perceived nonconsumptive uses of natural resources (Newsome et al., 2019). To name just a few, examples include lemur- and birdwatching tourism in Andasebe National Park (Newsome and Hassell, 2014), gorilla and chimpanzee tourism in Uganda (Newsome and Hughes, 2016), whale-watching tourism in the Antarctic (Williams and Crosbie, 2007), and shark and ray tourism in the Bahamas and Fiji (Haas et al., 2017, Macdonald et al., 2017). These examples illustrate how the employment of local tour guides and the provision of other services by local communities can result in economic success and growth (Newsome et al., 2019). Linked with an increased public awareness for endangered species, these economic transitions can also benefit the focal species and their surrounding ecosystems by promoting conservation efforts (Apps et al., 2018, Newsome and Hassell, 2014). However, previous studies have indicated detrimental effects of wildlife tourism activities (Barnett et al., 2016, Newsome et al., 2004, Semeniuk and Rothley, 2008, Green and Giese, 2004), and the resulting impaired individual fitness and survival has been documented in terrestrial (Orams, 2002), avian (Steven et al., 2011) and aquatic species (Williams and Crosbie, 2007). For example, close proximity and interactions of tourists with Adélie penguins, *Pygoscelis adeliae*, has resulted in as much as an 80% reduction in chick survival (Giese, 1996). Behavioural changes due to wildlife tourism can also lead to decreased survival, such as with coastal damselfish, *Chromis chromis* (Milazzo et al., 2006), or can increase aggressive behaviours towards humans, such as with yellow baboons, *Papio cynocephalus*, in Kenya and dingoes, *Canis lupus dingo*, on Fraser Island, Australia (Altmann and Muruthi, 1988, Burns and Howard, 2003).

Recent studies on elasmobranchs have shown that wildlife tourism can have a wide range of effects on focal and nonfocal species, including changes in seasonality, residency, abundance (Meyer et al., 2009, Clarke et al., 2011), space use (Huveneers et al., 2013, Bruce and Bradford, 2013, Brunnschweiler and Barnett, 2013, Fitzpatrick et al., 2011), activity (Huveneers et al., 2018, Corcoran et al., 2013) and diet (Meyer et al., 2020, Brunnschweiler et al., 2018). Such effects are often accentuated by feeding the focal species to ensure reliable and close encounters (Gallagher et al., 2015, Brena et al., 2015). For instance, Brunnschweiler and Barnett (2013) demonstrated changes in the fine-scale movement patterns of bull sharks, *Carcharhinus leucas*, that are frequently exposed to wildlife feeding operations in Fiji. They further found evidence suggesting significant intraspecific variation with regard to the effects of wildlife feeding (Brunnschweiler and Barnett, 2013). Besides the growing body of knowledge on the effects of feeding activities on the behaviours of elasmobranch species, few studies have investigated tourism-related changes in energy expenditure of sharks (e.g. Barnett et al., 2016, Huveneers et al., 2018). This information is, however, crucial to understand the effects of wildlife tourism (Brown et al., 2004, Wilson et al., 2006, Gallagher and Huveneers, 2018, Brunnschweiler et al., 2018). A study on whitetip reef sharks, *Triaenodon obesus*, at Osprey Reef (Great Barrier Reef, Australia) demonstrated that sharks subjected to regular feeding events showed elevated activity levels during the day when they would normally rest, resulting in a ca. 6.4% increase in metabolic rates. Similarly, white sharks, *Carcharodon carcharias*, are more active when interacting with cage-diving operators (Huveneers et al., 2018).

A common limitation in studies investigating the effects of direct feeding (hereafter referred to as ‘feeding’) is the lack of baseline data prior to establishing a feeding site (Semeniuk and Rothley, 2008, Huveneers et al., 2013). While a population that is not being fed can act as a control, observed differences can be due to site-specific factors. Instead, a more reliable approach for assessing the effects of feeding is one where baseline data can be collected prior to initiation of wildlife tourism activities. If feeding activities can occur for a finite period of time, it further provides the opportunity to investigate behaviour after they stop to assess how soon behaviours return to baseline levels. Therefore, gaining an understanding of a target species' behavioural ecology prior to the establishment of a new feeding site will help wildlife tourism managers to implement sustainable strategies, minimizing the negative impacts of these activities. Ultimately, this will provide further insight about the timing, location and frequency of feeding activities, as well as limits on the number of boats and people allowed to participate, similar to some of the guidelines developed for the whale-watching industry (Mallard, 2019).

Here, we first established a feeding site in Bimini (Bahamas), targeting juvenile lemon sharks, *Negaprion brevirostris*, in a known nursery area where wildlife tourism has not occurred (Guttridge et al., 2012). We chose juvenile lemon sharks as our model species due to their high abundance around Bimini and their well-known ecology and diurnal movement patterns. Lemon sharks are also often targeted by wildlife tourism operators, making this species relevant to the context of understanding the influence of cognitive behaviour and learning in relation to wildlife tourism practices. We used acoustic telemetry and accelerometery to assess daily activity, metabolic rates and spatial distribution patterns before, during and after a 1-month (daily) feeding regime. The aim of this study was to investigate whether daily feeding of juvenile lemon sharks at an aggregation site (1) resulted in anticipatory behaviour, as measured by changes in burst activity and fine-scale distribution, (2) increased activity, thereby affecting metabolic rate, and (3) affected the sharks' spatial distribution. We hypothesized that daily feeding would (1) lead to sharks anticipating feeding events, (2) increase daily activity and metabolic rates and (3) constrict space use of juvenile lemon sharks.

5.3 Methods

5.3.1 Study Site and Species

The study was conducted in Bimini (25°44’N, 79°16’W), Bahamas (island group located ca. 90 km east of Florida, U.S.A.; Figure 5.1) at a tidally mediated refuge site for juvenile lemon sharks known locally as ‘Aya’s Spot’. The area (ca. 200 m long and 20 m wide) is a shallow water mangrove inlet that becomes inaccessible during low tides and provides protection from predators over the high tides due to the narrow (< 1 m), shallow entrance (Guttridge et al., 2012). Juvenile lemon sharks use the area daily, with 7 ± 4 (mean ± SD) sharks aggregating in the northern part of the refuge at and around the high tide (Guttridge et al., 2011, Guttridge et al., 2010).

5.3.2 Acoustic Monitoring

Ten acoustic receivers (model VR2W–69Hz, Vemco, Halifax, Nova Scotia) were deployed in a nonoverlapping array within 2.5 km of Aya’s Spot (Figure 5.1). One receiver was positioned at the single access point of Aya’s Spot (refuge entrance), providing acoustic coverage of sharks entering or departing. Receivers were deployed inside a PVC housing with a concrete base (see Guttridge et al. (2012) for more details on the housing). Range testing using three stationary transmitters and two receivers placed at <1 m depth in a similar habitat to the present study showed a detection range of 50–100 m, depending on the tidal phase (Murchie et al., 2010, Guttridge et al., 2017). This range is relatively small compared to the range of receivers in open environments (Huveneers et al., 2016) and probably due to the shallow habitat. Temperature loggers (HOBO Water Temperature Pro v2 Data Logger, Onset Computer Corporation, Bourne, MA, U.S.A.) were placed at four of the 10 receivers (Figure 5.1), which recorded the ambient water temperature at 30 min intervals. In addition to the 10 receivers placed around Aya’s Spot, 55 receivers had already been deployed around Bimini (Figure 5.1) as part of a long-term acoustic monitoring project (Guttridge et al., 2017).

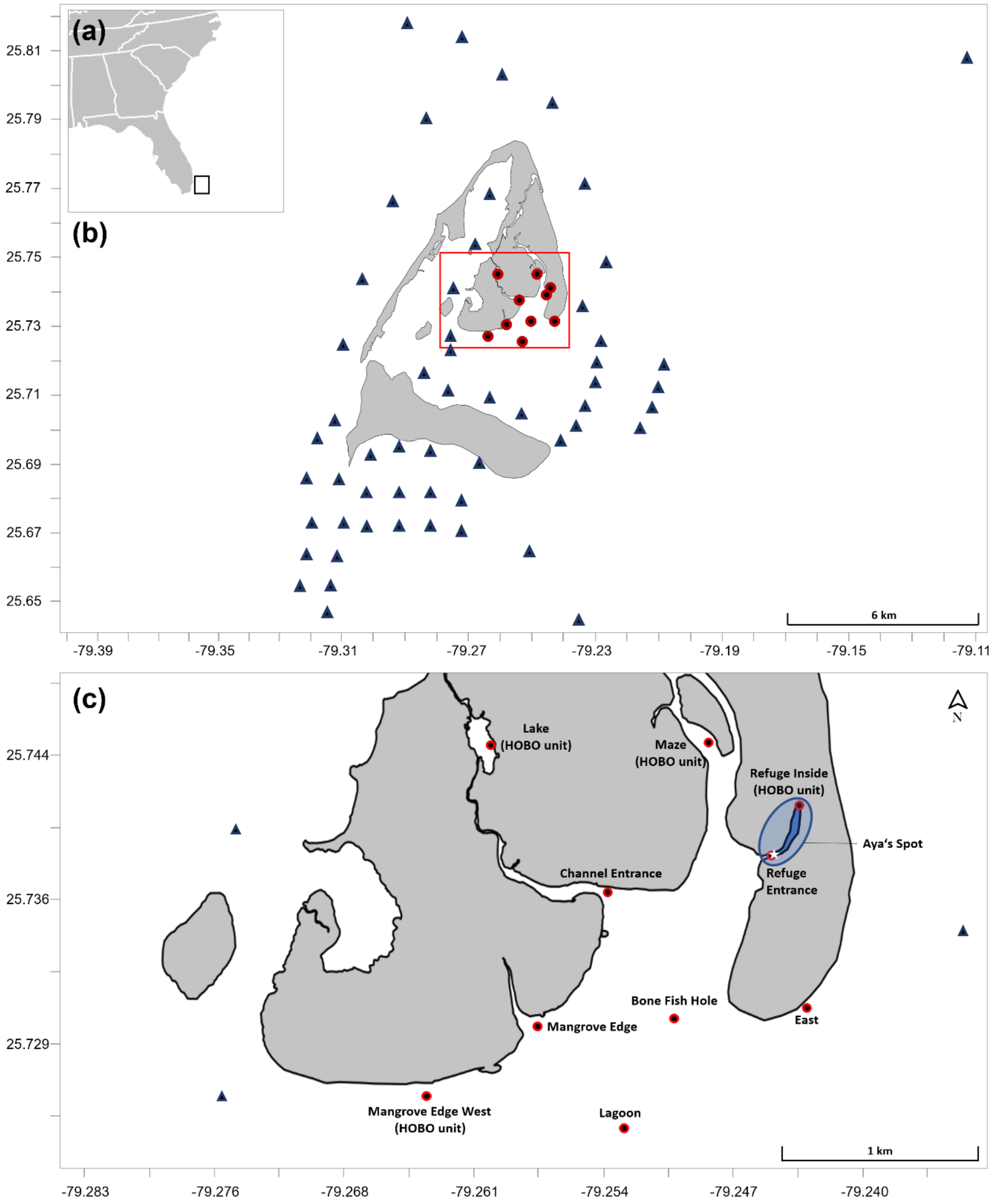


Figure 5.1 Maps of the study site showing (a) the position of the Bimini island group relative to Florida, U.S.A. and (b) an overview of the Bimini island group and the distribution of VR2W acoustic receivers around Bimini. Each marker represents one receiver. Blue triangles mark the locations of the receivers that are part of a long-term acoustic monitoring project. Red dots mark the receivers that were deployed for this study. (c) Map of the study site and the Aya's Spot refuge (blue polygon). Locations of HOBO water temperature loggers are also indicated as 'HOBO unit' underneath each receiver name. The white star next to the Refuge Entrance receiver marks the feeding site.

Juvenile lemon sharks were trapped using two seine nets (10 cm monofilament stretch mesh) that closed off Aya’s Spot during the last hour of the rising tide. Juvenile lemon sharks were caught individually using dip nets as they attempted to leave Aya’s Spot during the falling tide. Each shark was processed immediately upon capture and released before the next individual was caught. Upon capture, individual sharks were immediately transferred into a 200-litre tank where precaudal length was measured to the nearest millimetre and sex was determined (Dhellemmes et al., 2020). Nine sharks (five females and four males, between 1 and 3 years old) were equipped with an acoustic activity tag (V13AP, Vemco, Halifax, Nova Scotia) that was surgically implanted into the peritoneal cavity through a small (30–40 mm) incision along the ventral side of the shark. The incision was closed with interrupted absorbable sutures (Ethicon braided coated Vicryl) that were tied using a surgeon’s knot (Wagner et al., 2000). All procedures combined took less than 8 min per individual with the surgery being completed while sharks were held by hand in tonic immobility (Kessel and Hussey, 2015). This is a coma-like state that is achieved by turning sharks quickly on their backs. One member of the research team handheld the shark in this position, while another performed the surgical implantation. It is widely recognized as an effective alternative to chemical anaesthetics, providing several benefits, both from a practical and an animal welfare perspective (reviewed in Kessel and Hussey, 2015). Besides a rapid induction and recovery, which optimizes the surgical procedure, there is no risk of overdose and minimal disruption to respiration, thereby reducing the risk for negative sublethal impacts (Kessel and Hussey, 2015). Each shark was further marked with a unique second, external tag (colour coded T-bar tag; Floy Tag Manufacturing, Seattle, WA, U.S.A.) through the first or second dorsal fin. Following processing, all nine sharks swam away vigorously and were later detected via acoustic receivers or visual observations, thereby confirming survival.

Acceleration and depth data were transmitted at random intervals of 120–180 s. Acceleration (± 4.9 m/s^2 range) was sampled at 5 Hz for 20 s four times every five transmission cycles (10 - 15 min). Activity was calculated as an average root mean square (RMS) value for all three axes (activity = $[X^2 + Y^2 + Z^2]^{0.5}$) and transmitted as an 8-bit digital value. The static contribution to the overall acceleration (*g*) was filtered out prior to RMS calculation. RMS acceleration resolution was 0.0191 m/s^2 (Payne et al., 2011).

5.3.3 Experimental Protocol

Sharks were given 10 days to recover from capture-associated stress (Bullock et al., 2015) and surgery (Chin et al., 2015). Following this recovery period, they were not disturbed for an additional 30 days, which provided time to record natural behaviours. Sharks were then attracted to the feeding site using a bait box filled with fish scraps and target-fed daily for 27 days using feeding poles to ensure minimal physical interactions between the feeders and the sharks. We ensured each shark received the same amount of food, approaching their estimated daily ration in the wild (i.e. up to 2% of their body mass;(Sims, 1996). This amount was chosen based on learning trials on captive juvenile lemon sharks where feeding motivation was maintained across several months (Guttridge et al., 2013). Each day, the feeding event commenced 1 h before the daytime high tide. This schedule was used because juvenile lemon sharks in this area were already known to use Aya's Spot as a refuge during this time (Guttridge et al., 2012). The feeding area at Aya's Spot was marked with three vertical PVC pipes that were painted with black and white stripes and anchored to the substrate using steel bars. The PVC pipes acted as landmarks to help sharks learn to associate the tide cycle and the feeding event. Black and white panels have been used previously to demonstrate social learning in juvenile lemon sharks in Bimini (Guttridge et al., 2013). Feeding duration was 1 h until the peak high tide was reached. For each feeding event we recorded time of first arrival for each individual, as well as the maximum number of sharks that arrived. We were unable to feed on four occasions due to inclement weather (days 15, 23, 24 and 26). Sharks were no longer fed after the 27-day period, but acoustic receivers remained deployed for an additional 90 days to record changes in behaviour once the daily feeding events had ceased.

5.3.4 Data Analyses

5.3.4.1 Anticipation

Anticipatory behaviour was investigated using the proportion of high-activity events (Burst Activity; >0.9 m/s^2) across feeding times (1 h before [prefeeding], during [feeding] and 1 h after [postfeeding]). We expected that anticipation would lead to increased Burst Activity during prefeeding. We selected the threshold of 0.9 m/s^2 to represent periods of elevated activity that appeared to be associated with the feeding events based on the frequency distribution of activity measurements and the prominent shift of the distribution towards higher levels of activity during the feeding period (Appendix Figure A5.1). We also evaluated whether anticipatory behaviour was occurring by assessing the proportion of detections around the daytime high tides at the refuge entrance receiver located next to the feeding site (Spatial Attraction). The proportion was obtained by dividing the number of detections at the refuge entrance receiver by the total number of detections from both Aya's Spot refuge receivers. We used Spatial Attraction as a measure of anticipation, since previous observations showed that sharks spend most of their time inside Aya's Spot during high tide (Guttridge et al., 2012). An increase in Spatial Attraction would indicate that the sharks moved closer to the feeding site in anticipation of the upcoming feeding event. This enabled us to determine how much time sharks were spending in proximity to the feeding site. Spatial Attraction was compared across feeding times. To investigate the point in time when the sharks started to anticipate the feedings, we visually compared the time of first arrival and the maximum number of sharks present during the feeding events across days. We defined a learning criterion to identify the approximate time it took for sharks to start anticipating the feeding events. A shark had reached the criterion once it arrived at the feeding site within 6 min after the beginning of the feeding event on 3 consecutive days. We also extended the recovery period to 90 days to determine when the anticipatory behaviour ceased. The 90-day recovery period was split into three subperiods of 30 days called recovery 30, recovery 60 and recovery 90, respectively.

5.3.4.2 Daily activity and metabolic rates

We investigated whether daily feeding events affected mean activity (obtained from the activity tag) and metabolic rate. Given that the formula used to calculated metabolic rate ($\dot{M}_{O_2}$) requires the overall dynamic body acceleration (ODBA) in *g* (1 *g* = 9.80665 m/s^2), we first converted the activity tag values by dividing them by the Earth's gravitational pull (9.81 m/s^2). Metabolic rate ($\dot{M}_{O_2}$) was estimated following Lear et al. (2017):

$$\dot{\mathrm{M}}_{\mathrm{O}_2} = \alpha(\mathrm{ODBA}) + b, \tag{1}$$

where α is the slope and b the intercept of the ODBA–$\dot{\mathrm{M}}_{\mathrm{O}_2}$ relationship. While being species specific, α remains independent of environmental influences. The intercept *b*, however, varies with environmental factors, in our case water temperature, which scales the intercept as follows:

$$b = b_c \times S^{\frac{T_b - T_c}{10}}, \tag{2}$$

where T_b is the temperature at which the intercept b is calculated. The intercept of the ODBA–$\dot{\mathrm{M}}_{\mathrm{O}_2}$ relationship b_c is the intercept that was assessed during the calibration at temperature T_c. S is the scaling factor that was determined for the species, also known as the Q_{10}. All constant variables (α, b_c, T_c and S) were obtained from Lear et al. (2017), who calibrated the ODBA–$\dot{\mathrm{M}}_{\mathrm{O}_2}$ relationship at two different temperatures in three different species of sharks, including lemon sharks. Water temperatures were calculated as the mean temperatures from four temperature loggers (HOBO Water Temperature Pro v2 Data Logger, Onset Computer Corporation) deployed at the study site (Figure 5.1).

5.3.4.3 Spatial distribution

For our assessment of the spatial distribution of juvenile lemon sharks across the five periods (baseline, feeding, recovery 30, recovery 60 and recovery 90), we assumed that the detection efficiency was homogeneous across the receivers deployed in the study, which was reasonable, given the identical depth and habitat type. We used the packages VTrack (Campbell et al., 2012) and Animal Tracking Toolbox (Udyawer et al., 2018) in R v.4.0.0 (R-Core-Team, 2020) to create Brownian Bridge kernel utilization density models (BB-KUD) based on all detections recorded during each of the five periods. These models account for the movement paths between two fixed locations when calculating the movement range of an animal, using random walks between two successive locations based on the distance between the locations and the time it took an animal to cover this distance (Horne et al., 2007, Fischer et al., 2013). Calculating the BB-KUDs enabled us to quantitatively compare the activity space of the sharks and to statistically assess whether the daily feedings significantly affected said activity space (Fischer et al., 2013, Udyawer et al., 2018). We calculated the 50% (BB-KUD50 or core area hereafter) and 95% (BB-KUD95 or home range hereafter) ranges of the BB-KUDs and compared them statistically using a linear mixed model (LMM). Visualizing the extents of the BB-KUDs, we further investigated whether the core area shifted between periods.

In summary, we used five response variables to assess the effects of daily feeding events on shark behaviour: (1) Burst Activity, (2) Spatial Attraction, (3) daily activity, (4) daily metabolic rates, and (5) core area and home range. Burst Activity and Spatial Attraction were estimated for each prefeeding, feeding and postfeeding event; daily activity and daily metabolic rates were estimated for each day of the study; core area and home range were estimated for each period (data were collected 24 h a day) calculated for the baseline, feeding and three recovery periods). As feeding events only occurred during the day, the analyses of anticipatory behaviours (i.e. Burst Activity and Spatial Attraction) were restricted to daytime high tides. Night-time behaviour was also assessed and is presented in the Appendix.

Generalized linear mixed models (GLMMs) were used with Period (baseline, feeding, recovery 30, recovery 60, recovery 90) as a fixed factor; Time (prefeeding, feeding, postfeeding; fixed factor) was also included in the models assessing anticipatory behaviour

(Appendix Figure A5.2). We further included Temperature (continuous, fixed factor) for the analyses of mean daily activity and metabolic rates because of its known impact on metabolic rates (Lear et al., 2017). Day and Shark ID were included as random factors in all GLMMs to account for the lack of independence in the behaviour of each shark and across days. We included a random slope grouped by Shark ID in the GLMM assessing the effects of feeding on the daily activity measurements. For the remaining models we were unable to include a random slope due to convergence issues. The error structure of GLMM corrects for nonindependence of statistical units due to shared temporal structure (Zuur et al., 2009). We determined the most appropriate statistical family and error distribution for each analysis by examining the distribution of the response variables and visually inspecting the residuals for the saturated models in accordance with Zuur et al. (2010). Data transformations were used where appropriate to improve model fit as follows: (1) Burst Activity and Spatial Attraction models: binomial (logit) distribution, no transformations; (2) daily activity and metabolic rate models: Gaussian (identity) distribution with Box–Cox transformations. For the comparison of the BB-KUD ranges between periods, we used an LMM with a restricted maximum likelihood fit, no transformations, and Shark ID as a random factor. We used a dredge function from the R package MuMIn (Barton, 2020) to identify more parsimonious nested models based on their relative probabilities using Akaike's information criterion corrected for small sample size (AIC_c(Burnham et al., 2011). We used the AIC_c weights ($wAIC_c$) of all the potential models to calculate the relative variable importance values (RVI) to assess the impact of each variable on the statistical outcome. For all models we took a two-tailed approach, accepting significant results regardless of the direction of change. We used post hoc Tukey tests to test for statistical differences between periods. We did not run post hoc Tukey tests on the model outcomes investigating the daily activity and the metabolic rates due to the interaction between a categorical (Period) and a continuous (Temperature) variable. All analyses were performed in R v.4.0.0 (R-Core-Team, 2020) using the packages glmmTMB (Brooks et al., 2017) and lsmeans (Lenth, 2016). Graphs were created in R v.4.0.0 (R-Core-Team, 2020) using the packages ggplot2 (Wickham, 2016) and plotly (Sievert et al., 2017).

5.3.5 Ethical Note

The research was approved by the Flinders University Animal Welfare Committee (E452/17) and by the Ministry of Agriculture and Marine Resources, Nassau, Bahamas (Marine Scientific Research Permit).

The sample size was reduced as much as possible, while still ensuring meaningful results (Sequeira et al., 2019). All handling, including the capture of the sharks with dip nets, was done as quickly as possible to minimize stress. Sharks were caught individually and immediately processed and released. As a result, no shark was confined and handled for more than 8 min.

5.4 Results

Of the nine juvenile lemon sharks tagged, two were only detected for 9 days post tagging and not during the feeding or recovery periods. Therefore, we omitted these two individuals from further analyses. The remaining seven sharks were regularly detected at Aya's Spot until the end of the study on 13 January 2018. We documented 4530 ± 650 detections per shark with ca. 94% of these occurring at the two Aya's Spot receivers. None of the tagged sharks were detected on receivers outside the 10 receivers deployed for this project (Figure 5.1). Of the seven sharks successfully tracked, six frequently participated in the feeding events. The seventh shark was frequently detected on Aya's Spot receivers but did not participate in the feeding events and was therefore omitted from the analyses. Between the tagged and untagged sharks that frequented Aya's Spot, on average 7 ± 2 participated in the feedings. Based on personal observations we made during this study (maximum number of sharks observed inside Aya's spot was 11) and the maximum daily number of sharks that was reported by Guttridge et al. (2012) to visit Aya's Spot, we estimate that 70–90% of the sharks that frequented Aya's Spot during the time of our experiment participated in the feeding events.

5.4.1 Anticipation: Prefeeding Burst Activity and Spatial Attraction

Burst Activity was affected by the interaction between Period and Time ($wAIC_c$ = 1.00; Table 5.1, Appendix Table A5.1). It increased significantly during the 1 h leading up to the feeding events (post hoc Tukey: $\beta = -0.909$, $t_{9223} = -3.806$, $P = 0.012$) but dropped back to baseline levels within the first 30 days of recovery (post hoc Tukey: recovery 30: $\beta = 0.126$, $t_{9223} = 0.495$, $P = 1.000$; Figure 5.2, Table 5.1). At night, there were no signs of anticipation based on Burst Activity (Appendix Figure A5.3, Appendix Tables A5.2, A5.3).

Spatial Attraction was also affected by the interaction between Period and Time ($wAIC_c$ = 1.00; Table 5.1, Appendix Table A5.1). It increased for the 1 h of prefeeding during the feeding month (post hoc Tukey: $\beta = -5.568$, $t_{11447} = -5.003$, $P = 0.0001$; Figure 5.2) and remained significantly higher during the 1 h of prefeeding throughout the 90 days of recovery compared to the baseline period (post hoc Tukey: recovery 30: $\beta = -7.440$, $t_{11447} = -6.746$, $P < 0.0001$; recovery 60: $\beta = -8.000$, $t_{11447} = -7.342$, $P < 0.0001$; recovery 90: $\beta = -3.885$, $t_{11447} = -3.477$, $P = 0.038$; Figure 5.2). However, prefeeding Spatial Attraction started to decrease during recovery 90 and was significantly lower compared to recovery 60 (post hoc Tukey: $\beta = 4.115$, $t_{11447} = 4.033$, $P = 0.005$). During the 1 h of postfeeding, Spatial Attraction was significantly higher during the feeding month (post hoc Tukey: $\beta = -11.257$, $t_{11447} = -7.467$, $P < 0.0001$; Figure 5.2). Despite an initial drop during the first 30 days of recovery, the postfeeding Spatial Attraction remained significantly higher than the baseline level during recovery 60 (post hoc Tukey: $\beta = -5.602$, $t_{11447} = -4.520$, $P = 0.0006$; Figure 5.2).

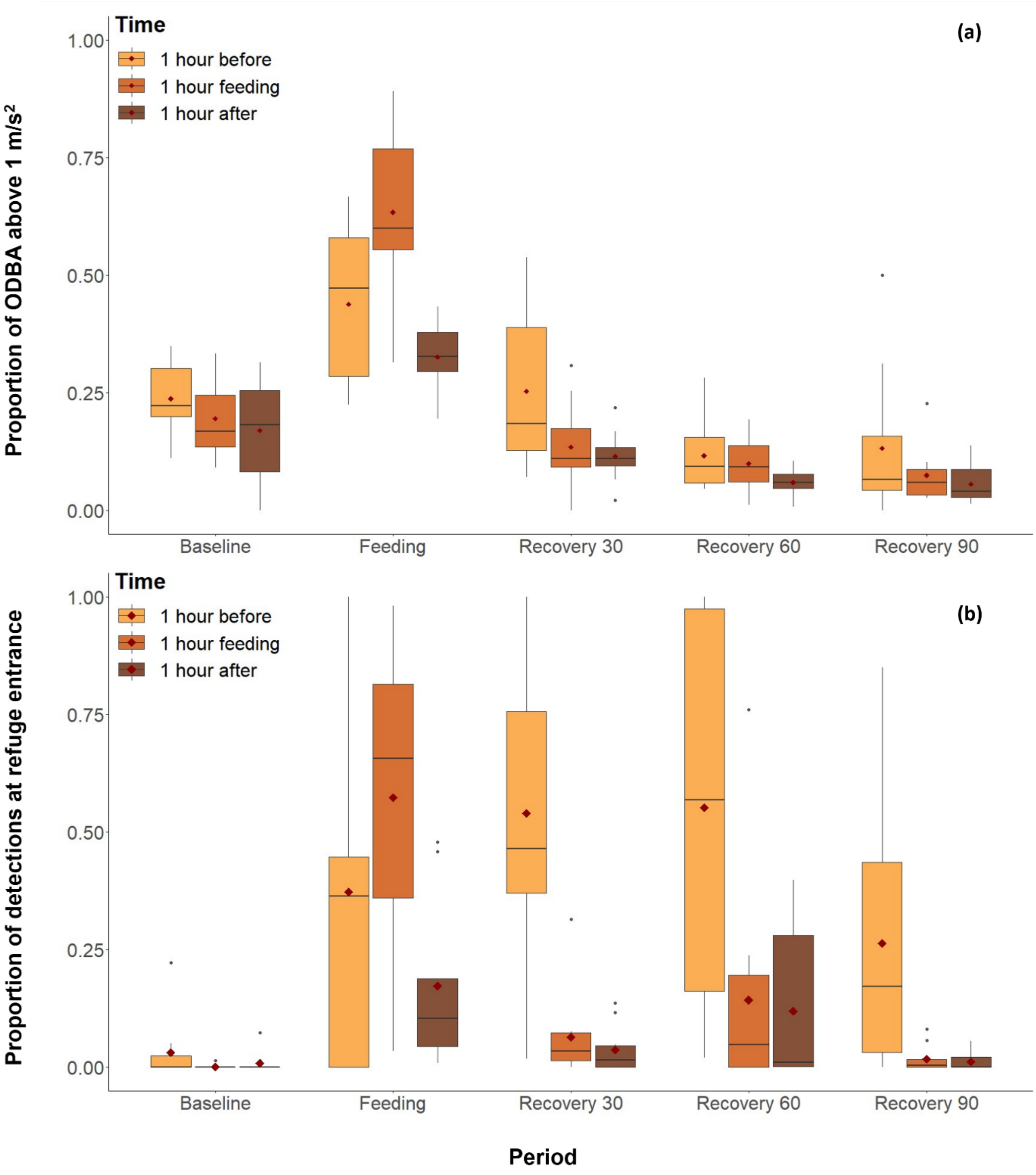


Figure 5.2 Box plot illustrating (a) the proportion of overall dynamic body acceleration (ODBA, m/s^2) above 0.9 m/s^2 (Burst Activity), and (b) the proportion of detections made at the refuge entrance (number of detections at refuge entrance/(number of detections at refuge entrance + number of detections inside refuge)) (Spatial Attraction) plotted against the Period. The horizontal lines inside the boxes mark the median and the red diamonds the mean for 1 h before, during and after feeding events. The upper and lower boundaries of the box represent the third and first quartiles. The whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers.

Table 5.1 Summary table for the models investigating the Period and Time effects on Burst Activity and Spatial Attraction

Effect	*β*	SE	*z*	*P*
Burst Activity				
(Intercept)	-1.390	0.235	-5.920	<0.001
Period (feeding)	0.885	0.245	3.609	<0.001
Period (recovery 30)	-0.166	0.264	-0.630	0.529
Period (recovery 60)	-0.888	0.241	-3.684	<0.001
Period (recovery 90)	-1.010	0.276	-3.661	<0.001
Time (during)	0.051	0.164	0.309	0.757
Time (after)	0.056	0.176	0.318	0.750
Period (feeding) * Time (during)	0.873	0.238	3.663	<0.001
Period (recovery 30) * Time (during)	-0.429	0.277	-1.549	0.121
Period (recovery 60) * Time (during)	-0.286	0.255	-1.120	0.263
Period (recovery 90) * Time (during)	-0.418	0.299	-1.396	0.163
Period (feeding) * Time (after)	-0.464	0.249	-1.866	0.062
Period (recovery 30) * Time (after)	-0.609	0.276	-2.203	0.028
Period (recovery 60) * Time (after)	-0.750	0.265	-2.827	0.005
Period (recovery 90) * Time (after)	-0.731	0.309	-2.362	0.018
Conditional R^2				0.30
Spatial Attraction				
(Intercept)	-6.789	0.965	-7.037	<0.001
Period (feeding)	5.568	1.113	5.003	<0.001
Period (recovery 30)	7.440	1.103	6.746	<0.001
Period (recovery 60)	8.000	1.090	7.342	<0.001
Period (recovery 90)	3.885	1.117	3.477	<0.001
Time (during)	-4.016	1.134	-3.541	<0.001
Time (after)	-3.305	0.773	-4.277	<0.001
Period (feeding) * Time (during)	5.689	1.160	4.904	<0.001
Period (recovery 30) * Time (during)	-0.522	1.187	-0.439	0.660
Period (recovery 60) * Time (during)	-1.450	1.177	-1.232	0.218
Period (recovery 90) * Time (during)	-0.365	1.242	-0.294	0.769
Period (feeding) * Time (after)	1.140	0.812	1.404	0.160
Period (Recovery 30) * Time (after)	-3.372	0.869	-3.880	<0.001

Period (recovery 60) * Time (after)	-2.398	0.829	-2.892	0.004
Period (recovery 90) * Time (after)	-1.735	0.904	-1.919	0.055
Conditional R^2				0.88

Estimated Period and Time effect coefficients (β) and their standard errors (SE), z values and the individual coefficient type I error estimate (P). Time (during) = 1 h of feeding, Time (after) = 1 h postfeeding, Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month. For the factor Time, the baseline level was the 1 h prefeeding.

Spatial learning and anticipation appear to have commenced after 9 days. This estimate is based on the number of sharks observed during the feeding events and the significant drop in latencies to first arrival (linear regression: Latency to first arrival ~ Day: β = -0.637, t_{119}= -4.215, $P < 0.0001$; Figure 5.3). Five of the six sharks included in the analysis reached the learning criterion and did so in 10.8 ± 0.37 days (Figure 5.3). In contrast to Burst Activity, the Spatial Attraction 1 h prior to the feeding events started to increase significantly during night-time high tides (slack tide was reached between sunset and 1 h after sunrise) during the feeding period and was retained for the first 60 days of recovery (Appendix Figure A5.3, Appendix Tables A5.2, A5.3).

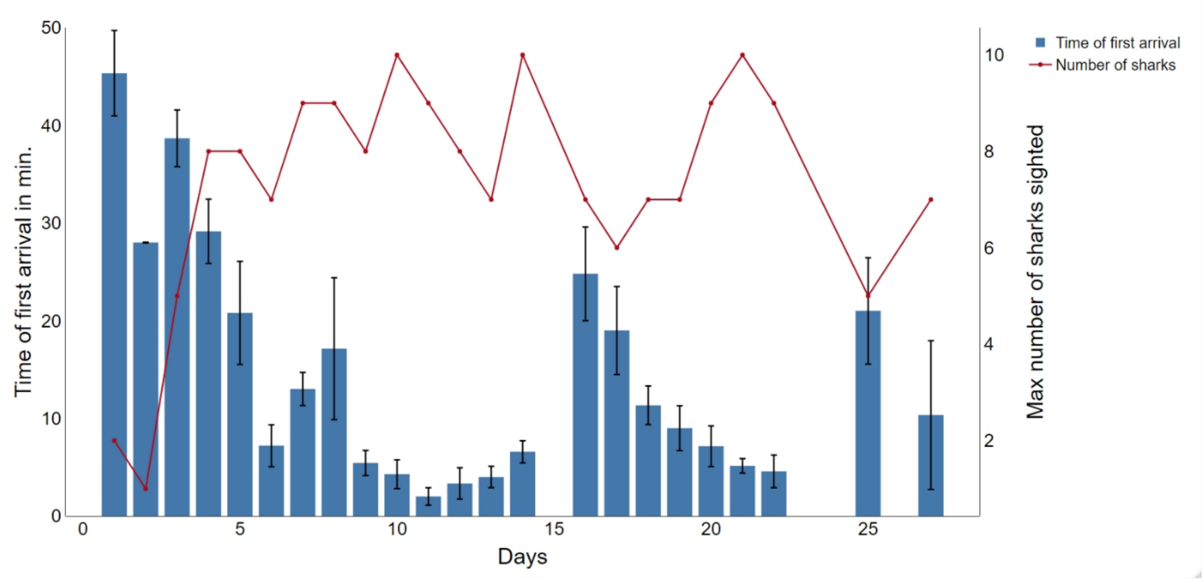


Figure 5.3 Illustration of the daily mean time of first arrival ± SE (min) following the introduction of the bait box at the feeding site, and the daily maximum number of sharks present during the feeding events.

Table 5.2 Summary table for the models investigating the Period and Time effects on the mean daily activity and the mean daily metabolic rates

Effect	β	SE	*z*	*P*
Mean daily activity				
(Intercept)	-0.271	0.224	-1.212	0.226
Period (feeding)	0.371	0.282	1.316	0.188
Period (recovery 30)	0.416	0.247	1.690	0.091
Period (recovery 60)	-0.608	0.238	-2.555	0.011
Period (recovery 90)	-0.660	0.239	-2.766	0.006
Temperature	-0.006	0.008	-0.729	0.466
Period (feeding) * Temperature	-0.015	0.011	-1.396	0.162
Period (recovery 30) * Temperature	-0.036	0.009	-3.823	<0.001
Period (recovery 60) * Temperature	0.00002	0.009	0.003	0.998
Period (recovery 90) * Temperature	0.008	0.009	0.883	0.377
Conditional R^2				0.16
Mean daily metabolic rate				
(Intercept)	2.303	0.026	88.79	<0.001
Period (feeding)	0.075	0.033	2.28	0.022
Period (recovery 30)	0.008	0.028	0.28	0.778
Period (recovery 60)	-0.146	0.027	-5.34	<0.001
Period (recovery 90)	-0.132	0.027	-4.83	<0.001
Temperature	0.033	0.001	35.14	<0.001
Period (feeding) * Temperature	-0.003	0.001	-2.20	0.028
Period (recovery 30) * Temperature	-0.002	0.001	-1.45	0.147
Period (recovery 60) * Temperature	0.004	0.001	3.74	<0.001
Period (recovery 90) * Temperature	0.004	0.001	3.45	<0.001
Conditional R^2				0.50

Estimated Period and Temperature effect coefficients (β) and their standard errors (SE), z values of factors included in the model averaging (Appendix Table A5.4), and the individual coefficient type I error estimate (*P*). Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month.

5.4.2 Daily Activity (Overall Dynamic Body Acceleration) and Metabolic Rates

Daily overall dynamic body acceleration was significantly affected by the interaction between Period and Temperature ($wAIC_c = 1.00$; Table 5.2, Appendix Table A5.4). Although it increased with temperature during the baseline and feeding months, the slope was very small (Figure 5.4, Table 5.2). It remained consistent during the recovery month (Figure 5.4, Table 5.2). Metabolic rates were significantly affected by the interaction between Period and Temperature ($wAIC_c = 1.00$; Table 5.2, Appendix Table A5.4). Across all periods, the metabolic rates increased significantly with temperature. However, they increased at a slower rate during the feeding period than the baseline period. In contrast, they increased significantly faster with temperature during recovery 60 and recovery 90 compared to the baseline period. Despite the significant effect of Period and of the interaction between Period and Temperature, variations were small compared to changes with temperature (Figure 5.4).

5.4.3 Changes in Spatial Distribution

The top-ranked model for the comparison of the core area was the Null model ($wAIC_c = 0.93$; Appendix Table A5.5). The top-ranked model investigating the extents of the home range included Period as a fixed factor ($wAIC_c = 0.93$; Appendix Table A5.5). While there was no significant difference in core areas between periods (Period-RVI = 0.07; Table 5.3), home range was significantly larger during the first 30 days of recovery compared to the baseline period as well as the last 30 days of recovery (Period–RVI = 0.93; post hoc Tukey: baseline–recovery 30: $\beta = -309718$, $t_{21} = -3.508$, $P = 0.016$; Figure 5.5, Table 5.3).

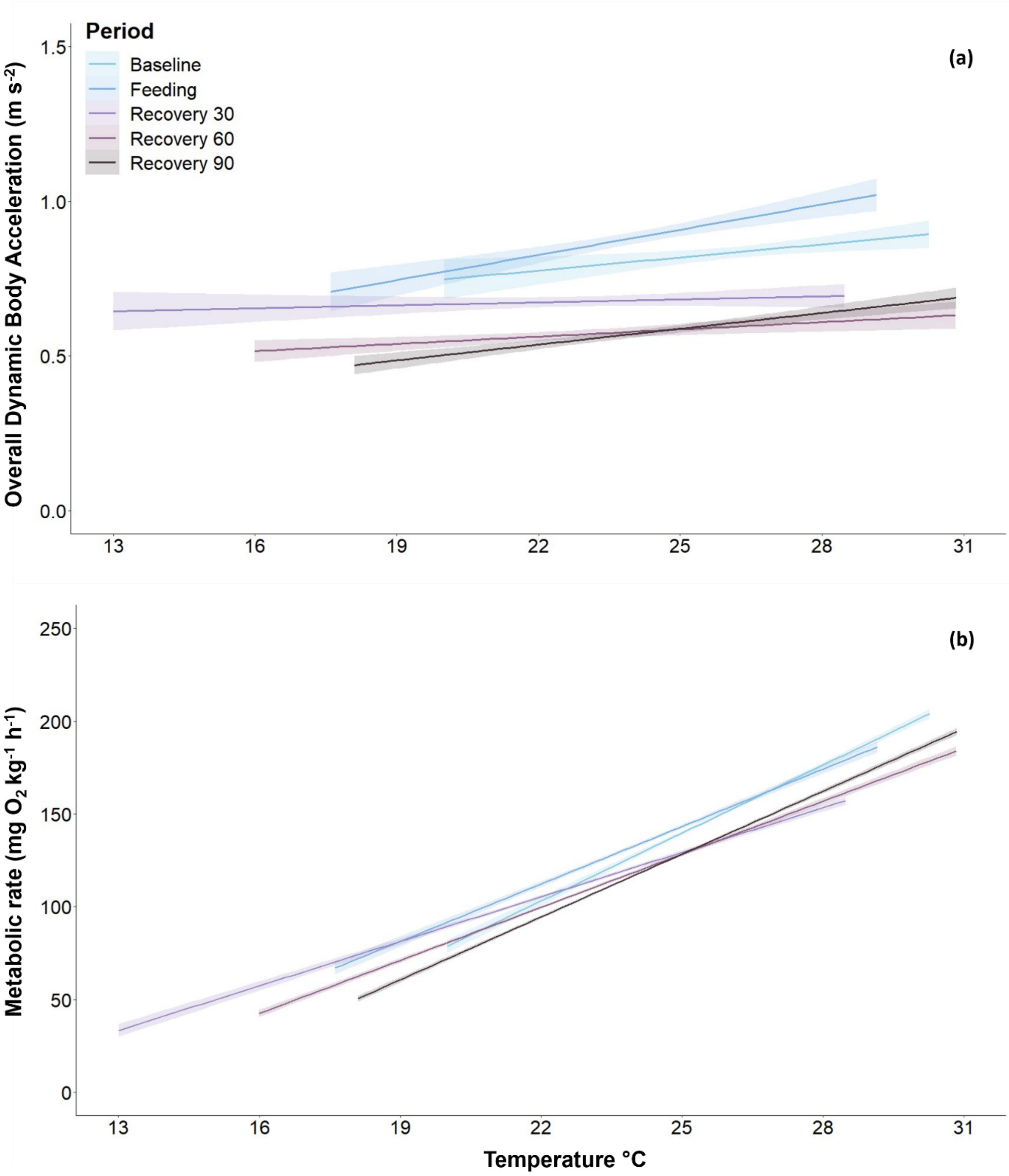
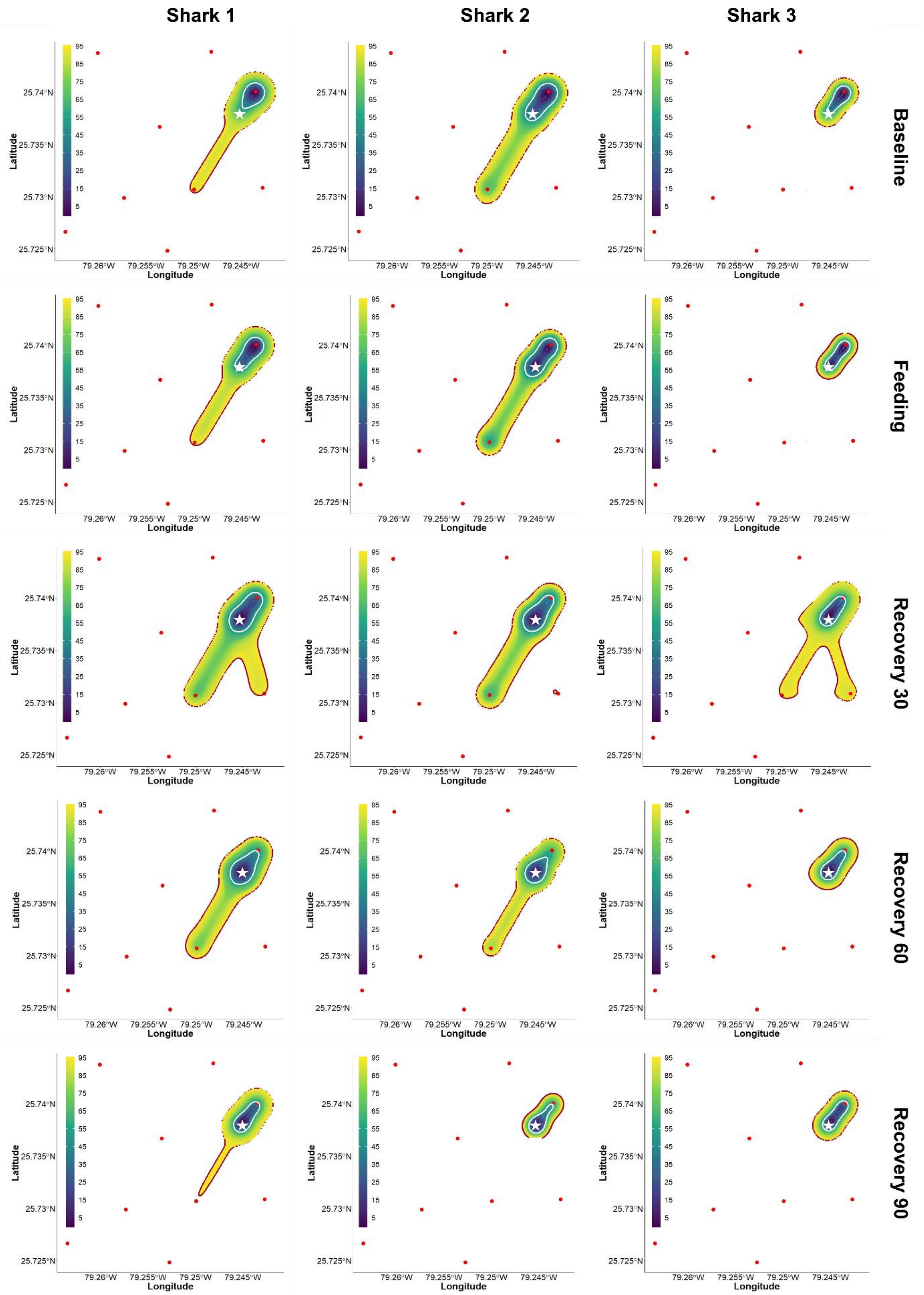


Figure 5.4 Relationship between (a) the mean daily overall dynamic body acceleration (m/s^2) and (b) the mean daily metabolic rate (mg O$_2$/kg per h) and water temperature. Lines of best fit were applied with 95% confidence intervals for each of the five periods.


Shark 1
Shark 2
Shark 3
Baseline
Feeding
Recovery 30
Recovery 60
Recovery 90
95
85
75
65
55
45
35
25
15
5
Latitude
25.74°N
25.735°N
25.73°N
25.725°N
79.26°W
79.255°W
79.25°W
79.245°W
Longitude

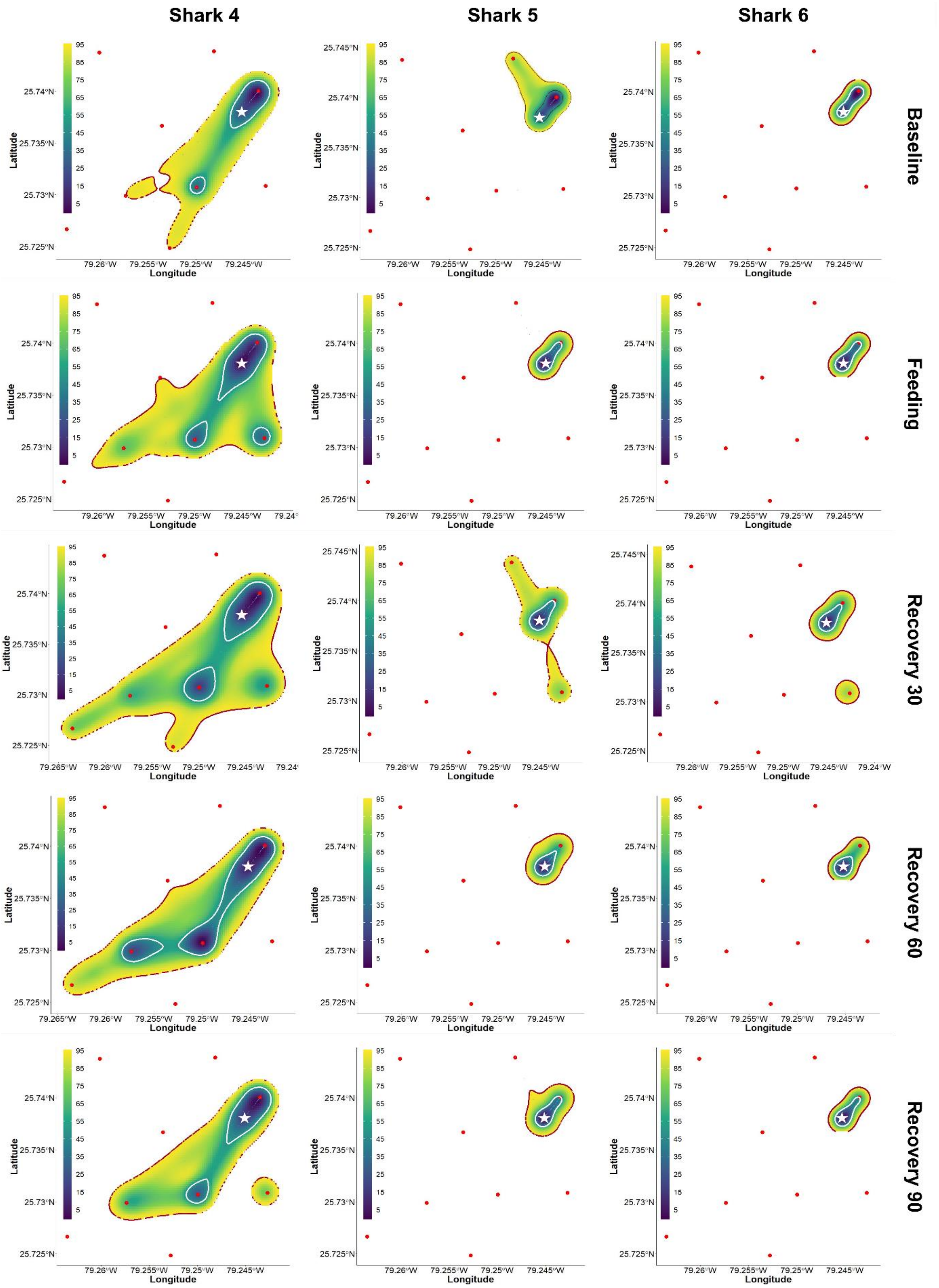


Figure 5.5 Brownian Bridge kernel utilization density estimates for each individual (sharks 1–6) and each period (baseline, feeding, recovery 30, recovery 60 and recovery 90). The white star marks the receiver located at the feeding site and the red dots mark the positions of the remaining nine receivers. The contours illustrate the core area (white) and the home range (red).

Table 5.3 Summary table for the models investigating the Period and Time effects on the core areas and home ranges

Effect	β	SE	*t*	*P*
Core area				
(Intercept)	88782.69	39492.24	2.248	0.035
Period (feeding)	15955.50	21183.56	0.753	0.460
Period (recovery 30)	32208.30	21183.56	1.520	0.143
Period (recovery 60)	37656.63	20775.68	1.813	0.084
Period (recovery 90)	-1869.02	20775.68	-0.0900	0.929
Conditional R^2				0.15
Home range				
(Intercept)	572388.7	252480.50	2.267	0.034
Period (feeding)	62213.9	88286.02	0.705	0.489
Period (recovery 30)	309717.7	88286.02	3.508	0.002
Period (recovery 60)	81309.6	86651.35	0.938	0.359
Period (recovery 90)	-57849.9	86651.35	-0.668	0.512
Conditional R^2				0.32

Estimated Period effect coefficients (β) and their standard errors (SE), *t* values of factors and the individual coefficient type I error estimate (*P*). Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month.

5.5 Discussion

We investigated the impact of daily feeding events on the spatial distribution, activity (as a proxy for energy expenditure) and metabolic rates of juvenile lemon sharks at a site previously unfrequented by humans. Data collection started prior to any feeding events taking place providing the opportunity to collect baseline data, which is often missing in studies assessing the effects of wildlife tourism. Using data collected daily during the 1 h prefeeding, feeding and postfeeding events, we have shown evidence for anticipatory behaviour during prefeeding. Sharks exhibited elevated levels of Burst Activity and spent more time at the feeding site within 27 days of daily feeding events. However, overall daily activity, energy expenditure and broadscale spatial distribution were not affected. Burst

Activity returned to baseline levels after the first 30-day recovery period during which feeding events had ceased. In contrast, the spatial association of the feeding site remained for at least 90 days postfeeding. Our findings reveal shark feeding operations may not affect the daily activity and metabolic rates of focal species or their broad distribution but can alter fine-scale spatial distribution patterns. We further found evidence for intraspecific variability with one individual, while being frequently detected on both refuge receivers, never participating in the feeding events. This variability is possibly due to differences in personality (Dhellemmes et al., 2020, Finger, 2019). Similar intraspecific variability with regard to wildlife feeding operations has previously been demonstrated in bull sharks in Fiji (Brunnschweiler and Barnett, 2013).

5.5.1 Anticipatory Behaviour

Anticipation is often used to investigate time–place learning capabilities of organisms by assessing behavioural changes preceding the introduction of a reward or punishment (Gómez-Laplaza and Morgan, 2005, Reebs, 1999). While juvenile lemon sharks are known to naturally use the shallow water inlet at Aya’s Spot during high tides (Guttridge et al., 2012), we observed a shift in space use inside Aya’s Spot as a result of daily feeding events. In the 1 h preceding the feeding event, sharks changed from being predominantly inside Aya’s Spot to spending more time at its entrance where feeding events took place. This anticipatory behaviour usually indicates spatial learning linked to a temporal component marked by the rising of the tide. Fish and other organisms are capable of tracking time by reference to an internal clock, which is often set by light cycles (i.e. circadian rhythm) and is maintained even in the absence of external cues (Amaral et al., 2014, Bell-Pedersen et al., 2005, de Almeida Moura et al., 2017). Marine organisms, including sharks, are, however, also predisposed to linking behaviour to tidal cycles (Gibson, 2003, Shepard et al., 2006); as a result, associating this natural cycle with feeding for our experiment potentially made it easier for sharks to anticipate the timing of feeding events. Regarding the spatial component of the learning process, juvenile lemon sharks possess an innate homing ability and can successfully return to their home range after being displaced by up to 16 km (Edrén and Gruber, 2005). Many shark species are known to return to a resided-in area after making long-distance movements (e.g. Port Jackson sharks, *Heterodontus portusjacksoni*: (Bass et al., 2017), display site fidelity (Bond et al., 2012, Vianna et al., 2013) or return to their exact birth place (i.e. natal philopatry; e.g. lemon sharks: (Chapman et al., 2015, Feldheim et al., 2014). Spatial learning has also been demonstrated on a much smaller spatial scale, with grey bamboo sharks, *Chiloscyllium griseum*, being able to locate a goal position inside a maze using a directional strategy or spatial learning based on visual landmarks (Fuss et al., 2014). Given the visual landmarks used in our study, juvenile lemon sharks might have also used a combination of direction strategy and spatial learning to locate the feeding site.

The shift in space use inside Aya's Spot during the 1 h of prefeeding shows that sharks moved close to the feeding site in anticipation of the upcoming feeding event. This is further supported by the simultaneous increase in Burst Activity. Based on time at first arrival and number of sharks sighted, juvenile lemon sharks required 5–10 days to start anticipating feeding events and ca. 11 days to reach the learning criterion. This learning period is similar to that of bamboo sharks trained to locate a goal inside a maze and Port Jackson sharks trained in a spatial task. Anticipation of a feeding event and subsequent spatial learning have also been demonstrated in bull sharks (Brunnschweiler and Barnett, 2013) and short-tail stingrays, *Bathytoshia brevicaudata* (Pini-Fitzsimmons et al., 2018). Following ca. 30 years of fish frames being frequently discarded at a boat ramp cleaning station, short-tail stingrays consistently visited the boat ramp in late afternoon when fish cleaning typically occurred, regardless of whether fish cleaning was taking place. This suggests that these rays anticipate afternoon feeds as a result of a learnt time–place association (Pini-Fitzsimmons et al., 2018).

Despite small declines in anticipation following periods of inclement weather when we were unable to feed sharks, some anticipation was still observed well after the feeding events had fully ceased. For instance, Burst Activity was still significantly higher than baseline levels during the first 30 days of recovery. Spatial anticipation lasted even longer, with sharks still spending more time at the feeding site during the 1 h feeding event after 60–90 days of recovery compared to the baseline period. Our results suggest that a learnt spatial association can be retained for extended periods in wild populations of elasmobranchs. Indeed, laboratory studies on memory retention capacities demonstrated that sharks can memorize learnt behaviours for long periods of time. Juvenile grey bamboo sharks remembered a learnt visual discrimination task for up to 50 weeks in the absence of reinforcement (Fuss and Schluessel, 2015). Similarly, Port Jackson sharks retained a learnt association with air bubbles for up to 40 days (Guttridge and Brown, 2013). However, the extent of memory retention and extinction rates in elasmobranchs requires further studies in more controlled environments.

5.5.2 Daily Activity and Metabolic Rates

The increased activity prior to and during the feeding period was not sufficient to affect sharks' daily overall dynamic body acceleration and metabolic rate. These results contrast with a previous study showing that wildlife tourism targeting reef sharks increased the amount of time whitetip reef sharks spent swimming and subsequently their daily overall dynamic body acceleration (Barnett et al., 2016). The discrepancy between Barnett et al.'s (2016) study and ours is probably because whitetip reef sharks typically spend most of the day resting on the sea floor (Barnett et al., 2016, Fitzpatrick et al., 2011), similar to other benthic sharks like the Port Jackson shark (Kadar et al., 2019). The increased activity of whitetip reef sharks due to tourism occurs during the day when these sharks would normally be resting. If they had been fed in the evening when they are naturally active, feeding events would probably have had little perceivable impact on their activity levels and hence energy budget. Although lemon sharks are able to rest on the benthos, they spend most of their time swimming (Bouyoucos et al., 2018), and the slight increase in activity during periods when sharks are normally active and swimming was not sufficient to substantially affect routine metabolic rates. This suggests that species that are naturally more active, such as pelagic species, are less likely to be affected by wildlife tourism events at the level of their metabolic rates than benthic species that would naturally rest during the day.

In the absence of a significant effect on shark activity, the observed differences in metabolic rates were driven primarily by changes in ambient water temperatures. This was expected, as temperature is the most important external factor determining energy expenditure in ectotherms like sharks, with basal or standard metabolic rates of ectotherms doubling to tripling with every 10 °C increase in temperature (Clarke and Johnston, 1999, Gillooly et al., 2001). While the interaction between temperature and period seemed important in the model, the effect size was relatively small and did not exceed the variation in metabolic rate that occurs throughout the day in lemon sharks (Bouyoucos et al., 2018). Given the extent of the temperature effect on the metabolic rates of sharks, it appears unlikely that short, daily feeding events (ca. 1 h) is substantial relative to the temperature effect. This line of reasoning leads us to conclude that the effect of feeding events will be most apparent at times of stable ambient temperatures.

5.5.3 Spatial Distribution and Fine-Scale Movement Patterns

The broad space use (core area and home range) of juvenile lemon sharks was not influenced by 1 h daily feeding events. Within Aya's Spot, however, there was a clear shift in space use towards the feeding site. The shift lasted for at least 90 days after feeding cessation, further illustrating an extended learning extinction period in these sharks. It also demonstrates that a feeding period as short as 27 days can have significant, long-lasting effects on the fine-scale space use of juvenile sharks. Previous studies support our results, even though most were carried out at well-established feeding sites (Brunnschweiler and Baensch, 2011, Clua et al., 2010, Corcoran et al., 2013). For instance, Brunnschweiler and Barnett (2013) demonstrated a change in the fine-scale movements of bull sharks at a feeding site in Fiji with sharks visiting the feeding site for more extended periods on feeding days than on nonfeeding days (Brunnschweiler and Barnett, 2013). The extended home range during the first 30 days of recovery could indicate that sharks were searching for the former feeding site landmarks in the area surrounding Aya’s Spot in case the feeding site had moved. Once they were satisfied that the feeding location was nowhere to be found their home ranges returned to baseline levels.

The number of sharks present at the feeding site increased from only three at the start of the daily feeding events (two tagged, one untagged) to ca. 10 within 10 days of feeding. Of the maximum number of 10 sharks we observed during feeding, six were tagged and four were untagged. The recruitment we observed at Aya’s Spot during the feeding events might be linked to social learning, which has previously been demonstrated in elasmobranchs (Vila Pouca et al., 2020, Thonhauser et al., 2013), including juvenile lemon sharks (Guttridge et al., 2013). Increases in shark abundance are commonly reported at feeding sites, such as with bull sharks in Fiji (Brunnschweiler et al., 2014) and southern stingrays, *Dasyatis americana*, in the Grand Cayman Islands (Corcoran et al., 2013, Semeniuk and Rothley, 2008). These changes in abundance can have severe, detrimental effects on the health of the animals, including increased parasite loads, increased conspecific bites and a reduced overall condition (Corcoran et al., 2013, Semeniuk and Rothley, 2008). However, the southern stingray is the only species of elasmobranchs that has so far been reported to suffer such severe detrimental effects from wildlife tourism operations (Trave et al., 2017). Furthermore, given the natural group-living conditions that juvenile lemon sharks are exposed to daily inside Aya’s Spot, it is unlikely they would suffer from the same effects, at least not to the extent witnessed in southern stingrays.

5.6 Conclusion

The short feeding period of only 27 days had a significant impact on the fine-scale movement pattern and the Burst Activity of juvenile lemon sharks, indicating that sharks are able to learn associations at a similar rate in natural and laboratory conditions (Fuss and Schluessel, 2015, e.g. Guttridge and Brown, 2013). Daily feeding events may result in quick behavioural changes via conditioning, which may be retained for at least 90 days in the absence of any further reinforcement. These responses, in particular the changes in fine-scale spatial distribution, appear to be similar across different species (Barnett et al., 2016, Corcoran et al., 2013, Brunnschweiler and Barnett, 2013). In contrast, the physiological response, metabolic rate, was not substantially affected, and this was probably due to feeding events occurring at times when lemon sharks are naturally active. The ability to collect baseline data prior to the initiation of daily feeding events replicating standard tourism operations provided a unique opportunity to assess how wildlife tourism affects shark behaviour. Our study shows that, while changes in shark behaviour were observed, these changes were spatially restricted and only occurred close to the feeding event. Some of the observed local effects were, however, long lasting. In conclusion, our experiment had minimal effects on the metabolic rate of juvenile lemon sharks, but managers of wildlife tourism industries should consider the potential impacts of long-lasting changes in shark behaviour, even if they are likely to be localized to the feeding site and time. However, the small sample size of our study needs to be taken into consideration. While our results indicate possible effects of feeding activities on the behavioural ecology and spatial distribution of sharks they do not necesseraily apply to all species and indiviuals equally. This is shown by the individual we tagged that, despite being present inside Aya’s Spot on feeding days, never participated in any of the feeding events. We therefore recommend to run small-scale pilot studies on target species/populations before establishing new feeding operations to ensure the most sustainable strategies are applied and the negative impacts are minimized.

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Chapter 6

General discussion

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6.1 Overview

The number of studies investigating the cognition ability of elasmobranchs has been increasing over the past two decades (Guttridge et al., 2009, Schluessel, 2015). As the most ancient group of extent jawed vertebrates, elasmobranchs hold a key phylogenetic position to understanding the evolutionary history of the vertebrate brain and its cognitive toolbox. However, research on the cognitive abilities of this taxonomic group have historically been underrepresented, partially due to an overshadowing interest in groups that share a neurocortex structure similar to our own, i.e. birds and mammals (Brown et al., 2011, Schluessel, 2015). This bias was further fuelled by the misconception of a linear evolutionary pathway from simpler to more advanced forms, presuming that fish and sharks would lack many of the cognitive abilities found in younger, more "advanced" taxonomic groups (Brown et al., 2011). Contradicting this long-lasting belief, recent research has demonstrated that elasmobranchs can master a wide variety of cognitive tasks, including the construction of a cognitive map and the use of tools (Guttridge et al., 2009, Schluessel, 2015). This thesis adds to our growing understanding of shark cognition, with the following key findings:

- Juvenile Port Jackson sharks habituate to a food-related olfactory cue, which appears to be a mechanism to optimise foraging efforts and avoid energetically wasteful behaviours (Chapter 2);
- The frequency of reinforcement has a stronger impact on the rate of learning in juvenile Port Jackson sharks than the size of the reward (Chapter 3):
- Juvenile lemon sharks were unable to learn a time-place association between two locations and times of day inside a semi-captive enclosure over 41 days (Chapter 4); and
- Free-ranging juvenile lemon sharks can anticipate daily feedings that took place one hour before the high tide after 11 days. A shift in the fine-scale movement patterns toward the feeding location was detected. Increased activity during feeding was temporally too constrained to affect daily energy expenditure (Chapter 5).

6.2 Advances in elasmobranch cognition research

Studies investigating the cognitive abilities of elasmobranchs have increased in the last two decades. Nonetheless, we still know relatively little about simple cognitive functions and factors that affect the rates at which sharks and rays can learn novel tasks. Being the most ancient extant taxonomic group of jawed vertebrates, elasmobranchs hold a unique phylogenetic position with regards to our understanding of the evolutionary history of the vertebrate brain. Therefore, uncovering similarities in the learning capacities of sharks, bony fishes and mammals paired with more invasive studies investigating the specific brain areas responsible for certain types of learning will help us better understand at what point during the evolutionary process the vertebrate brain began to develop into different directions. It will allow for a better understanding of how long the vertebrate brain was subject to a joint evolutionary development and which areas of the brain developed after different taxonomic groups split off. It will further shed light on the influence of environmental factors on the convergent evolution of analogous brain areas in different taxonomic groups that end up serving a similar purpose. This study furthered our understanding of the cognitive capacities of sharks, using two model species with different ecological needs. Studying cognition in different species of elasmobranchs is crucial to developing a representative baseline for this taxonomic group. Therefore, the first two studies of my thesis aimed to investigate the rate of habituation to a strong appetive olfactory stimulus (Chapter 2) and the influence of training frequency and reward size on the rate of learning in Port Jackson sharks (*Heterodontus portusjacksoni*, Chapter 3).

Although considered to be the simplest form of learning, habituation can directly benefit individual fitness by freeing up resources that can then be used in fitness related activities (Groves and Thompson, 1970, Thompson and Spencer, 1966, Rankin et al., 2009). In case of foraging-related stimuli, habituation can free up time and energy to search for richer foraging sites or prey items, optimising an animal’s foraging behaviour (Boyd et al., 1997, Haswell et al., 2018, Charnov, 1976, Watanabe et al., 2014). In my study, Port

Jackson sharks habituated to a strong food-related olfactory cue, with responses dropping following a single day of unrewarded exposure (Chapter 2), which is similar to the habituation rate of small-spotted catsharks to a weak electrical current resembling a natural prey (Kimber et al., 2014). The differences in foraging responses we observed between the smell, partial feeding, and feeding groups appear to be governed by the marginal value theorem, which describes an animal's need to abandon a foraging patch once the rate of food gain drops below a certain threshold (Charnov, 1976). As the food availability within a food patch diminishes, the theorem predicts an individual should abandon the patch and move on to the next (Charnov, 1976). For example, there was a positive correlation between patch residence time (dive duration) of Adélie penguins (*Pygoscelis adeliae*) and individual patch quality (rate of food gain) (Watanabe et al., 2014). Although the marginal value theorem describes a trade-off based on diminishing return rates within a foraging patch, it is not necessarily mutually exclusive from habituation. The process of habituation in response to a food-related stimulus is also based on a trade-off decision each time it is exposed to the stimulus. The decision process is thereby based on the current situation and previous experience. Therefore, habituation may contribute to optimise foraging efficiency, not just in sharks, but other taxonomic groups as well. The results of this thesis suggest that short-term habituation could be one of the mechanisms driving the marginal value theorem. Sharks also demonstrated signs of intra-session or short-term habituation, i.e. a drop in their responses within a single day or training session. This could be the mechanism that allows for quicker transitions from one feeding patch to the next. Hence, the rate of an animal's short-term habituation to a food-related stimulus could determine the threshold of the food return rate at which it will decide to move on to the next foraging patch.

Studies on the effects of training frequency and reward size on the learning and extinction rates (the rate at which an animal unlearns a previously learnt behavioural response) of animals have previously focused on mammals (*Rattus norvegicus domestica*, Bouton et al., 2014, Devan et al., 2003, *Didelphis albiventris* and *Lutreolina crassicaudata*, Papini et al., 1988), birds (*Columba livia*, Roberts et al., 1963), amphibians (*Rhinella arenarum*, Muzio et al., 1992, Schmajuk et al., 1981), and fishes (*Carassius auratus*, Gonzalez and Bitterman, 1967, Gonzalez et al., 1974). While the focus of these past studies often lay on the extinction phase of an experiment, my study assessed which of frequency or size of the reward had a stronger influence on the learning performance of Port Jackson sharks during the acquisition phase (Chapter 3). Sharks generally learn at rates similar to other vertebrate groups (Guttridge et al., 2009, Schluessel, 2015). Overall, Port Jackson sharks required between 13 and 18 training sessions to learn the simple spatial cognitive task provided. This is similar to the 15 sessions required by grey bamboo sharks (*Chiloscyllium griseum*) and coral cat sharks (*Atelomycterus marmoratus*) to learn to locate a fixed goal position inside a maze (Fuss et al., 2014) or the 17 sessions it took the ocellate river stingray (*Potamotrygon motoro*) to learn a similar spatial cognitive task (Schluessel and Ober, 2018). The results of this study further suggest that the frequency at which sharks are trained has a stronger influence on the overall learning performance compared to the size of the reward they receive (Chapter 3). Similar to Long-Evans hooded rats (*Rattus norvegicus domestica*) that were trained to navigate and escape a water maze or goldfish (*C. auratus*) that were trained to press a target to gain access to a food reward, sharks trained at a higher frequency learnt faster compared to those trained at a lower frequency (Gonzalez and Bitterman, 1967, Devan et al., 2003). In contrast, unlike goldfish, there was no correlation between the learning performance of sharks and the reward size (Gonzalez and Bitterman, 1967). This could be due to the overall size of the food reward used during this study. Whilst the large reward provided was 200% the amount of the small reward, the absolute weight difference was only 0.2 g per trials. Larger differences in reward sizes might be needed to provide a strong enough incentive for one group to learn faster compared to the other groups.

A confounding factor during this study may have been the choice between the left and the right pathway, and a potential brain lateralisation in Port Jackson sharks, which may have resulted in a side bias (Byrnes et al., 2016a, Byrnes et al., 2016b, Vila Pouca et al., 2019, Vila Pouca et al., 2018). The difference in performances between sharks that were trained to use the right door and those trained to use the left door showed pronounced brain lateralisation in some of the sharks tested. Brain lateralisation has also been shown to be advantageous when performing certain cognitive tasks (Chivers et al., 2017, Dadda and Bisazza, 2006, Sovrano et al., 2005). However, during this two-choice experiment an individual with a strong preference to either side may have appeared to be an incredibly quick learner or unable to learn the task, depending on the door it was trained toward. A previous study on the cognitive ability of the poeciliid *Brachyrhaphis episcopi* found strongly lateralised individuals performed significantly worse compared to weakly lateralised individuals, likely due to them failing to overcome their turn bias (Brown and Braithwaite, 2005). Therefore, future studies investigating spatial cognitive abilities and the impact of certain environmental factors should consider side preference tests on each individual prior to the actual experiment to ensure an even distribution of lateralised individuals across treatment groups.

Although the results for the Port Jackson sharks cannot be directly extrapolated to the most popular focal species of shark tourism activities, they are somewhat representative of other benthic species. For example, the grey nurse shark (*Carcharias taurus*) or the whitetip reef shark (*Triaenodon obesus*) are popular species among tourists and tourism operators (Barnett et al., 2016, Huveneers et al., 2017, Smith et al., 2010). Our results may provide some indication as to how shark tourism activities may affect these species. Furthermore, since sharks are highly diverse and many of the species targeted by wildlife tourism operators are difficult to work with, it is important to investigate behavioural traits related to wildlife tourism activities in different species to comprehensively understand the range of behavioural responses that may then be used to inform tourism management.

Moving towards more complex cognitive tasks, Chapter 4 aimed to investigate the ability of elasmobranchs to learn time-place associations involving two different locations and two distinct times of day. During 41 training days within a semi-captive environment, juvenile lemon sharks were unable to learn a circadian-based time-place association. Time-place learning is wide-spread throughout the animal kingdom and has been demonstrated in insects (Gould, 1987, Schatz et al., 1994, Schatz et al., 1999, Moore et al., 1989), teleost fishes (Gómez-Laplaza and Morgan, 2005, Reebs, 1996, Brännäs, 2014, Reebs, 1999), birds (Biebach et al., 1989, Daan and Koene, 1981, Falk et al., 1992, Tello-Ramos et al., 2015, Shettleworth and Plowright, 1992), and mammals (Carr and Wilkie, 1997, Mulder et al., 2013b, Mulder et al., 2015, Van der Zee et al., 2008). Time-place learning can shape the diurnal behaviours of animals with the learnt associations being based on an animal's endogenous circadian clock or external environmental factors such as tides, light cycles, precipitation, and temperature. It enables animals to optimise their behaviour during resource localisation and exploitation, as well as predator avoidance (Daan and Koene, 1981, Fraser et al., 1993, Guttridge et al., 2012, Heupel and Simpfendorfer, 2008, Mulder et al., 2013a). Time-place learning thereby increases energy efficiency and survivability, directly benefiting individual fitness (Crystal, 2009, Kuhlman et al., 2018, Mulder et al., 2013a). Although my finding contradicts most studies on time-place learning based on circadian clocks, convict cichlids were also unable to make similar time-place associations following 30 days of training (Reebs, 1993). It is possible that the relatively small size of the enclosures used during this experiment did not provide enough incentive for the sharks to learn the time-place association. The distance of 9 m between the two feeding locations may have been too short to be ecologically relevant to the sharks, given their relatively large space use in the wild of up to 93 km^2 (Gruber et al., 1988, Guttridge et al., 2012). Consequently, time-place learning may simply not be operating on such a fine spatial scale. A study on cichlid angelfish (*Pterophyllum scalare*) that showed successful time-place learning inside small aquaria was carried out using captive-bred individuals (Gómez-Laplaza and Morgan, 2005), which could explain why these fish viewed their environment differently and the incentive to learn the association was strong enough despite the small size of the enclosures. Therefore, future studies should be carried out across a significantly larger arena relative to the size of the sharks or consider using captive-hatched and reared individuals. Field studies that do not restrict shark movement might be necessary to provide a strong enough incentive to learn circadian-based time-place associations. However, it can be difficult to control for external factors and ensure sufficient interaction with individual sharks in such free-ranging studies.

Furthermore, investigating the learning abilities of different shark species will help us better understand, which environmental factors drive diurnal behaviours. For example, sharks living in more stable environments may have their diurnal behaviour dictated mainly by the time of day, hence their internal circadian clocks. In contrast, sharks living in highly fluctuating environments may need to time their daily behaviours based on other environmental factors, such as the tides, to avoid larger predators, find suitable prey, or conserve energy. Juvenile lemon sharks were unable to learn time-place associations based on their circadian clock. Given their ecological need to seek refuge from larger predators during high tides, they were instead able to learn the association between the high tides and daily feeding events. This shows how ecological requirements may dictate how well sharks and other animals may learn certain tasks and associations and which types of learning are more important. Living in highly fluctuating environments may further pose the risk of a sensual overload (Houslay et al., 2019). Being able to learn to ignore irrelevant stimuli could be crucial to these species to maintain available cognitive resources. Learning in sharks may also influence the behaviour of sharks on a broader geographic scale, as seen in tiger sharks timing their long-distance migrations with the reproductive cycles of turtles and albatross (Meyer et al., 2010, Hammerschlag et al., 2016).

Another reason for the lack of successful time-place learning could be the strong dependency of juvenile lemon sharks to the diurnal tidal cycles in Bimini (Guttridge et al., 2012). During high tides, some areas around Bimini become accessible to larger predatory sharks. To seek protection from these predators, juvenile lemon sharks take refuge inside shallow water mangrove inlets until the tides begin to fall and the predators are forced out

(Guttridge et al., 2012). The need to move with the tides may have suppressed the ability of these sharks to form an association based on their endogenous circadian clocks. The periodicity shown in my data appears to match the tidal cycle but starts to diminish after approximately 30 days. It is possible that a prolonged experiment could have resulted in the elimination of the tide association and allowed sharks to learn the time-place association instead.

Building up on the results from Chapter 4 where it seemed like tides were impacting learning, the final study of my thesis investigated the ability of juvenile lemon sharks to associate daily feedings with the last hour of the rising tide (Chapter 5). I further aimed to investigate the impacts of daily feedings on lemon shark spatial distribution and energy expenditure. With the feedings linked to the final hour of the rising tide, sharks started to anticipate feedings within 5 – 10 days. This anticipation manifested in part as a temporal increase in the sharks' activity. However, unlike whitetip reef sharks, which were previously demonstrated to suffer from an increased daily energy expenditure on days they were fed by tourism operators (Barnett et al., 2016), the short activity increases observed in juvenile lemon sharks had no effect on their daily energy needs. Following the termination of the feeding activities the anticipatory behaviour was retained for up to 90 days, supporting results of previous studies on the long memory retention capacities of elasmobranchs (Fuss and Schluessel, 2015, Guttridge and Brown, 2013). Juvenile grey bamboo sharks remembered a learnt visual discrimination task for up to 50 weeks (Fuss and Schluessel, 2015). Given the wide range of known memory windows in elasmobranchs (40 days to 50 weeks; Fuss and Schluessel, 2015, Guttridge and Brown, 2013), further research is required to better understand how long certain tasks are remembered for and how this varies across species.

In addition to the short-term increase in activity, shark anticipation was also seen from sharks being at the feeding site 1 hour prior to the start of the feedings. This resulted in a fine-scale shift in space use, which lasted throughout the 90 days of recovery. Although previous studies have been mostly undertaken at established feeding sites, their observed changes in shark movement patterns support our results (Brunnschweiler and Baensch, 2011, Clua et al., 2010, Corcoran et al., 2013). For example, bull sharks remained at a feeding site in Fiji for extended periods on feeding days compared to non-feeding days (Brunnschweiler and Barnett, 2013). However, the broad movement of juvenile lemon sharks was not influenced by the daily feedings, with one exception – the home range extended during the first 30 days of recovery. This extension could be due to the sharks searching for the former feeding site landmarks in the area surrounding Aya's Spot, in case the site had moved. Once satisfied that the feeding location was nowhere to be found, the home range returned to its original size.

Results from Chapters 4 and 5 in conjunction with those of previous studies on the behaviours of juvenile lemon sharks around Bimini indicate that sharks' ability to learn circadian-based time-place associations can be suppressed by their dependency on the tidal cycles (Gruber et al., 1988, Guttridge et al., 2012). This may change throughout the lifetime of an individual, highlighting the need to investigate ontogenetic effects on cognitive abilities. Juvenile lemon sharks around Bimini need to enter the shallow water mangrove inlets during high tides to seek protection from larger predators that are now able to move into areas that are inaccessible to them during low tides (Guttridge et al., 2012). This changes once the sharks outgrow the refuges and their larger size protects them from most predators (Guttridge et al., 2012). Ontogenetic changes in ecological requirements may affect the role of learning and the ability of sharks to master certain cognitive traits. Since my research involved juveniles exclusively, the ontogenetic development of the role of learning and the cognitive abilities of sharks was not explored in my thesis but warrants research in future endeavours.

6.3 Implications for wildlife tourism

The provision of food is often used by tourists and tourism operators to attract popular, but elusive species, and to increase the likelihood of a close and thrilling encounter (Knight, 2009). The possible behavioural effects of feeding wildlife for tourism purposes is, however, often raised by opponents of this practice in an attempt to stop it (Orams, 2002). Faced with the challenge to ensure sustainable practices (Apps et al., 2018, Apps et al., 2017, Brena et al., 2015, Orams, 2002) and keep their social license (Cullen-Knox et al., 2017), shark-diving operators may not be allowed to feed sharks. For example, Hawaii, Florida, and New Caledonia have all prohibited any activities that involved shark feeding (FFWCC, 2002, Johansen, 2013, Techera, 2012). Whilst the banning of shark feeding operations will most likely prevent sharks from learning any harmful associations, it will also result in less reliable shark encounters and potentially decrease customer satisfaction. In order to achieve a sustainable industry that protects both, the focal species and its ecosystem as well as socio-economic benefits (Haas et al., 2017, Macdonald et al., 2017, Newsome et al., 2019, Williams and Crosbie, 2007), less drastic management strategies are needed.

The first study of this thesis (Chapter 2) tested whether smell as a daily attractant will eventually result in habituation if sharks are never rewarded. Despite the ecological relevance of the stimulus, the response of juvenile Port Jackson sharks to the squid smell started to decline following the first day of unrewarded exposure. Similar to most cognitive functions, habituation rates are likely species-specific and depend on other factors, like the frequency of exposure to the stimulus (Rankin et al., 2009, Thompson, 2009). For example, the South Australian white shark cage-diving industry use a near-constant odour corridor of berley (or chum) comprising mix of minced southern bluefin tuna (*Thunnus maccoyii*) products including offal, oil, and blood to attract sharks present to the cage-diving vessel. Tethered baits of tuna sections or gills and entrails of up to several kilograms are used to improve client experience by keeping sharks within visual range of divers in the cage.

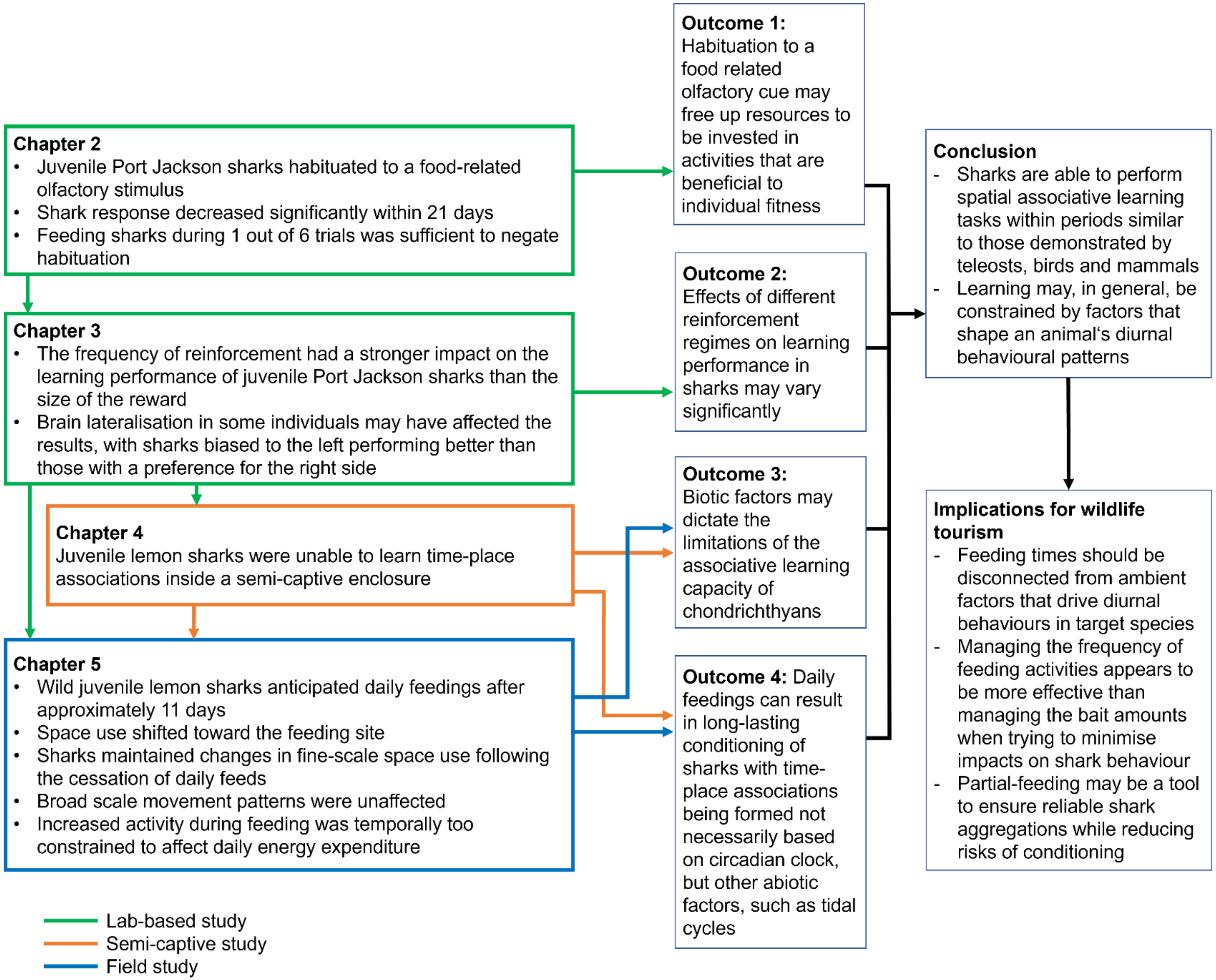
Although operators are not allowed to deliberately feed sharks and receive a 15-minute penalty if a shark accidentally consumes the bait (DEWNR, 2016), the bait handler is not always fast enough to retract the bait. The frequency of baits being taken is dependent on the speed of shark approach, visibility, and vigilance of the bait handler. In addition, white sharks only reside at the Neptune Islands for relatively short periods of time (Nazimi et al., 2018, Schilds et al., 2019). This automatically limits the maximum exposure of each individual shark to the berley and unrewarded food stimulus. Since white sharks at the Neptune Islands occasionally get the bait, the South Australian cage-diving industry most resembled the partial-feeding group of my study (Chapter 2). As a result, white sharks at the Neptune Islands are unlikely to become habituated to the berley used by cage-diving operators, similar to the Port Jackson sharks in the partial-feeding group. In contrast, whitetip reef sharks at Osprey Reef are frequently fed for wildlife tourism purposes (Barnett et al., 2016, Fitzpatrick et al., 2011). These sharks have a high site fidelity, staying at Osprey Reef year-round (Fitzpatrick et al., 2011). Therefore, individual sharks will interact tourism activities and be fed much more frequently than the Neptune Island's white sharks. If feeding at Osprey Reef was prohibited and operators were only allowed to use fish scent to attract sharks, it is possible that these sharks would become habituated to the stimulus and be less likely to respond to the scent used. In areas where dive operators strongly depend on shark encounters, sharks habituating to attracting stimuli could threaten local tourism businesses, potentially driving operators back to a consumptive use of natural resources (Newsome et al., 2019). An alternative for species like the whitetip reef sharks could be occasional feedings, which provided a stable level of attraction, without leading to a conditioned association (Chapter 2). Furthermore, my results suggest that managing the frequency of feedings will be a more effective tool to manage the susceptibility of sharks to learn potentially detrimental associations compared to managing the bait amount (Chapter 3). Future studies should also investigate habituation in relation to flight response to humans. Sharks may also start to lose their fear from humans and water-craft, which could result in bolder behaviours and an elevated risk of dangerous interactions between humans and sharks (Burgess, 1998). Such reduced fear and increase boldness have previously been observed as a result of terrestrial wildlife tourism (Burns and Howard, 2003). For example, dingo (*Canis lupus Dingo*) feeding during tourism activities on Fraser Island (Queensland, Australia) is believed to have led to an increase of dingo bites on humans (Burns and Howard, 2003). Following the first human death from a dingo attack , the Queensland government ordered a cull in which 32 dingoes were killed that year (Burns and Howard, 2003, O'Neill et al., 2017). In Western Australia, six fatalities from shark bites occurred along a 400 km stretch of the coastline between 2011 and 2013 (ASAF, 2014). The occurrence of fatal shark bites within a relatively short period on a small stretch of the coast led the Western Australian Government to run a drum-line programme intending to catch and kill white sharks, tiger sharks, and bull sharks that were more than three metres total length. These examples illustrate how human-wildlife conflicts can result in retaliations and culling of wildlife. In the case of Western Australia, the proposed drum-line programme became controversial because white sharks are considered a threatened marine species and protected by Commonwealth (*Environment Protection and Biodiversity Conservation Act 1999*), by state legislations, and by international laws such as the Convention on International Trade in Endangered Species (CITES) and the Convention on Migratory Species (CMS). This led to conflicts between supporters of the drum-line programme who perceived that the introduced measures would increase public safety and opponents who felt that the measures would have no tangible benefits to ocean users and would undermine the protected status and welfare of white sharks (Gibbs and Warren, 2015).

Sharks and rays may also learn and remember the place and the time of daily feedings and may start to anticipate the arrival of food, resulting in a potentially detrimental change in behaviour. For example, southern stingrays around the Cayman Islands changed their solitary lifestyle to a group-living behaviour, aggregating around the daily feeding site. This resulted in an overall decline in individual health condition, an increase in conspecific bite marks, and an increase in parasite loads (Corcoran et al., 2013, Semeniuk and Rothley, 2008). However, time-place learning does not always have to be based on the circadian rhythm but may also be linked to the diurnal patterns of environmental factors, such as the tidal cycles. For instance, whilst juvenile lemon sharks were unable to learn the circadian-based time-place association within a semi-captive experimental setup, they quickly learnt to anticipate daily feedings, once the feeding activities were relocated to target a wild population in their natural habitat (Chapters 4 & 5). The factors used by sharks to learn time-place associations may depend on the level of environmental fluctuation a species or population experiences within their natural habitats. Sharks living in fluctuating environments may be more likely to learn time-place associations based on environmental factors, such as the tides rather than their endogenous circadian clocks (Chapters 4 & 5). In contrast, sharks living in relatively stable conditions may be more likely to learn associations based on their circadian clocks. However, since I was unable to demonstrate this ability during my experiments, further research is needed to evaluate the capacity of sharks to learn circadian-based time-place associations. For the management of wildlife tourism activities, it would be generally advisable to disconnect the timing of these activities from the factors the target species are most likely to base their time-place associations upon, requiring a good understanding of the ecology and diurnal behaviour of the target species.

Future studies investigating the effects of prolonged wildlife tourism activities on focal species in the field should be done during the pilot phase of a newly developed tourism site whenever possible. This will provide the opportunity to collect baseline behavioural data prior to the establishment of novel wildlife tourism sites to allow for a direct comparison between behaviours prior and post establishment. In areas where tourism is already well established, studies should focus on the comparison between fed populations and unfed populations, as it has been done in studies before (Corcoran et al., 2013, Semeniuk et al., 2009, Semeniuk and Rothley, 2008). Ideally data would be collected on more than one control population to account for physical differences between locations, including substrates, depth, tidal fluctuations, prey availabilities, predation pressure and competition (Huveneers et al., 2021).

6.4 Conclusion

My thesis aimed to advance our understanding of elasmobranchs cognitive abilities and to provide further insights on how certain forms of learning will drive behavioural alterations of sharks and rays in response to frequent exposure to wildlife tourism activities. This was achieved by: 1) investigating the viability of only using smell without feeding to attract sharks for wildlife tourism purposes (Chapter 2); 2) assessing the limitations of associative learning in sharks and the effects of biotic factors on shark learning performance (Chapters 3, 4, and 5); and 3) investigating the effects of regular feeding on the behaviour of sharks, to inform management strategies (Chapters 2, 3, 4 & 5). This thesis demonstrates significant impacts of different biotic factors on the cognitive abilities of sharks (Chapters 3, 4 & 5), highlighting the importance of understanding the ecology and diurnal behaviour of species targeted by wildlife tourism to implement sustainable management strategies. Findings from my PhD provide new information about the learning abilities of sharks in the context of wildlife tourism. As wildlife tourism and shark tourism increase in popularity, so does our responsibility to sustainably manage this activity and ensure minimal impacts on the species it targets. The frequent use of food to attract target species and propensity of animals to learn and make associations also raise concerns about the relationship between wildlife tourism and increased risk to humans, leading to various human-wildlife conflicts. My PhD used laboratory, semi-captive, and wild studies to test the capacity of wildlife tourism to affect shark behaviour and provides recommendations to reduce or manage potential behavioural changes. As my thesis focused on juveniles Port Jackson and lemon sharks, future studies should assess the generality of my findings by undertaking similar studies on adult sharks to test ontogenetic changes in cognitive abilities and in the role of learning, and on other species, including rays, to determine inter-species differences and investigate phylogenetic variations linked to sharks’ and rays’ evolution. Overall, my PhD thesis advances our understanding of the cognitive capacities and limitations of elasmobranchs, which helps fill a knowledge gap in the fields of biology, neurophysiology, ethology, and ecology.



Chapter 2
- Juvenile Port Jackson sharks habituated to a food-related olfactory stimulus
- Shark response decreased significantly within 21 days
- Feeding sharks during 1 out of 6 trials was sufficient to negate habituation

Chapter 3
- The frequency of reinforcement had a stronger impact on the learning performance of juvenile Port Jackson sharks than the size of the reward
- Brain lateralisation in some individuals may have affected the results, with sharks biased to the left performing better than those with a preference for the right side

Chapter 4
Juvenile lemon sharks were unable to learn time-place associations inside a semi-captive enclosure

Chapter 5
- Wild juvenile lemon sharks anticipated daily feedings after approximately 11 days
- Space use shifted toward the feeding site
- Sharks maintained changes in fine-scale space use following the cessation of daily feeds
- Broad scale movement patterns were unaffected
- Increased activity during feeding was temporally too constrained to affect daily energy expenditure

Outcome 1: Habituation to a food related olfactory cue may free up resources to be invested in activities that are beneficial to individual fitness

Outcome 2: Effects of different reinforcement regimes on learning performance in sharks may vary significantly

Outcome 3: Biotic factors may dictate the limitations of the associative learning capacity of chondrichthyans

Outcome 4: Daily feedings can result in long-lasting conditioning of sharks with time-place associations being formed not necessarily based on circadian clock, but other abiotic factors, such as tidal cycles

Conclusion
- Sharks are able to perform spatial associative learning tasks within periods similar to those demonstrated by teleosts, birds and mammals
- Learning may, in general, be constrained by factors that shape an animal's diurnal behavioural patterns

Implications for wildlife tourism
- Feeding times should be disconnected from ambient factors that drive diurnal behaviours in target species
- Managing the frequency of feeding activities appears to be more effective than managing the bait amounts when trying to minimise impacts on shark behaviour
- Partial-feeding may be a tool to ensure reliable shark aggregations while reducing risks of conditioning

Figure 6.1 Overall conclusion and outcomes of the thesis and the contribution of each chapter to the main outcomes.
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Appendix

Chapter 2

R-Code A2.1

```
Loading dataframe

df <- read.table("D:/Dennis Heinrich/Dropbox/PhD Dennis/Lab Experiments/Sy
dney/Chapter 5 - Habituation Pjs/Analysis/Data sheet/time budget test.csv"
, sep = ",", dec = ".", header = TRUE, fill = TRUE, stringsAsFactors = FAL
SE)

Loading required packages

library(lme4)
library(MCMCglmm)
library(tidyverse)
library(broom)
library(nadiv)
library(Hmisc)

Testing our predictions using the four response variables in question: 1)
latency to leave starting compartment (lat.sec.5), 2) the percentage of ti
me spent resting (Resting), 3) the latency to locate and initiate the inve
stigation of the stimulus box (lat.invest), and 4) the percentage of time
spent investigating the stimulus box (Investigate).

#creating the prior
prior_E_B_fit_1px = list(R = list(R = list(V = 1, nu = 0.002)),
                         G = list(G1 = list(V = diag(2), nu = 0.002, alpha
.mu = c(0,2), alpha.V = diag(2)*25^2)))

#running the MCMCglmm model
df <- df %>%
  mutate(Day_start = Day - 1,
         assay_rep_cen = assay_rep - 2,
         Weight_z = (Weight - mean(Weight))/sd(Weight))

mcmc_E_B_fit <- MCMCglmm(lat.sec.5 ~ (Day_start + tg + assay_rep_cen)^3 +
                           Weight_z+
                           Sex,
                         random =~ us(1 + Day_start):Shark.ID,
                         rcov =~ units,
                         family = c("gaussian"),
                         prior = prior_E_B_fit_1px,
                         nitt=100000,
                         burnin=20000,
                         thin=20,
                         verbose = TRUE,
                         pr = TRUE,
                         data = as.data.frame(df))

#replace 'lat.sec.5' with other response variables to test remaining predi
ctions
```

```r
#creating model summary
summary(mcmc_E_B_fit)

#testing model fit
plot(mcmc_E_B_fit$VCV)
```

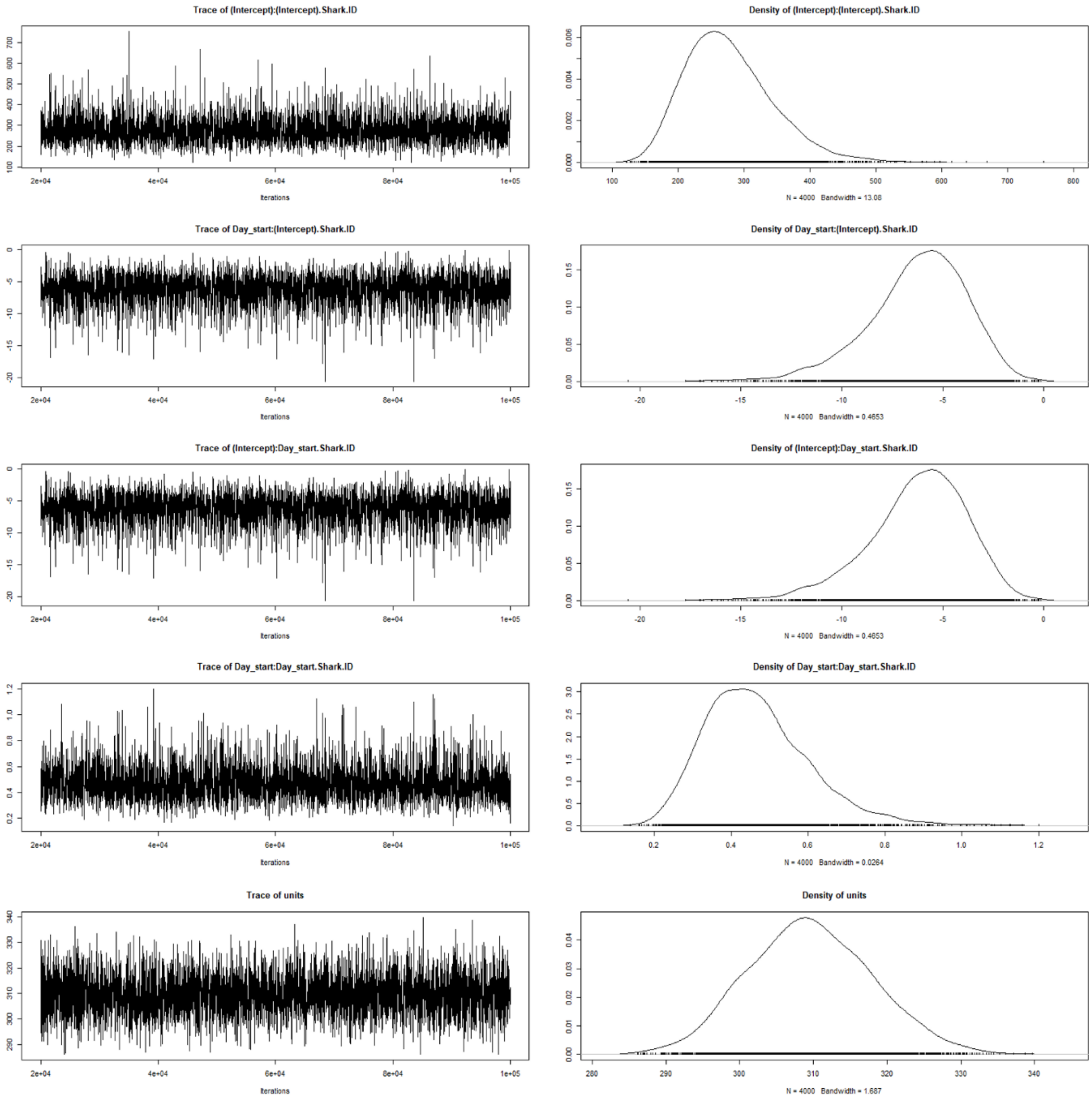

Figure A2.1 Trace plots of the applied Monte Carlo Markov Chains and the respective density plots for the effective sample size of n = 4000.

Chapter 3

Table A3.1 Ranking of the best models ($\Delta AIC_c<2$) assessing the effect of *reward magnitude* and *reinforcement frequency* on the latency to passing the correct door. df = degrees of freedom, AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top ranked model, $wAIC_c$ = model probability. Shark ID nested within holding tank was included as a random factor in all models. For models that contain an interaction, single factors were also included, but not listed separately in the table below.

Model	df	AIC_c	ΔAIC_c	$wAIC_c$
Pass rate ~Day+Magnitude+Side	6	1042.21	0.00	0.46
Pass rate ~Day+Magnitude	5	1042.87	0.66	0.33
Pass rate ~Day*Magnitude+Side	7	1043.69	1.48	0.22

Table A3.2 Estimated latency to pass coefficients (*β*) and their standard errors (SE), *z*-values of factors included in the model averaging (Table A3.1), and the individual coefficient Type I error estimate (*P*).

Effect	*β*	SE	*z*	*P*
(Intercept)	0.327	0.719	0.455	0.649
Day	0.163	0.034	4.759	<0.001
Magnitude	-1.582	0.600	2.634	0.008
Side	-0.624	0.380	1.640	0.101
Day* Magnitude	0.042	0.057	0.738	0.460

Table A3.3 Ranking of the best models ($\Delta AIC_c<2$) assessing the effect of *reward magnitude* and *reinforcement frequency* on the latency to passing the correct door. df = degrees of freedom, AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top ranked model, $wAIC_c$ = model probability. Shark ID nested within holding tank was included as a random factor in all models. For models that contain an interaction, single factors were also included, but not listed separately in the table below.

Model	**df**	**AIC_c**	**ΔAIC_c**	**$wAIC_c$**
Approach index ~Day*Magnitude+Side	8	1123.67	0.00	0.18
Approach index ~Day*Frequency+Day*Magnitude+Side	10	1123.75	0.09	0.17
Approach index ~ Magnitude+Side	6	1124.96	1.30	0.09
Approach index ~Day*Frequency+Magnitude+Side	9	1125.07	1.41	0.09
Approach index ~Day+Magnitude+Side	7	1125.11	1.44	0.09
Approach index ~ Frequency*Magnitude+ Day*Frequency+Day*Magnitude+Side	11	1125.11	1.44	0.09
Approach index ~ Side	5	1125.15	1.48	0.08
Approach index ~ Day+Side	6	1125.27	1.60	0.08
Approach index ~Day*Frequency+Side	8	1125.28	1.61	0.08
Approach index ~Day*Magnitude+Frequency+Side	9	1125.64	1.97	0.07

Table A3.4 Estimated latency to pass coefficients (β) and their standard errors (SE), *z*-values of factors included in the model averaging (Table A3.3), and the individual coefficient Type I error estimate (*P*).

Effect	**β**	**SE**	***z***	***P***
(Intercept)	0.595	0.101	5.882	<0.001
Day	0.003	0.006	0.576	0.564
Frequency	0.166	0.132	1.252	0.211
Magnitude	-0.200	0.136	1.470	0.142
Side	-0.154	0.048	3.185	0.001
Frequency*Magnitude	0.076	0.092	0.833	0.405
Day*Frequency	-0.014	0.007	1.986	0.047
Day* Magnitude	0.012	0.007	1.850	0.064

Table A3.5 Ranking of the best models ($\Delta AIC_c<2$) assessing the effect of *reward magnitude* and *reinforcement frequency* on the latency to passing the correct door. df = degrees of freedom, AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top ranked model, $wAIC_c$ = model probability. Shark ID nested within holding tank was included as a random factor in all models. For models that contain an interaction, single factors were also included, but not listed separately in the table below.

Model	**df**	**AIC_c**	**ΔAIC_c**	**$wAIC_c$**
Latency Pass ~Day*Frequency*Magnitude	11	5425.69	0.00	0.62
Latency Pass ~Day*Frequency*Magnitude+Side	12	5426.69	1.00	0.38

Table A3.6 Estimated latency to pass coefficients (β) and their standard errors (SE), *z*-values of factors included in the model averaging (Table A3.5), and the individual coefficient Type I error estimate (*P*).

Effect	**β**	**SE**	***z***	***P***
(Intercept)	3.527	0.259	13.583	<0.001
Day	-0.032	0.014	2.235	0.025
Frequency	0.315	0.418	0.753	0.451
Magnitude	1.092	0.419	2.604	0.009
Side	0.047	0.095	0.495	0.621
Frequency* Magnitude	-2.152	0.643	3.342	<0.001
Day*Frequency	-0.007	0.025	0.290	0.772
Day*Magnitude	-0.039	0.024	1.658	0.097
Day*Frequency* Magnitude	0.113	0.038	2.972	0.003

Table A3.7 Ranking of the best models ($\Delta AIC_c<2$) assessing the effect of *reward magnitude* and *reinforcement frequency* on the latency to passing the correct door. df = degrees of freedom, AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top ranked model, $wAIC_c$ = model probability. Shark ID nested within holding tank was included as a random factor in all models. For models that contain an interaction, single factors were also included, but not listed separately in the table below.

Model	**df**	**AIC_c**	**ΔAIC_c**	**$wAIC_c$**
Success Correct Pass ~Day*Frequency+Magnitude+Side	8	1557.65	0.00	0.39
Success Correct Pass ~Day*Frequency+Day*Magnitude+ Side	9	1558.62	0.97	0.24
Success Correct Pass ~Day*Frequency+ Frequency*Magnitude+Side	9	1558.92	1.26	0.21
Success Correct Pass ~Day*Frequency+Side	7	1559.45	1.79	0.16

Table A3.8 Estimated latency to pass coefficients (β) and their standard errors (SE), *z*-values of factors included in the model averaging (Table A3.7), and the individual coefficient Type I error estimate (*P*).

Effect	**β**	**SE**	***z***	***P***
(Intercept)	0.098	0.578	0.170	0.865
Day	0.067	0.029	2.299	0.022
Frequency	1.390	0.722	1.923	0.055
Magnitude	-0.799	0.534	1.494	0.135
Side	-1.294	0.279	4.636	<0.001
Frequency* Magnitude	0.660	0.700	0.942	0.346
Day*Frequency	-0.109	0.041	2.662	0.008
Day*Magnitude	0.041	0.040	1.028	0.304

Chapter 4

Table A4.1: Estimated parametric *Event * Tides* effect coefficients (*β*), assessing the proportion of images taken within three body lengths of the feeding location and their standard errors (SE), *z*-values, and the individual coefficient Type I error estimate (*P*). The baseline level for the different events is the feeding event of the control sharks.

Effect	***β***	**SE**	***t***	***P***
20 minutes prior to feedings				
(Intercept)	-1.301	0.122	-10.651	< 0.001
Feeding Trained Morning	-0.720	0.255	-2.826	0.005
Feeding Trained Afternoon	0.144	0.239	0.601	0.548
No Feeding Control	0.522	0.220	2.377	0.017
No Feeding Trained	0.393	0.226	1.738	0.082
Tides	0.013	0.036	0.373	0.709
Feeding Trained Morning * Tides	0.150	0.089	1.691	0.091
Feeding Trained Afternoon * Tides	-0.144	0.076	-1.895	0.058
No Feeding Control * Tides	-0.247	0.069	-3.582	< 0.001
No Feeding Trained * Tides	-0.211	0.069	-3.047	0.002
10 minutes prior to feedings				
(Intercept)	-1.826	0.151	-12.073	< 0.001
Feeding Trained Morning	0.596	0.255	2.336	0.019
Feeding Trained Afternoon	0.878	0.233	3.769	< 0.001
No Feeding Control	1.189	0.221	5.372	< 0.001
No Feeding Trained	1.688	0.244	6.921	< 0.001
Tides	0.105	0.040	2.622	0.009
Feeding Trained Morning * Tides	-0.153	0.082	-1.870	0.061
Feeding Trained Afternoon * Tides	-0.301	0.066	-4.552	< 0.001
No Feeding Control * Tides	-0.329	0.069	-4.748	< 0.001
No Feeding Trained * Tides	-0.517	0.069	-7.498	< 0.001
3 minutes prior to feedings				
(Intercept)	-0.88016	0.13994	-6.290	< 0.001
Feeding Trained Morning	-1.19578	0.24742	-4.833	< 0.001
Feeding Trained Afternoon	0.49463	0.24616	2.009	0.04451
No Feeding Control	0.66100	0.22261	2.969	0.00299
No Feeding Trained	0.35632	0.23869	1.493	0.13550

Tides	-0.11097	0.03553	-3.123	0.00179
Feeding Trained Morning * Tides	0.34332	0.07726	4.444	< 0.001
Feeding Trained Afternoon * Tides	-0.11648	0.07064	-1.649	0.09919
No Feeding Control * Tides	-0.30354	0.07087	-4.283	< 0.001
No Feeding Trained * Tides	-0.17110	0.06606	-2.590	0.00959

Table A4.2: Estimated parametric *Event* * *Tides* effect coefficients (β), assessing the distance from the feeding location and their standard errors (SE), *z*-values, and the individual coefficient Type I error estimate (*P*). The baseline level for the different events is the feeding event of the control sharks.

Effect	**β**	**SE**	***t***	***P***
20 minutes prior to feedings				
(Intercept)	4.732	0.132	35.736	< 0.001
Feeding Trained Morning	0.482	0.223	2.163	0.031
Feeding Trained Afternoon	-0.235	0.250	-0.943	0.346
No Feeding Control	-0.715	0.216	-3.309	< 0.001
No Feeding Trained	-0.476	0.229	-2.085	0.037
Tides	0.009	0.037	0.245	0.807
Feeding Trained Morning * Tides	-0.142	0.074	-1.933	0.053
Feeding Trained Afternoon * Tides	0.208	0.076	2.734	0.006
No Feeding Control * Tides	0.296	0.067	4.394	< 0.001
No Feeding Trained * Tides	0.279	0.067	4.181	< 0.001
10 minutes prior to feedings				
(Intercept)	5.311	0.167	31.803	< 0.001
Feeding Trained Morning	-0.086	0.265	-0.323	0.747
Feeding Trained Afternoon	-0.197	0.287	-0.686	0.492
No Feeding Control	-1.526	0.216	-7.053	< 0.001
No Feeding Trained	-1.424	0.268	-5.318	< 0.001
Tides	-0.097	0.037	-2.632	0.008
Feeding Trained Morning * Tides	-0.058	0.073	-0.798	0.425
Feeding Trained Afternoon * Tides	0.087	0.076	1.148	0.251
No Feeding Control * Tides	0.441	0.067	6.547	< 0.001
No Feeding Trained * Tides	0.441	0.066	6.715	< 0.001
3 minutes prior to feedings				
(Intercept)	4.115	0.168	24.443	< 0.001
Feeding Trained Morning	1.383	0.259	5.345	< 0.001
Feeding Trained Afternoon	-0.744	0.282	-2.633	0.008
No Feeding Control	0.201	0.206	0.977	0.329
No Feeding Trained	0.096	0.267	0.361	0.718
Tides	0.114	0.036	3.184	0.001

Feeding Trained Morning * Tides	-0.454	0.067	-6.753	< 0.001
Feeding Trained Afternoon * Tides	0.135	0.072	1.882	0.060
No Feeding Control * Tides	0.121	0.064	1.885	0.059
No Feeding Trained * Tides	0.147	0.063	2.333	0.020

Table A4.3: Estimated *Day* and *Event* effect coefficients (*β*), assessing the KDE-95 and KDE-50 ranges and their standard errors (SE), *z*-values, and the individual coefficient Type I error estimate (*P*). The baseline for the factor *Event* was Feeding Control.

KDE-95

Effect	***β***	**SE**	***z***	***P***
20 minutes prior to feedings				
Intercept	7.070	0.0845	83.66	<0.001
Event (Feeding Trained)	-0.210	0.120	-1.76	0.0791
Event (No Feeding Control)	0.109	0.124	0.88	0.380
Event (No Feeding Trained)	0.0884	0.139	0.63	0.526
10 minutes prior to feedings				
Intercept	7.170	0.127	56.24	<0.001
Day	-0.00291	0.00359	-0.81	0.418
Event (Feeding Trained)	-0.298	0.131	-2.27	0.023
Event (No Feeding Control)	0.0801	0.126	0.63	0.526
Event (No Feeding Trained)	0.0733	0.150	0.49	0.625
3 minutes prior to feedings				
Intercept	7.173	0.124	57.79	<0.001
Day	-0.00722	0.00382	-1.89	0.0586
Event (Feeding Trained)	-0.640	0.116	-5.50	<0.001
Event (No Feeding Control)	0.0807	0.134	0.60	0.548
Event (No Feeding Trained)	-0.121	0.140	-0.86	0.388

KDE-50

Effect	***β***	**SE**	***z***	***P***
20 minutes prior to feedings				
Intercept	3.700	0.120	30.72	<0.001
Day	0.00487	0.00365	1.33	0.183
Event (Feeding Trained)	-0.176	0.115	-1.53	0.127
Event (No Feeding Control)	0.135	0.128	1.05	0.29,
Event (No Feeding Trained)	0.196	0.137	1.43	0.152
10 minutes prior to feedings				
Intercept	3.841	0.0888	43.28	<0.001
Event (Feeding Trained)	-0.284	0.126	-2.26	0.0235
Event (No Feeding Control)	0.110	0.127	0.87	0.386

Event (No Feeding Trained)	0.138	0.145	0.95	0.343
3 minutes prior to feedings				
Intercept	4.307	0.0951	45.28	<0.001
Day	-0.00910	0.00296	-3.08	0.00210
Event (Feeding Trained)	-0.303	0.0879	-3.45	0.000563
Event (No Feeding Control)	0.139	0.104	1.33	0.182
Event (No Feeding Trained)	0.0692	0.106	0.65	0.515

Chapter 5

Table A5.1 Ranking of all models assessing the Burst Activity and Spatial Attraction

Model	***df***	**AIC$_c$**	**ΔAIC$_c$**	**wAIC$_c$**
Burst Activity				
Period * Time	17	6784.22	0.00	1.00
Period + Time	9	6837.05	52.83	0.00
Period	7	6901.02	116.79	0.00
Time	5	6973.35	189.13	0.00
(Null)	3	7040.76	256.53	0.00
Spatial Attraction				
Period * Time	17	3201.46	0.00	1.00
Period + Time	9	3734.01	532.54	0.00
Time	5	3803.25	601.79	0.00
Period	7	5236.55	2035.09	0.00
(Null)	3	5298.49	2097.03	0.00

AIC$_c$ = Akaike's information criterion corrected for small sample size, ΔAIC$_c$ = the difference in AIC$_c$ between the current and the top-ranked model, wAIC$_c$ = model probability. Shark ID and Day were included as random factors in all models. For models that contained an interaction, single factors were also included, but not listed in the table.

Table A5.2 Ranking of all possible models assessing the Burst Activity and Spatial Attraction around the night-time high tides

Model	***df***	**AIC$_c$**	**ΔAIC$_c$**	**wAIC$_c$**
		Burst Activity		
Period + Time	9	6737.80	0.00	0.9
Period * Time	17	6742.23	4.43	0.1
Period	7	6753.85	16.05	0.0
Time	5	6833.71	95.91	0.0
(Null)	3	6849.95	112.15	0.0
		Spatial Attraction		
Period * Time	17	3292.33	0.00	1.0
Period + Time	9	3335.21	42.88	0.0
Time	5	3374.58	82.25	0.0
Period	7	4525.02	1232.69	0.0
(Null)	3	4561.03	1268.70	0.0

AIC$_c$ = Akaike's information criterion corrected for small sample size, ΔAIC$_c$ = the difference in AIC$_c$ between the current and the top-ranked model, wAIC$_c$ = model probability. Shark ID and Day were included as random factors in all models. For models that contained an interaction, single factors were also included, but not listed separately in the table.

Table A5.3 Summary table for the models investigating the Period and Time effects on the Burst Activity and Spatial Attraction around night-time high tides

Effect	***β***	**SE**	***z***	***P***
		Burst Activity		
(Intercept)	-0.923	0.227	-4.072	<0.001
Period (feeding)	0.130	0.243	0.534	0.593
Period (recovery 30)	-0.765	0.227	-3.368	<0.001
Period (recovery 60)	-1.006	0.226	-4.449	<0.001
Period (recovery 90)	-1.401	0.253	-5.542	<0.001
Time (during)	-0.165	0.176	-0.942	0.346
Time (after)	-0.232	0.180	-1.288	0.198
Period (feeding) * Time (during)	0.0549	0.263	0.209	0.834
Period (recovery 30) * Time (during)	0.0423	0.268	0.158	0.875
Period (Recovery 60) * Time (during)	-0.174	0.272	-0.640	0.522
Period (recovery 90) * Time (during)	-0.00973	0.285	-0.034	0.973
Period (feeding) * Time (after)	-0.499	0.270	-1.850	0.064
Period (recovery 30) * Time (after)	0.0127	0.256	0.050	0.960
Period (recovery 60) * Time (after)	-0.176	0.261	-0.676	0.499
Period (recovery 90) * Time (after)	-0.0272	0.284	-0.096	0.924
Conditional R^2				0.16
		Spatial Attraction		
(Intercept)	-2.437	0.854	-2.853	0.004
Period (feeding)	0.768	1.012	0.759	0.448
Period (recovery 30)	4.044	0.953	4.243	<0.001
Period (recovery 60)	3.781	0.940	4.023	<0.001
Period (recovery 90)	-0.531	0.982	-0.541	0.589
Time (during)	-4.101	0.546	-7.516	<0.001
Time (after)	-5.029	0.572	-8.798	<0.001
Period (feeding) * Time (during)	0.137	0.713	0.193	0.847
Period (recovery 30) * Time (during)	-0.637	0.675	-0.943	0.345
Period (recovery 60) * Time (during)	1.348	0.590	2.284	0.022
Period (recovery 90) * Time (during)	0.502	0.672	0.746	0.456
Period (feeding) * Time (after)	-0.858	0.871	-0.984	0.325
Period (recovery 30) * Time (after)	-0.644	0.694	-0.927	0.354

Period (recovery 60) * Time (after)	1.977	0.603	3.280	0.001
Period (recovery 90) * Time (after)	0.924	0.656	1.408	0.159
Conditional R^2				0.86

Estimated effect coefficients (β) and their standard errors (SE), *z* values and the individual coefficient type I error estimate (*P*). Time (during) = 1 h of feeding, Time (after) = 1 h postfeeding, Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month. For the factor Time, the baseline level was the 1 h prefeeding before the feeding event.

Table A5.4 Ranking of all possible models assessing the mean daily activity and mean daily metabolic rates

Model	***df***	**AIC_c**	**ΔAIC_c**	**wAIC_c**
	Mean daily activity			
Period * Temperature	27	111285.3	0.00	1.00
Period + Temperature	23	111326.4	41.19	0.00
Temperature	19	111342.5	57.22	0.00
Period	22	111350.9	65.65	0.00
(Null)	18	111366.9	81.67	0.00
	Mean daily metabolic rates			
Period * Temperature	13	-80668.84	0.00	1.00
Period + Temperature	9	-80584.77	84.07	0.00
Temperature	5	-80473.31	195.53	0.00
Period	8	-68614.08	12054.76	0.00
(Null)	4	-68534.66	12134.18	0.00

AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top-ranked model, wAIC_c = model probability. Shark ID was included as a random factor in all models. For models that contained an interaction, single factors were also included, but not listed separately in the table.

Table A5.5 Ranking of all possible models assessing the core areas and home ranges

Model	***df***	**AIC_c**	**ΔAIC_c**	**$wAIC_c$**
Core area				
Null	3	795.68	0.00	0.93
Period	7	800.78	5.1	0.07
Home range				
Period	7	898.66	0.00	0.93
Null	3	903.77	5.1	0.07

AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top-ranked model, $wAIC_c$ = model probability. Shark ID was included as a random factor in all models.

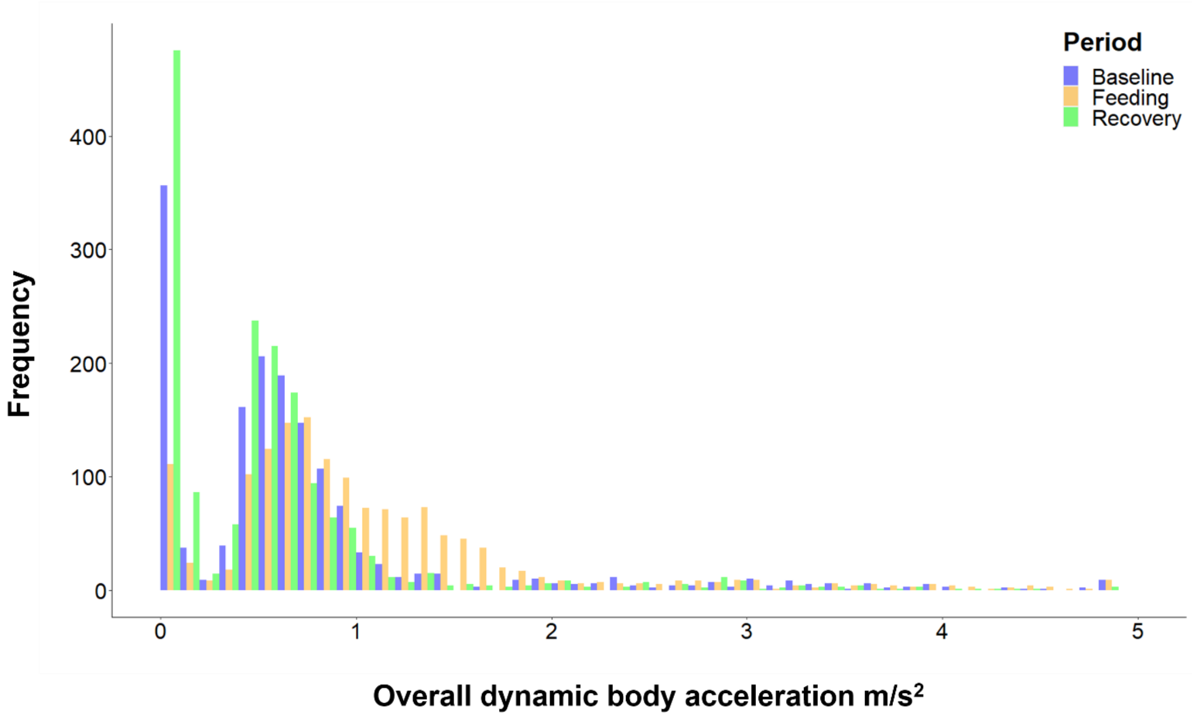

Figure A5.1 Frequency distribution of shark overall dynamic body acceleration across the baseline, feeding and combined recovery periods. The data used only included the three 1 h windows of the daytime high tides we focused on during our investigation of the anticipatory behaviour of the sharks.

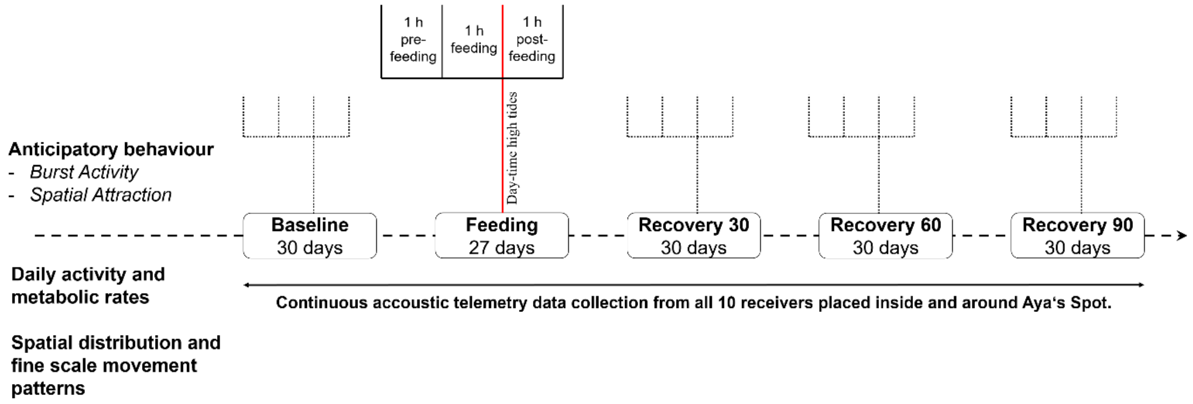

Figure A5.2 Experimental timeline, showing the length of each of the five periods and illustrating the 3 h window around the high tides.

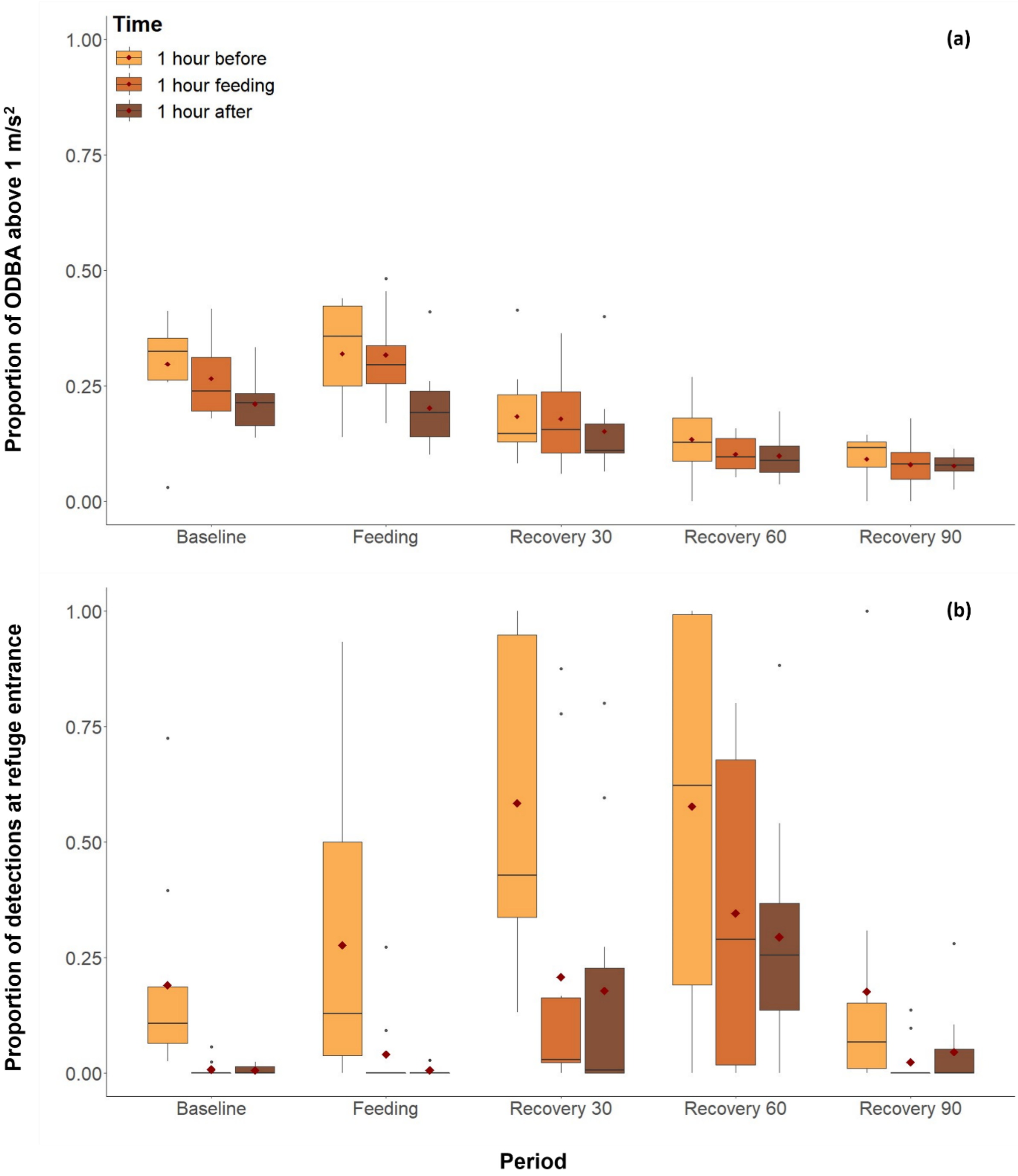

Figure A5.3 Box plot illustrating (a) the proportion of overall dynamic body acceleration (ODBA, m/s^2) above 1 m/s^2, and (b) the proportion of detections made at the refuge entrance during prefeeding, feeding and postfeeding around the night-time high tides plotted against the Period. The horizontal lines inside the boxes mark the median and the red diamonds the mean for 1 h before, during and after feeding events. The upper and lower boundaries of the box represent the third and first quartiles. The whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers.

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ORIGINAL PAPER

Effects of reward magnitude and training frequency on the learning rates and memory retention of the Port Jackson shark *Heterodontus portusjacksoni*

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Abstract

The development of adaptive responses to novel situations via learning has been demonstrated in a wide variety of animal taxa. However, knowledge on the learning abilities of one of the oldest extant vertebrate groups, Chondrichthyes, remains limited. With the increasing interest in global wildlife tourism and shark feeding operations, it is important to understand the capacities of these animals to form associations between human activities and food. We used an operant conditioning regime with a simple spatial cognitive task to investigate the effects of reinforcement frequency and reward magnitude on the learning performance and memory retention of Port Jackson sharks (*Heterodontus portusjacksoni*). Twenty-four Port Jackson sharks were assigned one of four treatments differing in reward magnitude and reinforcement frequency (large magnitude–high frequency; large magnitude–low frequency; small magnitude–high frequency; small magnitude–low frequency). The sharks were trained over a 21-day period to compare the number of days that it took to learn to pass an assigned door to feed. Sharks trained at a high reinforcement frequency demonstrated faster learning rates and a higher number of passes through the correct door at the end of the trials, while reward magnitude had limited effects on learning rate. This suggests that a reduction in reinforcement frequency during tourism-related feeding operations is likely to be more effective in reducing the risk of sharks making associations with food than limiting the amount of food provided.

Keywords Cognition · Conditioning · Elasmobranchs · Management · Tourism

Introduction

Learning has been demonstrated in a wide variety of animal taxa, including invertebrates (e.g., Schatz et al. 1994; Kawecki 2010; Srinivasan 2010) and vertebrates (e.g., Gruber and Schneiderman, 1975; Brown et al. 2008; Taylor et al. 2010; Fuss et al. 2014a). The ability to learn enables individuals to develop adaptive responses to changing conditions and novel situations within an individual's lifetime. This is particularly important for animals living in highly fluctuating and complex environments, such as fishes living in some coastal areas (Dill 1983; Dodson 1988). The learning abilities in fishes range from complex spatial learning tasks to object discrimination, and recognition of individuals of the same and different species (Brown et al. 2008; Brown 2015). However, knowledge on the learning capacities of elasmobranchs remains limited. Early studies demonstrated the ability of sharks to learn (Gruber and Schneiderman 1975), with recent studies, showing that elasmobranchs are capable of solving spatial cognitive tasks, recognising and discriminating between objects (Aronson et al. 1967; Graeber and Ebbesson 1972; Fuss et al. 2014b), and use different orientation strategies and spatial memory systems to navigate during long- and short-distance migrations (O'Gower 1995; Edrén and Gruber 2005; Schluessel and Bleckmann

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2005, 2012; Meyer et al. 2010; Papastamatiou et al. 2011). Furthermore, sharks can associate artificial sounds with food rewards (Pouca and Brown 2018), discriminate different quantities (Pouca et al. 2019), and engage in social learning (Guttridge et al. 2013; Pouca et al. 2020). Previous studies have shown that learning rate can be influenced by reinforcement frequency, i.e., the number of rewarded trials per training session (Lauer and Estes 1955; Kerpelman and Himmelfarb 1971; van den Akker et al. 2014) and reward magnitude, i.e., the size of the reward per trial (Neuringer 1967; Gonzalez et al. 1974; Muzio et al. 1992). However, despite the growing body of knowledge on the cognitive abilities of elasmobranchs, the factors that influence the rate of learning in this taxonomic group are yet to be investigated.

Wildlife tourism is considered one of the fastest growing sectors of the tourism industry (Scheyvens 1999), generating billions of dollars annually globally (Corcoran et al. 2013). Many of the targeted species, including large marine predators, are difficult to observe due to their shy and elusive nature (Bres 1993; Burgess 1998). These animals are often fed to maximise interactions with humans and produce reliable and good viewing opportunities. This increasingly popular practice is known as provisioning and it has been subject to scrutiny in recent decades (Orams 2002; Newsome et al. 2004). The deliberate feeding of large predators is suspected to lead to detrimental effects on the target animals, their environments, and humans (Dobson 2006; Newsome and Rodger 2008; Hammerschlag et al. 2012). These effects can range from decreased physiological condition to behavioural alterations that could cause cascading effects throughout the marine ecosystem, or increase the risk for humans, resulting in injuries due the learnt association between humans and food (Orams 2002; Huveneers et al. 2013; Gallagher and Huveneers 2018).

Due to these potential risks, a few regions have banned shark feeding (e.g., New Caledonia, Florida, Hawaii) (FFWCC 2002; Techera 2012; Johansen 2013). A bill was also introduced into the US Congress in 2016 (3099 "Access to Sportfishing Act of 2016") that would render shark feedings illegal in all United States federal waters (Nelson 2016). Currently, there are a few individual states, e.g., Florida (FFWCC 2002) and Hawaii (Techera 2012), that have already banned shark feeding operations in federal waters. In the case of the white shark (*Carcharodon carcharias*) cage-diving industry, the use of bait and berley is strictly regulated in all locations where cage-diving occurs (Bruce and Tasmania 2015). However, these regulations differ between locations in terms of the amounts of bait and berley that can be used. In California, baiting and the use of berley to attract white shark are prohibited. In South Africa, bait and berley have a maximum daily limit of 25 kg, whereas in South Australia, operators have a fortnight limit of 1000 kg. To further strengthen the incentive to remove the bait before the shark can take it, South Australia also introduced a 15-min penalty during which no bait or berley can be used when a shark takes the bait. Compliance to regulations and limits is monitored and legislated by the South Australian Department for Environment and Water (DEW) using a logbook system and remote camera placed on one of the islands. While not stipulated in policy documents, limits on the amount of food-based attractant and number of baits used minimise the risk of learning or making an association between humans and food while interacting with cage-diving vessels. While associative learning can be influenced by reward frequency or magnitude in a range of taxa (Neuringer 1967; Kerpelman and Himmelfarb 1971; Muzio et al. 1992; van den Akker et al. 2014), it is currently unknown whether the number of baits or size of baits (i.e., amount of food) most affect the rate at which sharks learn or which should be regulated to reduce potential associations being made.

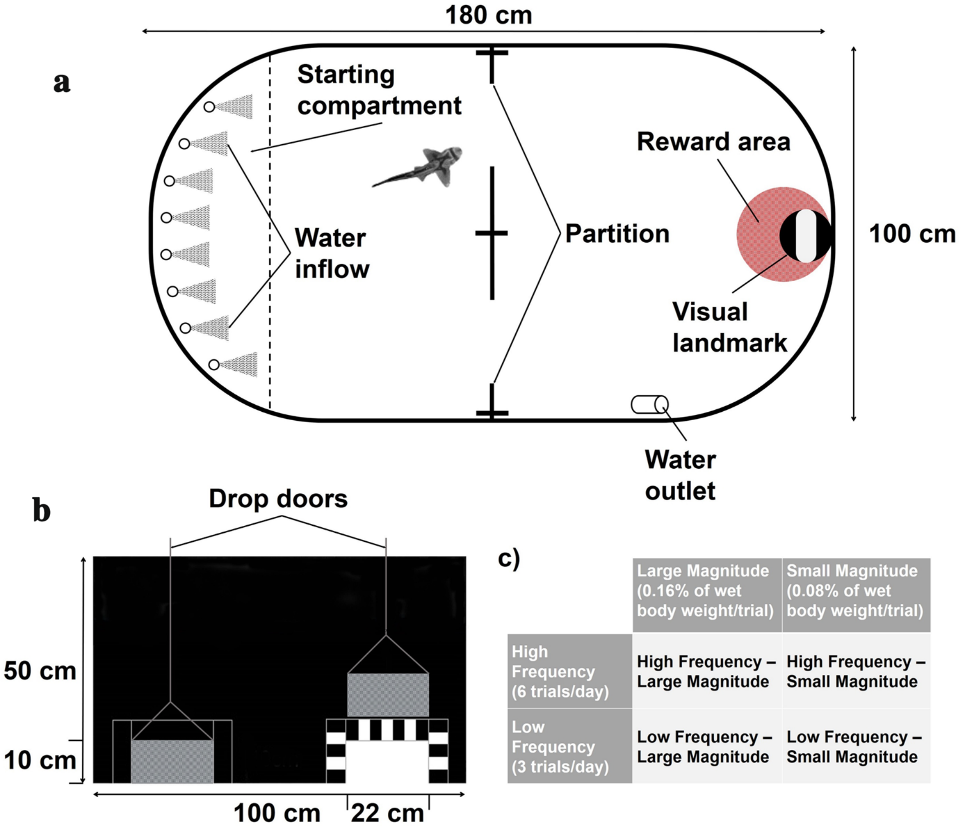
Here, we investigate the effect of reinforcement frequency and reward magnitude on the rate of learning in the benthic Port Jackson shark (*Heterodontus portusjacksoni*). The experiment consisted of a simple operant conditioning regime based on a spatial task in a controlled laboratory environment. We hypothesised that sharks subjected to higher reinforcement frequencies and larger reward magnitudes would learn the given task at a significantly faster rate than those trained at a low frequency and a small reward.

Methods

Port Jackson sharks (*H. portusjacksoni*) are benthic elasmobranchs that are endemic to the temperate waters of Australia. Its small size, hardiness, and high abundance along the New South Wales coast make it a suitable model species for shark-related research performed under controlled laboratory conditions (Powter and Gladstone 2009; Byrnes and Brown 2016; Byrnes et al. 2016a). Moreover, the logistical difficulties holding and conducting experiments on species typically targeted by shark-diving tourism in captivity are prohibitive. Twenty-four Port Jackson shark eggs were opportunistically collected from rocky reef substrates by hand in Jervis Bay. They were transported to the Sydney Institute for Marine Science (SIMS, Mosman, NSW) where they were housed in four 500 L aquaria. Upon hatching, sharks were moved into three 1000 L aquaria where they were kept for the duration of the experiments. All tanks were continuously supplied with clean, aerated seawater from Chowder Bay, Sydney, NSW, at ambient temperatures (23.25 C° ± 2.75 C°) and received a natural Sydney photoperiod. Shelters, consisting of pieces of 100 mm PVC pipe and clay pots, were provided in each holding tank. Under the initial husbandry conditions, *H. portusjacksoni* were fed approximately 6% of their body mass in food three times per week with defrosted squid *Loligo opalescens* and *Nototodarus sloanii*, whiteleg shrimp *Litopenaeus vannamei*, and Basa fish *Pangasius bocourti*.

The total length of each individual was measured to the nearest 5 mm and the body mass was recorded to the nearest gram using a digital balance. Sex was determined by noting the presence of claspers in males. Each shark was sub-dermally implanted with a PIT tag at the base of the first dorsal fin to allow for easy identification. Sharks were assigned to one of four treatment groups, high frequency–large magnitude, high frequency–small magnitude, low frequency–large magnitude, and low frequency–small magnitude. We used pseudo-randomisation when assigning the sharks to the treatment groups to ensure an equal sex and mass distribution (one-way ANOVA for mass: $F_3 = 0.288$, $p = 0.834$). The frequency of the treatment groups represents the number of trials a shark received per day (high frequency = 6, low frequency = 3) and the magnitude represents the size of the reward each shark received upon completion of a trial (large = 0.16% and small = 0.08% of wet body weight equivalent). The reward magnitudes were small enough to ensure a strong feeding motivation during all trials. To further ensure equal feeding motivation across all treatments, each shark was fed a total of 2% wet body mass equivalent per day (Sims 1996). This included the rewards fed during the trials and the remaining 1–1.5% body mass depending on the treatment, which were fed after the completion of the daily training.

Experiments were carried out in an oval experimental arena measuring 180 × 100 cm with a water depth of 40 cm. The length of the arena was divided into two equally sized areas using a partition. Two equally sized doors were cut out of the wall, and two sheets of clear Perspex were used as guillotine doors to close the openings. Around the door frames, 5-cm-wide stripes of laminated paper showing two different patterns (black on the left-side door and black/white squares on the right-side door) were glued to the wall to assist sharks in distinguishing the two doors (Fig. 1). A partition made of black mesh was used to close off the starting area. On the opposite side of the tank, a black disc with a single white stripe in the middle was located behind the reward area to serve as a visual landmark to assist sharks in locating the food reward. To account for a potential side bias of the sharks they were assigned to either the right-hand or the left-hand door. This assignment was done using pseudo-randomisation to ensure that within each of the four treatment groups, three sharks were trained to the left and three sharks were trained to the right-hand door. Water inflow was provided from the side that held the starting compartment. This prevented the olfactory cues from being carried downstream toward the sharks inside the starting compartment,



	Large Magnitude (0.16% of wet body weight/trial)	Small Magnitude (0.08% of wet body weight/trial)
High Frequency (6 trials/day)	High Frequency – Large Magnitude	High Frequency – Small Magnitude
Low Frequency (3 trials/day)	Low Frequency – Large Magnitude	Low Frequency – Small Magnitude

Fig. 1 Schematic illustration of **a** a birds-eye view of the tank, and **b** a frontal view of the partitioning with the left-hand door displayed as being closed and the right-hand door being open, and **c** a table summarising the four different treatment groups (large-reward groups received 0.16% and small-reward groups received 0.08% of wet body mass per trial; high-frequency groups received 6 and low-frequency groups received 3 trials per day)

eliminating the possibility of sharks locating the food reward by smell rather than learning the association with the door.

Acclimation phase

Sharks were provided with a period of 3 days to acclimate to the experimental arena and the transfer between the holding and experimental tank. On each of the 3 acclimation days, sharks were transferred individually to the experimental basin, using a bucket filled with seawater, where they were free to explore the arena with both doors open for 15 min. The sharks were then fed the equivalent of 2% of their wet body mass in small pieces of squid using tongs. After a total of 30 min, the sharks were transferred back to their holding tanks.

Pre-training phase

Pre-training trials were run on days 1–10. Training occurred on 7 days a week and consisted of a single training session per day. The number of trials per day depended on the treatment group a shark was assigned to. Low-frequency training consisted of three trials per day and high-frequency training of six trials per day. Prior to the first trial, sharks were provided with 5 min of acclimation with both doors closed. Sharks were then gently ushered back to the starting area and provided with 30–60 s rest before the first trial commenced. At the start of the first trial, the treatment door was opened, whilst the control door was kept closed. The partition closing off the starting area was then removed, and the food was introduced on the opposite side of the door. The location of the food reward was moved further away from the door each day during the pre-training phase. At the end of the pre-training phase, the reward location had reached the opposite end of the tank (Fig. 1) where it remained throughout the training phase.

Following the removal of the start partition, sharks were provided 90 s to pass through the door before the trial was terminated and recorded as unsuccessful. When sharks passed the treatment door within 90 s, they were given another 30 s to consume the food reward. If the shark did not attempt to consume the reward within 30 s, the trial was terminated and recorded as a correct pass. The time limit of 30 s was used to ensure that sharks could make a link between the food reward and passing the correct door. At the end of a trial, the shark was gently ushered back to the starting area and provided 30–60 s of rest before the start of the next trial. Following the last trial, sharks were given 3–6 min of rest with both doors closed before being ushered back to the starting area where they were fed any left-over rewards and the remainder of the 2% wet body mass equivalent in food.

Training phase

The training phase occurred from days 10 to 21. The treatment protocol remained the same, but both doors were opened during the trials and the food reward was provided at the designated reward location (Fig. 1a). The number of daily trials depended on a shark's treatment group (low frequency = 3 trials; high frequency = 6 trials). An individual was considered to have learnt the task successfully once it passed through the treatment door within 90 s during 9 out of 12 consecutive trials (0.75 success rate).

Data analyses

All trials were video recorded and trial statistics were collected by one observer using video playback on VLC media player. It was not possible to record data blind, because our individuals have uniquely identifying markings. The response variables included: (i) pass rate, (ii) approach index, (iii) latency to pass the correct door, and (iv) success rate (Table 1). In cases when sharks did not pass any of the doors during the 90 s provided, we assigned a ceiling score of 270 s to the latency to pass variable, which was three times the maximum trial time. The pre-training phase was omitted from the learning analyses.

For each of the four response variables, Generalised Linear Mixed Models (GLMMs) were used to test the effects

Table 1 List of response variables used in the learning task and the corresponding distribution used for the GLMM

Variable	Definition	Distribution
Pass rate	Proportion of trials sharks passed through either door within a session. Pass rate ranged between 0 (never passed a door) and 1 (passed a door on all trials). A shark was considered to have passed a door when its head and pectoral fins had crossed the door	Binomial
Approach index	Percentage of approaches made toward the correct door ([number of correct approaches/total number of approaches] × 100). An approach was defined as a direct swim toward the door within one body length of the door	Gaussian
Latency to pass	Time taken for sharks to pass the correct door	Negative binomial
Success rate	Proportion of trials sharks passed through the correct door within a session. Success rate ranged between 0 (never passed the correct door) and 1 (passed the correct door on all trials)	binomial

of reinforcement frequency and reward magnitude on the learning performance of juvenile Port Jackson sharks. Explanatory variables included reinforcement frequency, reward magnitude, experimental day, treatment side (left or right), and the interaction between the reinforcement frequency, reward magnitude, and experimental day. Given the repeated measurements of individuals, individual sharks were included in the model as the random effect nested within holding tank. The error structure of GLMM corrects for non-independence of statistical units due to shared temporal structure and permits the random effect variance explained at different levels of clustering to be decomposed. We determined the most appropriate statistical family and error distribution for each analysis by examining the distribution of the response variables and visually inspecting the residuals for the saturated models in accordance with Zuur et al. (2010) (Table 1). We ran all models for all possible combinations of factors, and compared their relative probability using Akaike's information criterion corrected for small sample size (AIC_c) (Burnham and Anderson 2002). To identify the most influential drivers of shark learning behaviour, a dredge function (R package MuMIn; Barton 2019) was used to identify more-parsimonious nested models according to the AIC_c. A 'confidence set' of models with $\Delta AIC < 2$ were considered equivalent and included in model averaging (Burnham et al. 2011); from which the Relative Variable Importance values (RVI; calculated from the sum of AIC weights of models within the confidence set in which the parameter of interest appears) were used to identify important variables. All analyses were performed in R v.3.4.3 (R Core Team 2013) using the package glmmTMB (Wood et al. 2017). Graphs were created in R v.3.4.3 (R Core Team 2013) using the packages ggplot2 (Wickham 2016) and plotly (Sievert et al. 2017).

Results

The mean number of sessions required to reach the learning criterion (LC) ranged from 13.33 ± 0.88 in the low frequency–small magnitude, and 13.75 ± 1.44 in the high frequency–large magnitude group to 16.40 ± 0.75 in the low frequency–large magnitude, and 17.6 ± 0.81 in the high frequency–small magnitude group (Fig. 2). Neither frequency (ANOVA, $F = 0.149$, $p = 0.706$) nor magnitude (ANOVA, $F = 0.350$, $p = 0.564$) had a significant effect on the number of training sessions needed to reach LC. However, not all tested individuals reached the learning criteria within the provided 21 days of training (Fig. 2). Though the mean number of sessions required to reach the LC was highest in the high frequency–small magnitude group (17.6 ± 0.81), it also had the highest number of sharks to successfully reach the LC (5 out of 6) together with the low frequency–large magnitude group (Fig. 2). The performance of Port Jackson sharks with respect to the behavioural traits which we investigated was significantly influenced by reinforcement frequency and reward magnitude (Figs. 3, 4). However, reinforcement frequency had a stronger effect on the learning rate of sharks (Figs. 3, 4).

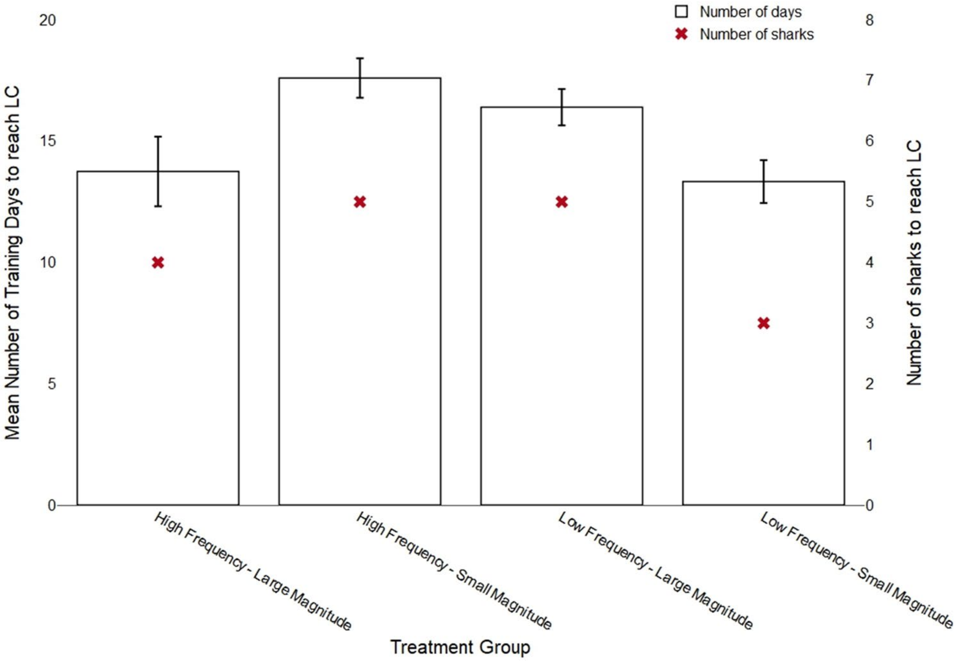


Fig. 2 The mean number of training days (bars) ± standard error which it took sharks to reach the learning criteria (LC). Red crosses represent the number of sharks that reached LC within the 21 days of the experiment. High-frequency–small-reward sharks took the longest to reach LC, but had the most individuals reaching LC within 21 days, together with the low-frequency–large-reward sharks (color figure online)

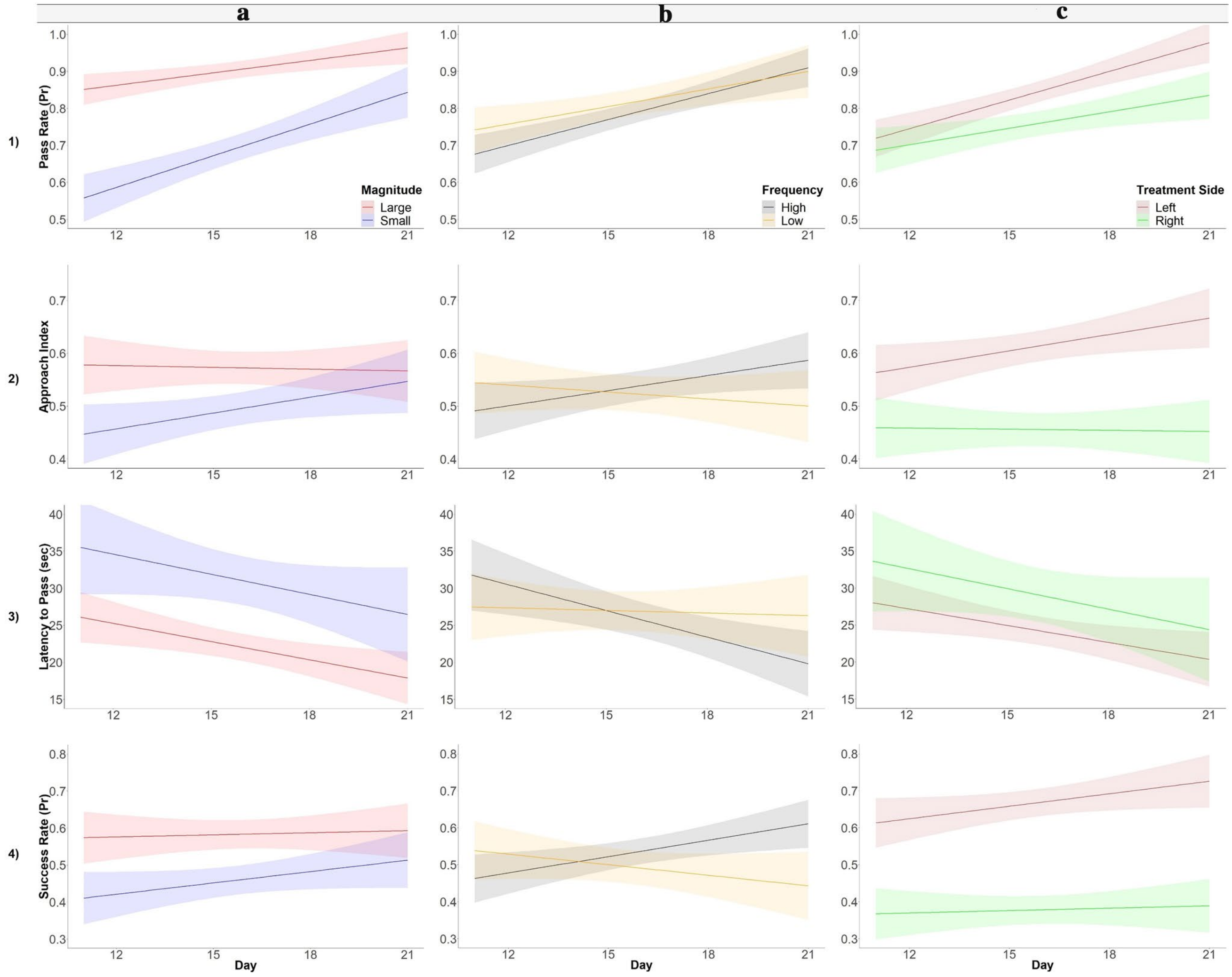


Fig. 3 Effects of reward magnitude (left), reinforcement frequency (centre), and treatment side (right) on (1) pass rate, (2) approach index, (3) latency to pass the correct door, and (4) success rate through time with 95% confidence bands. High-frequency sharks learnt at a faster rate with regards to the approach index and the success rate. Reward magnitude only affected the approach index with small-reward sharks learning at a faster rate

Pass rate

When looking at the proportion of trials that resulted in sharks passing either door, the RVI scores for the variables included in the model averaging ranged from 0.22 (day*magnitude) to 1.00 (day, magnitude, Table 2). Pass rate increased throughout the experiment by up to ~ 25% during the 10-day training period (Fig. 3). Individuals that received large rewards displayed a consistently higher pass rate throughout the experiment than individuals that received small rewards ($p = 0.0084$) (Fig. 3, Online Resource 1). However, the interaction between the reward magnitude and the experimental day had no significant effect on the pass rate ($p = 0.46$). The lack of any frequency effect and the non-significant effect of the magnitude*day interaction indicates that neither frequency nor magnitude had a significant impact on the learning rate of sharks with respect to the pass rate. Furthermore, sharks trained to pass the left door performed slightly better than those trained to pass through the right door ($p = 0.10$) (Fig. 3).

Approach index

When testing the effects of reward magnitude and reinforcement frequency on the approach index, the RVI scores range from 0.09 (frequency*magnitude) to 1.00 (side, Table 2). While sharks that received a small reward increased in performance, individuals trained with a large reward had a

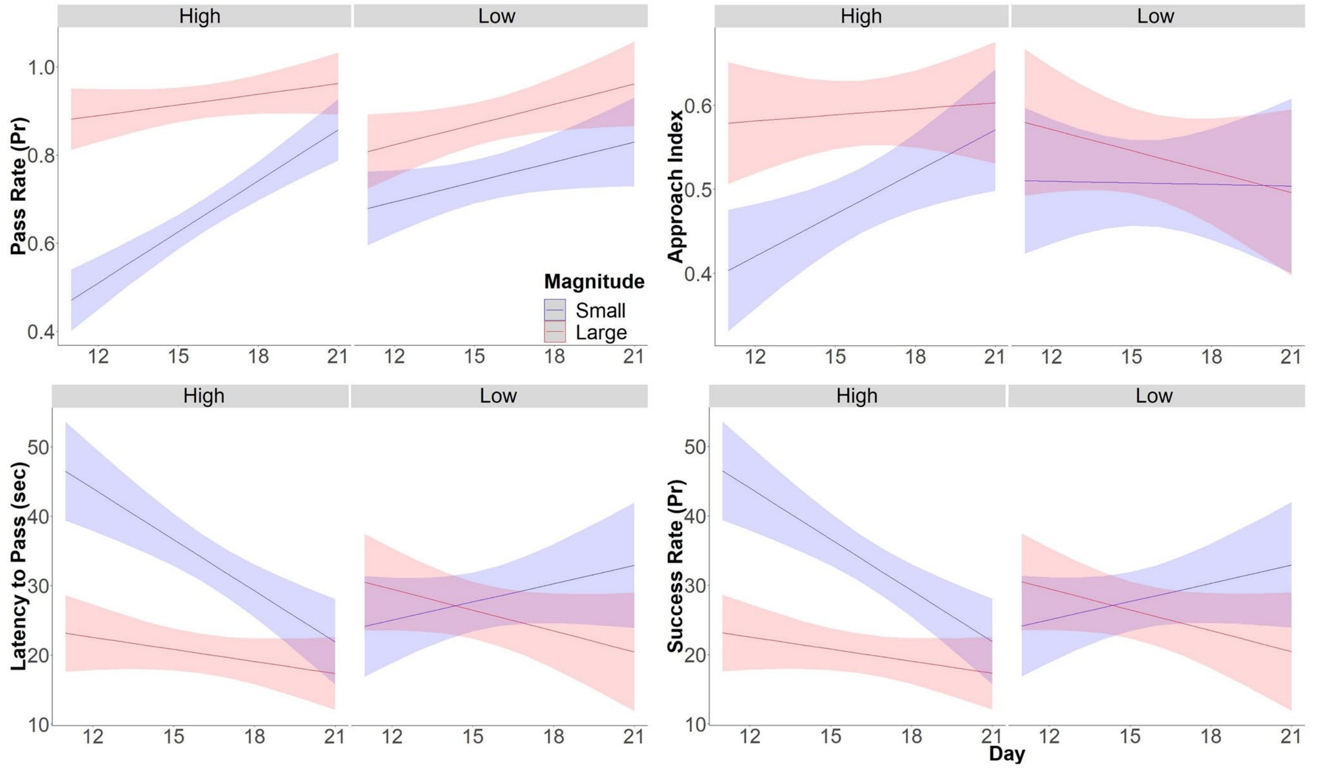


Fig. 4 Interaction effect between the reinforcement frequency (left: high, right: low), the reward magnitude (blue: small, red: large), and the experimental day on **a** pass rate, **b** approach index, **c** latency to pass the correct door, and **d** success rate with 95% confidence bands. Reinforcement frequency and reward magnitude had a significant interaction effect on the latency to pass with large-reward sharks learning significantly faster when subjected to a low reinforcement frequency (color figure online)

Table 2 The relative importance values for all explanatory variables

	Pass rate	Approach index	Latency to pass	Success rate
Day	1.00	0.85	1.00	1.00
Magnitude	1.00	0.78	1.00	0.84
Side	0.68	1.00	0.38	1.00
Frequency	0*	0.50	1.00	1.00
Day*Magn	0.22	0.51	1.00	0.24
Day*Freq	0*	0.43	1.00	1.00
Freq*Magn	0*	0.09	1.00	0.21
Day*Freq*Magn	0*	0*	1.00	0*

Values marked with * indicate that they were not included in the top-ranking models ($\Delta AIC_c < 2.0$) and, as a result, they were not part of the model averaging

relatively consistent approach index throughout the experiment ($p = 0.064$) (Fig. 3). In contrast, individuals trained at a high reinforcement frequency demonstrated an increasingly higher approach index, whereas the approach index decreased in sharks trained less frequently ($p = 0.047$) (Fig. 3, Online Resource 2). Treatment side also had a significant impact, with individuals trained to pass through the left door performing better than those trained to the right door ($p = 0.0015$) (Fig. 3, Table 2, Online Resource 2).

Latency to pass correct door

The latency to pass through the correct door was significantly affected by the three-way interaction between the reinforcement frequency, the reward magnitude, and the experimental day ($p = 0.0030$) (Table 2, Online Resource 3). When trained at a high reinforcement frequency, latency to pass the correct door decreased more rapidly in individuals that received small rewards than those receiving large rewards ($p = 0.0030$) (Fig. 4). In contrast, when trained at a low reinforcement frequency, sharks that received small rewards took progressively longer to pass the correct door, whereas sharks that received large rewards passed the correct door faster over time ($p = 0.0030$) (Fig. 4).

Success rate

The RVIs of the variables included in the top-ranked models (ΔAICc < 2, Online Resource 4) ranged from 0.21 (frequency*magnitude) to 1.00 (day*frequency, side, Table 2). Sharks trained at a high frequency performed increasingly better; whereas the proportion of correct passes decreased over time in sharks that were trained at a low frequency ($p = 0.0078$) (Fig. 3). Reward magnitude had no significant effect on the success rate of the sharks ($p = 0.14$). However, sharks trained to the left door performed significantly better than those trained to the right door ($p < 0.001$) (Fig. 3, Table 2, Online Resource 4).

Discussion

Our study revealed that reinforcement frequency affects the rate at which Port Jackson sharks learnt aspects of a spatial cognitive task in an operant conditioning regime. The effect of reinforcement frequency varied depending on the approach index, latency to pass, and success rate, but not the pass rate. In contrast, reward magnitude had little effect on the rate at which sharks improved with regards to the four behavioural traits assayed. Still, the overall performance was found to be consistently higher in sharks trained with a large reward. The results were influenced by lateralisation, with individuals trained to pass the left door performing better than those trained to pass the right door.

Port Jackson sharks learnt the spatial cognitive task in this study within a 13–18 sessions (or 40–106 trials) depending on the treatment group. This is similar to the 15 sessions taken by grey bamboo sharks (*C. griseum*) and coral cat sharks (*Atelomycterus. marmoratus*) to learn to locate a fixed goal position in a diamond-shaped maze from two different starting positions (Fuss et al. 2014a). Grey bamboo sharks and Port Jackson sharks also took a similar amount of time (18 sessions) to learn other cognitive tasks such as visual discrimination and association of two time-separated events respectively (Guttridge and Brown 2013; Fuss et al. 2014b). Besides sharks, learning has also been investigated in other elasmobranchs. The ocellate river stingray, (*Potamotrygon motoro*) for instance, learnt a spatial cognitive task within 17 session at a frequency of ten trials per session (Schluessel and Ober 2018) and the yellow stingray (*Urobatis jamaicensis*) learnt to discriminate magnetic stimuli within 13 session with four trials per session (Newton and Kajiura 2017). This suggests that, when trained daily, sharks and rays can learn a range of spatial and visual tasks relatively quickly. In comparison, teleost fish appear to outperform elasmobranchs on similar tasks. For instance, when trained at a frequency of 25 trials per day, goldfish (*Carassius auratus*) achieve a learning criterion of 80% successful trials when subjected to a similar spatial cognitive task within 3–4 days (Portavella and Vargas 2005). Three spined sicklebacks (*Gasterosteus aculeatus*) required 5–10 trials to reach the learning criterion (three successful consecutive trials) when trained at lower frequencies (one trial every 36–48 h) (Girvan and Braithwaite 1998). However, Fuss et al. (2018) recently carried out an object discrimination experiment comparing the cognitive abilities of juvenile grey bamboo sharks (*Chiloscyllium griseum*) and Malawi cichlids (*Pseudotropheus zebra*). They demonstrated that the sharks reached the LC within 15 sessions, whereas the cichlids required 32 sessions on average. Furthermore, sharks were able to apply previously gained knowledge to novel stimuli and use abstract relational concepts to categorise these novel objects. In contrast, cichlids failed to apply both concepts simultaneously.

Reinforcement frequency

Our findings show that sharks learnt faster when trained at a higher reinforcement frequency with regards to the approach index and the success rate. However, the pass rate, hence, the general association between passing the partition and receiving a food reward, was unaffected by the reinforcement frequency. Low reinforcement frequency also increased latency to pass when combined to small-reward size. Our findings are supported by the previous studies showing positive correlations between reinforcement frequency and performance during the acquisition of a task or behaviour (Gonzalez and Bitterman 1967; Muzio et al. 1992; Devan et al. 2003). Many of these studies, however, compared continuous (every trial is rewarded) to partial (only a certain percentage of trials is rewarded) reinforcement (Kerpelman and Himmelfarb 1971; Muzio et al. 1992; Bouton et al. 2014) and tested the active role of inhibition or frustration in addition to the passive role of nonreinforcement (Amsel 1958, 1962). Nonetheless, our findings show a positive correlation between reinforcement frequency and learning rates similar to previous studies on Argentine common toads (*Bufo arenarum*) and Fancy rats (*Rattus norvegicus domestica*) that were subjected to spatial cognitive tasks (Muzio et al. 1992; Devan et al. 2003), and goldfish (*Carassius auratus*) that were trained to press a target to gain access to a food reward (Gonzalez and Bitterman 1967).

Reward magnitude

The elevated performance (i.e., higher number of correct passes) in individuals that received a large reward was evident from the beginning of the experiments, suggesting that it might be due to individual variability rather than the effect of reward magnitude. Our findings showing that learning rate was not affected by reward magnitude contradict previous studies. For example, goldfish (*Carassius auratus*) and Argentine common toads (*Rhinella arenarum*) exhibited a positive correlation between reward magnitude and the rate at which they learnt a given task (Gonzalez et al. 1972; Muzio et al. 1992).

The lack of correlation observed in our study could be due to the size of the food rewards. Although the amount of food doubled between the low- and high-reward magnitude, the sizes of the rewards were small due to the number of trials per day and the need to maintain a consistent feeding motivation to the food stimulus during all trials. Therefore, we limited the amount of food provided during the trials to a total of 1% wet body weight equivalent. With the juvenile Port Jackson sharks only weighing ~ 175 g, the actual difference in the reward magnitude was ~ 0.2 g per trial, which may have been too small for sharks to learn at a different pace.

Treatment side and lateralisation

Brain lateralisation has recently been demonstrated in both juvenile and adult Port Jackson sharks (Byrnes et al. 2016a, b; Vila Pouca et al. 2018; Pouca et al. 2019), and it could explain the significant effects of treatment side that we observed during our study. While the ecological benefit of lateralisation has not been demonstrated in elasmobranch, cerebral and behavioural lateralisation are widespread and have been suggested to offer many selective advantages. For example, strongly lateralised fish perform simultaneous tasks more efficiently than non-lateralised individuals (Dadda and Bisazza 2006), enhance predator recognition learning (Chivers et al. 2017), and spatial reorientation (Sovrano et al. 2005), all of which likely to increase survival, especially in predator-rich environments (Stier et al. 2013; Ferrari et al. 2015). Since we could not test the laterality strength of the Port Jackson sharks tested, it was not possible to determine if performance was affected by the shark's lateralisation, other than noticing the strong bias towards the left door.

Implications to wildlife tourism

With the increasing popularity of shark-diving tourism, we are in need of management strategies that will ensure the sustainability of the industry (Gallagher and Huveneers 2018). The use of bait to attract sharks is a popular method to ensure reliable encounters with these elusive predators (Clua and Séret 2010). However, these practices can affect the behaviour of the targeted species (Huveneers et al. 2013), putting them at risk of getting caught in an ecological trap (Schlaepfer et al. 2002). Ecological traps occur when the habitat choices of organisms are negatively affected by cues that would normally be associated with habitat quality (Schlaepfer et al. 2002). For instance, the artificial feedings of southern stingrays (*Dasyatis americana*) off the Cayman Islands, Bahamas have caused these animals to change from a solitary to a group-living lifestyle (Semeniuk and Rothley 2008; Corcoran et al. 2013). Consequently, they suffer from reduced health conditions, increased parasite loads, and an increase in conspecific bite marks. These detrimental effects are due to the changes in residential behaviour caused by the poor habitat choices (Semeniuk and Rothley 2008; Corcoran et al. 2013). The white shark cage-diving industry in South Australia also affects the behaviour and diet of non-target species, e.g., smooth stingray (*Bathytoshia brevicaudata*), trevally (*Pseudocaranx spp.*), and yellowtail kingfish (*Seriola lalandi*) (Rizzari et al. 2017; Meyer et al. 2019).

Shark feeding operations may also cause an increased level of aggression toward conspecifics, other species of sharks, and humans (Burgess 1998; Gallagher and Huveneers 2018). An example can be found off Bimini, Bahamas where great hammerhead sharks (*Sphyrna mokarran*) have been provisioned since 2013. Tourism operators were observed dumping bait at the dive sites in between tours to keep the sharks nearby. During the second season of provisioning, bull shark (*Carcharhinus leucas*) had already overrun many of the dive sites exhibiting some aggression toward other shark species and humans (Gallagher and Huveneers 2018). Aside from the negative effects on the surrounding ecosystem and the safety of the divers, the presence of the bull sharks also impacted the industry itself, since they drove off the great hammerhead sharks (Gallagher and Huveneers 2018). The increased aggression toward humans is a major public concern. People are afraid that sharks will learn to associate food with humans when being fed for tourism purposes (Burgess 1998). Our results suggest that a reduction in the frequency of exposure to these operations would contribute towards avoiding behavioural alterations in targeted and non-targeted elasmobranch species.

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Author contributions All authors contributed to the study design. DH and CVP executed the experiments and collected the data. DH ran the statistical analysis and drafted the manuscript. All authors revised the manuscript, gave final approval, and are accountable for accuracy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Flinders University Animal Welfare Committee (E438/16) and the Macquarie University Animal Ethics Committee (ARA2014/003).

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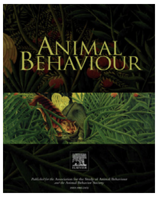

Short-term impacts of daily feeding on the residency, distribution and energy expenditure of sharks

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Tourism-related feeding of wildlife can result in detrimental, human-induced changes to the spatial distribution, social behaviour and health of target species. The feeding of sharks as part of shark-viewing activities has become increasingly popular in recent years to ensure reliable and consistent encounters. A common limitation in determining how feeding affects individuals or populations is the lack of baseline data prior to the establishment of a feeding site. Here, we documented the residency, spatial distribution, activity patterns and daily metabolic rates of juvenile lemon sharks, *Negaprion brevirostris*, prior to initiating daily feeding for 27 days to assess the effect of short-term feeding. We implanted acoustic transmitters equipped with accelerometers to record movement and activity in six lemon sharks. Sharks progressively anticipated the feeding events during the 27 days of daily feeding, as shown by a change in activity and increased time spent near the feeding site 1 h prior to feeding events. Shark behaviour did not fully return to baseline levels within the documented 90 days of postfeeding recovery. However, neither spatial distribution outside the refuge nor mean daily activity was affected by feeding. Sharks decreased their metabolic rates over the course of the study, but this was probably due to falling water temperature rather than the effect of feeding. Overall, our study shows that anticipatory behaviour in juvenile lemon sharks can occur within 11 days of daily feeding events, but behavioural changes seem confined to fine-scale movement patterns and may not affect these sharks' daily energy needs. The ability to assess the effects of daily feeding at a site where tourism has not been occurring previously provides new information for operators and managers of wildlife tourism to account for and minimize potentially detrimental effects on the target species.

In recent decades, wildlife tourism has become one of the fastest-growing sectors of the tourism industry (Scheyvens, 1999; Wearing & Neil, 2009), partly due to the increasing desire of people to reconnect with nature (Miller, 2005). Associated with a number of socioeconomic benefits (Apps, Dimmock, & Huveneers, 2018; Huveneers et al., 2017; Newsome, Rodger, Pearce, & Chan, 2019; Orams, 2002), wildlife tourism has been viewed as a tool to assist local economies transitioning from consumptive to perceived nonconsumptive uses of natural resources (Newsome et al., 2019). To name just a few, examples include lemur- and birdwatching tourism in Andasebe National Park (Newsome & Hassell, 2014), gorilla and chimpanzee tourism in Uganda (Newsome & Hughes, 2016), whale-watching tourism in the Antarctic (Williams & Crosbie, 2007), and shark and ray tourism in the Bahamas and Fiji (Haas, Fedler, & Brooks, 2017; Macdonald et al., 2017). These examples illustrate how the employment of local tour guides and the provision of other services by local communities can result in economic success and growth (Newsome et al., 2019). Linked with an increased public awareness for endangered species, these economic transitions can also benefit the focal species and their surrounding ecosystems by promoting conservation efforts (Apps

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et al., 2018; Newsome & Hassell, 2014). However, previous studies have indicated detrimental effects of wildlife tourism activities (Barnett, Payne, Semmens, & Fitzpatrick, 2016; Green & Giese, 2004; Newsome, Lewis, & Moncrieff, 2004; Semeniuk & Rothley, 2008), and the resulting impaired individual fitness and survival has been documented in terrestrial (Orams, 2002), avian (Steven, Pickering, & Castley, 2011) and aquatic species (Williams & Crosbie, 2007). For example, close proximity and interactions of tourists with Adélie penguins, *Pygoscelis adeliae*, has resulted in as much as an 80% reduction in chick survival (Giese, 1996). Behavioural changes due to wildlife tourism can also lead to decreased survival, such as with coastal damselfish, *Chromis chromis* (Milazzo, Anastasi, & Willis, 2006), or can increase aggressive behaviours towards humans, such as with yellow baboons, *Papio cynocephalus*, in Kenya and dingoes, *Canis lupus dingo*, on Fraser Island, Australia (Altmann & Muruthi, 1988; Burns & Howard, 2003).

Recent studies on elasmobranchs have shown that wildlife tourism can have a wide range of effects on focal and nonfocal species, including changes in seasonality, residency, abundance (Clarke, Lea, & Ormond, 2011; Meyer, Dale, Papastamatiou, Whitney, & Holland, 2009), space use (Bruce & Bradford, 2013; Brunnschweiler & Barnett, 2013; Fitzpatrick, Abrantes, Seymour, & Barnett, 2011; Huveneers et al., 2013), activity (Corcoran et al., 2013; Huveneers, Watanabe, Payne, & Semmens, 2018) and diet (Brunnschweiler, Payne, & Barnett, 2018; Meyer, Whitmarsh, Nichols, Revill, & Huveneers, 2020). Such effects are often accentuated by feeding the focal species to ensure reliable and close encounters (Brena, Mourier, Planes, & Clua, 2015; Gallagher et al., 2015). For instance, Brunnschweiler and Barnett (2013) demonstrated changes in the fine-scale movement patterns of bull sharks, *Carcharhinus leucas*, that are frequently exposed to wildlife feeding operations in Fiji. They further found evidence suggesting significant intraspecific variation with regard to the effects of wildlife feeding (Brunnschweiler & Barnett, 2013). Besides the growing body of knowledge on the effects of feeding activities on the behaviours of elasmobranch species, few studies have investigated tourism-related changes in energy expenditure of sharks (e.g. Barnett et al., 2016; Huveneers et al., 2018). This information is, however, crucial to understand the effects of wildlife tourism (Brown, Gillooly, Allen, Savage, & West, 2004; Brunnschweiler et al., 2018; Gallagher & Huveneers, 2018; Wilson et al., 2006). A study on whitetip reef sharks, *Triaenodon obesus*, at Osprey Reef (Great Barrier Reef, Australia) demonstrated that sharks subjected to regular feeding events showed elevated activity levels during the day when they would normally rest, resulting in a ca. 6.4% increase in metabolic rates. Similarly, white sharks, *Carcharodon carcharias*, are more active when interacting with cage-diving operators (Huveneers et al., 2018).

A common limitation in studies investigating the effects of direct feeding (hereafter referred to as 'feeding') is the lack of baseline data prior to establishing a feeding site (Huveneers et al., 2013; Semeniuk & Rothley, 2008). While a population that is not being fed can act as a control, observed differences can be due to site-specific factors. Instead, a more reliable approach for assessing the effects of feeding is one where baseline data can be collected prior to initiation of wildlife tourism activities. If feeding activities can occur for a finite period of time, it further provides the opportunity to investigate behaviour after they stop to assess how soon behaviours return to baseline levels. Therefore, gaining an understanding of a target species' behavioural ecology prior to the establishment of a new feeding site will help wildlife tourism managers to implement sustainable strategies, minimizing the negative impacts of these activities. Ultimately, this will provide further insight about the timing, location and frequency of feeding activities, as well as limits on the number of boats and people allowed to participate, similar to some of the guidelines developed for the whale-watching industry (Mallard, 2019).

Here, we first established a feeding site in Bimini (Bahamas), targeting juvenile lemon sharks, *Negaprion brevirostris*, in a known nursery area where wildlife tourism has not occurred (Guttridge et al., 2012). We used acoustic telemetry and accelerometry to assess daily activity, metabolic rates and spatial distribution patterns before, during and after a 1-month (daily) feeding regime. The aim of this study was to investigate whether daily feeding of juvenile lemon sharks at an aggregation site (1) resulted in anticipatory behaviour, as measured by changes in burst activity and fine-scale distribution, (2) increased activity, thereby affecting metabolic rate, and (3) affected the sharks' spatial distribution. We hypothesized that daily feeding would (1) lead to sharks anticipating feeding events, (2) increase daily activity and metabolic rates and (3) constrict space use of juvenile lemon sharks.

METHODS

Study Site and Species

The study was conducted in Bimini (25°44′N, 79°16′W), Bahamas (island group located ca. 90 km east of Florida, U.S.A.; Fig. 1) at a tidally mediated refuge site for juvenile lemon sharks known locally as 'Aya's Spot'. The area (ca. 200 m long and 20 m wide) is a shallow water mangrove inlet that becomes inaccessible during low tides and provides protection from predators over the high tides due to the narrow (<1 m), shallow entrance (Guttridge et al., 2012). Juvenile lemon sharks use the area daily, with 7 ± 4 (mean ± SD) sharks aggregating in the northern part of the refuge at and around the high tide (Guttridge, Gruber, Krause, & Sims, 2010; Guttridge et al., 2011).

Acoustic Monitoring

Ten acoustic receivers (model VR2W–69Hz, Vemco, Halifax, Nova Scotia) were deployed in a nonoverlapping array within 2.5 km of Aya's Spot (Fig. 1). One receiver was positioned at the single access point of Aya's Spot (refuge entrance), providing acoustic coverage of sharks entering or departing. Receivers were deployed inside a PVC housing with a concrete base (see Guttridge et al. (2012) for more details on the housing). Range testing using three stationary transmitters and two receivers placed at <1 m depth in a similar habitat to the present study showed a detection range of 50–100 m, depending on the tidal phase (Guttridge et al., 2017; Murchie et al., 2010). This range is relatively small compared to the range of receivers in open environments (Huveneers et al., 2016) and probably due to the shallow habitat. Temperature loggers (HOBO Water Temperature Pro v2 Data Logger, Onset Computer Corporation, Bourne, MA, U.S.A.) were placed at four of the 10 receivers (Fig. 1), which recorded the ambient water temperature at 30 min intervals. In addition to the 10 receivers placed around Aya's Spot, 55 receivers had already been deployed around Bimini (Fig. 1) as part of a long-term acoustic monitoring project (Guttridge et al., 2017).

Juvenile lemon sharks were trapped using two seine nets (10 cm monofilament stretch mesh) that closed off Aya's Spot during the last hour of the rising tide. Juvenile lemon sharks were caught individually using dip nets as they attempted to leave Aya's Spot during the falling tide. Each shark was processed immediately upon capture and released before the next individual was caught. Upon capture, individual sharks were immediately transferred into a 200-litre tank where precaudal length was measured to the nearest millimetre and sex was determined (Dhellemmes, Finger, Laskowski, Guttridge, & Krause, 2020). Nine sharks (five females

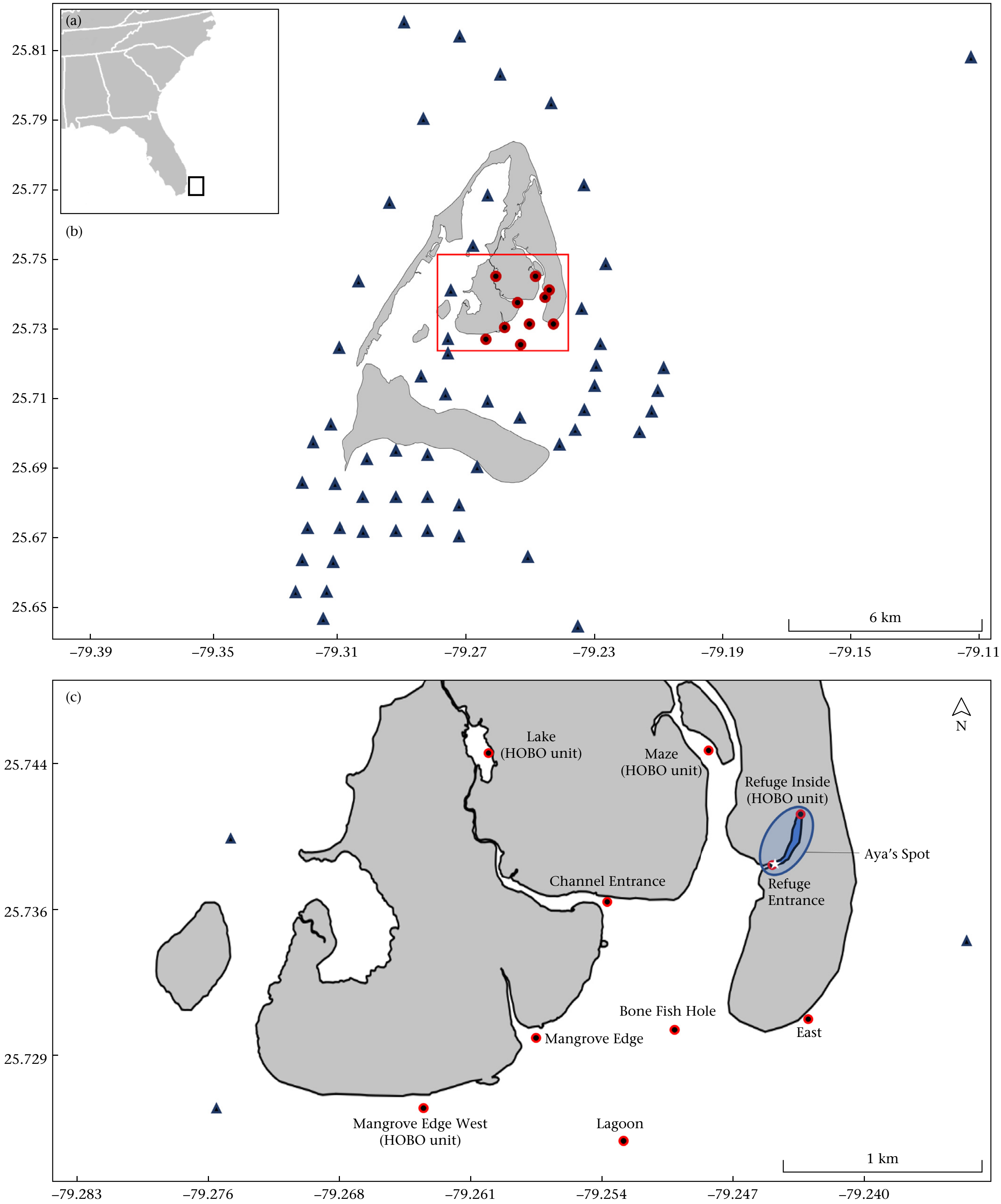

Figure 1. Maps of the study site showing (a) the position of the Bimini island group relative to Florida, U.S.A. and (b) an overview of the Bimini island group and the distribution of VR2W acoustic receivers around Bimini. Each marker represents one receiver. Blue triangles mark the locations of the receivers that are part of a long-term acoustic monitoring project. Red dots mark the receivers that were deployed for this study. (c) Map of the study site and the Aya's Spot refuge (blue polygon). Locations of HOBO water temperature loggers are also indicated as 'HOBO unit' underneath each receiver name. The white star next to the Refuge Entrance receiver marks the feeding site.

and four males, between 1 and 3 years old) were equipped with an acoustic activity tag (V13AP, Vemco, Halifax, Nova Scotia) that was surgically implanted into the peritoneal cavity through a small (30–40 mm) incision along the ventral side of the shark. The incision was closed with interrupted absorbable sutures (Ethicon braided coated Vicryl) that were tied using a surgeon's knot (Wagner, Stevens, & Byrne, 2000). All procedures combined took less than 8 min per individual with the surgery being completed while sharks were held by hand in tonic immobility (Kessel & Hussey, 2015). This is a coma-like state that is achieved by turning sharks quickly on their backs. One member of the research team handheld the shark in this position, while another performed the surgical implantation. It is widely recognized as an effective alternative to chemical anaesthetics, providing several benefits, both from a practical and an animal welfare perspective (reviewed in Kessel & Hussey, 2015). Besides a rapid induction and recovery, which optimizes the surgical procedure, there is no risk of overdose and minimal disruption to respiration, thereby reducing the risk for negative sublethal impacts (Kessel & Hussey, 2015). Each shark was further marked with a unique second, external tag (colour coded T-bar tag; Floy Tag Manufacturing, Seattle, WA, U.S.A.) through the first or second dorsal fin. Following processing, all nine sharks swam away vigorously and were later detected via acoustic receivers or visual observations, thereby confirming survival.

Acceleration and depth data were transmitted at random intervals of 120–180 s. Acceleration (±4.9 m/s^2 range) was sampled at 5 Hz for 20 s four times every five transmission cycles (10–15 min). Activity was calculated as an average root mean square (RMS) value for all three axes (activity $= [X^2 + Y^2 + Z^2]^{0.5}$) and transmitted as an 8-bit digital value. The static contribution to the overall acceleration (g) was filtered out prior to RMS calculation. RMS acceleration resolution was 0.0191 m/s^2 (Payne et al., 2011).

Experimental Protocol

Sharks were given 10 days to recover from capture-associated stress (Bullock, Guttridge, Cowx, Elliott, & Gruber, 2015) and surgery (Chin, Mourier, & Rummer, 2015). Following this recovery period, they were not disturbed for an additional 30 days, which provided time to record natural behaviours. Sharks were then attracted to the feeding site using a bait box filled with fish scraps and target-fed daily for 27 days using feeding poles to ensure minimal physical interactions between the feeders and the sharks. We ensured each shark received the same amount of food, approaching their estimated daily ration in the wild (i.e. up to 2% of their body mass; Sims, 1996). This amount was chosen based on learning trials on captive juvenile lemon sharks where feeding motivation was maintained across several months (Guttridge et al., 2013). Each day, the feeding event commenced 1 h before the daytime high tide. This schedule was used because juvenile lemon sharks in this area were already known to use Aya's Spot as a refuge during this time (Guttridge et al., 2012). The feeding area at Aya's Spot was marked with three vertical PVC pipes that were painted with black and white stripes and anchored to the substrate using steel bars. The PVC pipes acted as landmarks to help sharks learn to associate the tide cycle and the feeding event. Black and white panels have been used previously to demonstrate social learning in juvenile lemon sharks in Bimini (Guttridge et al., 2013). Feeding duration was 1 h until the peak high tide was reached. For each feeding event we recorded time of first arrival for each individual, as well as the maximum number of sharks that arrived. We were unable to feed on four occasions due to inclement weather (days 15, 23, 24 and 26). Sharks were no longer fed after the 27-day period, but acoustic receivers remained deployed for an additional 90 days to record changes in behaviour once the daily feeding events had ceased.

Data Analyses

Anticipation

Anticipatory behaviour was investigated using the proportion of high-activity events (Burst Activity; >0.9 m/s^2) across feeding times (1 h before [prefeeding], during [feeding] and 1 h after [postfeeding]). We expected that anticipation would lead to increased Burst Activity during prefeeding. We selected the threshold of 0.9 m/s^2 to represent periods of elevated activity that appeared to be associated with the feeding events based on the frequency distribution of activity measurements and the prominent shift of the distribution towards higher levels of activity during the feeding period (Fig. A1). We also evaluated whether anticipatory behaviour was occurring by assessing the proportion of detections around the daytime high tides at the refuge entrance receiver located next to the feeding site (Spatial Attraction). The proportion was obtained by dividing the number of detections at the refuge entrance receiver by the total number of detections from both Aya's Spot refuge receivers. We used Spatial Attraction as a measure of anticipation, since previous observations showed that sharks spend most of their time inside Aya's Spot during high tide (Guttridge et al., 2012). An increase in Spatial Attraction would indicate that the sharks moved closer to the feeding site in anticipation of the upcoming feeding event. This enabled us to determine how much time sharks were spending in proximity to the feeding site. Spatial Attraction was compared across feeding times. To investigate the point in time when the sharks started to anticipate the feedings, we visually compared the time of first arrival and the maximum number of sharks present during the feeding events across days. We defined a learning criterion to identify the approximate time it took for sharks to start anticipating the feeding events. A shark had reached the criterion once it arrived at the feeding site within 6 min after the beginning of the feeding event on 3 consecutive days. We also extended the recovery period to 90 days to determine when the anticipatory behaviour ceased. The 90-day recovery period was split into three subperiods of 30 days called recovery 30, recovery 60 and recovery 90, respectively.

Daily activity and metabolic rates

We investigated whether daily feeding events affected mean activity (obtained from the activity tag) and metabolic rate. Given that the formula used to calculated metabolic rate ($\dot{M}_{O_2}$) requires the overall dynamic body acceleration (ODBA) in g ($1\ g = 9.80665$ m/s^2), we first converted the activity tag values by dividing them by the Earth's gravitational pull (9.81 m/s^2). Metabolic rate ($\dot{M}_{O_2}$) was estimated following Lear et al. (2017):

$$\dot{M}_{O_2} = \alpha(\mathrm{ODBA}) + b, \tag{1}$$

where α is the slope and b the intercept of the ODBA–$\dot{M}_{O_2}$ relationship. While being species specific, α remains independent of environmental influences. The intercept b, however, varies with environmental factors, in our case water temperature, which scales the intercept as follows:

$$b = b_c \times S^{\frac{T_b - T_c}{10}}, \tag{2}$$

where T_b is the temperature at which the intercept b is calculated. The intercept of the ODBA–$\dot{M}_{O_2}$ relationship b_c is the intercept that was assessed during the calibration at temperature T_c. S is the scaling factor that was determined for the species, also known as the Q_{10}. All constant variables (α, b_c, T_c and S) were obtained from

Lear et al. (2017), who calibrated the ODBA–$\dot{M}_{O_2}$ relationship at two different temperatures in three different species of sharks, including lemon sharks. Water temperatures were calculated as the mean temperatures from four temperature loggers (HOBO Water Temperature Pro v2 Data Logger, Onset Computer Corporation) deployed at the study site (Fig. 1).

Spatial distribution

For our assessment of the spatial distribution of juvenile lemon sharks across the five periods (baseline, feeding, recovery 30, recovery 60 and recovery 90), we assumed that the detection efficiency was homogeneous across the receivers deployed in the study, which was reasonable, given the identical depth and habitat type. We used the packages VTrack (Campbell, Watts, & Dwyer, 2012) and Animal Tracking Toolbox (Udyawer et al., 2018) in R v.4.0.0 (R Core Team, 2013) to create Brownian Bridge kernel utilization density models (BB-KUD) based on all detections recorded during each of the five periods. These models account for the movement paths between two fixed locations when calculating the movement range of an animal, using random walks between two successive locations based on the distance between the locations and the time it took an animal to cover this distance (Fischer, Walter, & Avery, 2013; Horne, Garton, Krone, & Lewis, 2007). Calculating the BB-KUDs enabled us to quantitatively compare the activity space of the sharks and to statistically assess whether the daily feedings significantly affected said activity space (Fischer et al., 2013; Udyawer et al., 2018). We calculated the 50% (BB-KUD50 or core area hereafter) and 95% (BB-KUD95 or home range hereafter) ranges of the BB-KUDs and compared them statistically using a linear mixed model (LMM). Visualizing the extents of the BB-KUDs, we further investigated whether the core area shifted between periods.

In summary, we used five response variables to assess the effects of daily feeding events on shark behaviour: (1) Burst Activity, (2) Spatial Attraction, (3) daily activity, (4) daily metabolic rates, and (5) core area and home range. Burst Activity and Spatial Attraction were estimated for each prefeeding, feeding and postfeeding event; daily activity and daily metabolic rates were estimated for each day of the study; core area and home range were estimated for each period (data were collected (24 h a day) calculated for the baseline, feeding and three recovery periods). As feeding events only occurred during the day, the analyses of anticipatory behaviours (i.e. Burst Activity and Spatial Attraction) were restricted to daytime high tides. Night-time behaviour was also assessed and is presented in the Appendix.

Generalized linear mixed models (GLMMs) were used with Period (baseline, feeding, recovery 30, recovery 60, recovery 90) as a fixed factor; Time (prefeeding, feeding, postfeeding; fixed factor) was also included in the models assessing anticipatory behaviour (Fig. A2). We further included Temperature (continuous, fixed factor) for the analyses of mean daily activity and metabolic rates because of its known impact on metabolic rates (Lear et al., 2017). Day and Shark ID were included as random factors in all GLMMs to account for the lack of independence in the behaviour of each shark and across days. We included a random slope grouped by Shark ID in the GLMM assessing the effects of feeding on the daily activity measurements. For the remaining models we were unable to include a random slope due to convergence issues. The error structure of GLMM corrects for nonindependence of statistical units due to shared temporal structure (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). We determined the most appropriate statistical family and error distribution for each analysis by examining the distribution of the response variables and visually inspecting the residuals for the saturated models in accordance with Zuur, Ieno, and Elphick (2010). Data transformations were used where appropriate to improve model fit as follows: (1) Burst Activity and Spatial Attraction models: binomial (logit) distribution, no transformations; (2) daily activity and metabolic rate models: Gaussian (identity) distribution with Box–Cox transformations. For the comparison of the BB-KUD ranges between periods, we used an LMM with a restricted maximum likelihood fit, no transformations, and Shark ID as a random factor. We used a dredge function from the R package MuMIn (Barton, 2019) to identify more parsimonious nested models based on their relative probabilities using Akaike's information criterion corrected for small sample size (AIC$_c$; Burnham, Anderson, & Huyvaert, 2011). We used the AIC$_c$ weights (wAIC$_c$) of all the potential models to calculate the relative variable importance values (RVI) to assess the impact of each variable on the statistical outcome. For all models we took a two-tailed approach, accepting significant results regardless of the direction of change. We used post hoc Tukey tests to test for statistical differences between periods. We did not run post hoc Tukey tests on the model outcomes investigating the daily activity and the metabolic rates due to the interaction between a categorical (Period) and a continuous (Temperature) variable. All analyses were performed in R v.4.0.0 (R Core Team, 2013) using the packages glmmTMB (Brooks et al., 2017) and lsmeans (Lenth, 2016). Graphs were created in R v.4.0.0 (R Core Team, 2013) using the packages ggplot2 (Wickham, 2016) and plotly (Sievert et al., 2017).

Ethical Note

The research was approved by the Flinders University Animal Welfare Committee (E452/17) and by the Ministry of Agriculture and Marine Resources, Nassau, Bahamas (Marine Scientific Research Permit).

The sample size was reduced as much as possible, while still ensuring meaningful results (Sequeira et al., 2019). All handling, including the capture of the sharks with dip nets, was done as quickly as possible to minimize stress. Sharks were caught individually and immediately processed and released. As a result, no shark was confined and handled for more than 8 min.

RESULTS

Of the nine juvenile lemon sharks tagged, two were only detected for 9 days post tagging and not during the feeding or recovery periods. Therefore, we omitted these two individuals from further analyses. The remaining seven sharks were regularly detected at Aya's Spot until the end of the study on 13 January 2018. We documented 4530 ± 650 detections per shark with ca. 94% of these occurring at the two Aya's Spot receivers. None of the tagged sharks were detected on receivers outside the 10 receivers deployed for this project (Fig. 1). Of the seven sharks successfully tracked, six frequently participated in the feeding events. The seventh shark was frequently detected on Aya's Spot receivers but did not participate in the feeding events and was therefore omitted from the analyses. Between the tagged and untagged sharks that frequented Aya's Spot, on average 7 ± 2 participated in the feedings. Based on personal observations we made during this study (maximum number of sharks observed inside Aya's spot was 11) and the maximum daily number of sharks that was reported by Guttridge et al. (2012) to visit Aya's Spot, we estimate that 70–90% of the sharks that frequented Aya's Spot during the time of our experiment participated in the feeding events.

Anticipation: Prefeeding Burst Activity and Spatial Attraction

Burst Activity was affected by the interaction between Period and Time (wAIC$_c$ = 1.00; Table 1, Appendix Table A1). It increased significantly during the 1 h leading up to the feeding events (post hoc Tukey: $\beta = -0.909$, $t_{9223} = -3.806$, $P = 0.012$) but dropped back to baseline levels within the first 30 days of recovery (post hoc Tukey: recovery 30: $\beta = 0.126$, $t_{9223} = 0.495$, $P = 1.000$; Fig. 2, Table 1). At night, there were no signs of anticipation based on Burst Activity (Appendix Tables A2, A3, Fig. A3).

Spatial Attraction was also affected by the interaction between Period and Time ($wAIC_c = 1.00$; Table 1, Appendix Table A1). It increased for the 1 h of prefeeding during the feeding month (post hoc Tukey: $\beta = -5.568$, $t_{11447} = -5.003$, $P = 0.0001$; Fig. 2) and remained significantly higher during the 1 h of prefeeding throughout the 90 days of recovery compared to the baseline period (post hoc Tukey: recovery 30: $\beta = -7.440$, $t_{11447} = -6.746$, $P < 0.0001$; recovery 60: $\beta = -8.000$, $t_{11447} = -7.342$, $P < 0.0001$; recovery 90: $\beta = -3.885$, $t_{11447} = -3.477$, $P = 0.038$; Fig. 2). However, prefeeding Spatial Attraction started to decrease during recovery 90 and was significantly lower compared to recovery 60 (post hoc Tukey: $\beta = 4.115$, $t_{11447} = 4.033$, $P = 0.005$). During the 1 h of postfeeding, Spatial Attraction was significantly higher during the feeding month (post hoc Tukey: $\beta = -11.257$, $t_{11447} = -7.467$, $P < 0.0001$; Fig. 2). Despite an initial drop during the first 30 days of recovery, the postfeeding Spatial Attraction remained significantly higher than the baseline level during recovery 60 (post hoc Tukey: $\beta = -5.602$, $t_{11447} = -4.520$, $P = 0.0006$; Fig. 2).

Spatial learning and anticipation appear to have commenced after 9 days. This estimate is based on the number of sharks observed during the feeding events and the significant drop in latencies to first arrival (linear regression: Latency to first arrival ~ Day: $\beta = -0.637$, $t_{119} = -4.215$, $P < 0.0001$; Fig. 3). Five of the six sharks included in the analysis reached the learning criterion and did so in 10.8 ± 0.37 days (Fig. 3). In contrast to Burst Activity, the Spatial Attraction 1 h prior to the feeding events started to increase significantly during night-time high tides (slack tide was reached between sunset and 1 h after sunrise) during the feeding period and was retained for the first 60 days of recovery (Appendix Tables A2, A3, Fig. A3).

Daily Activity (Overall Dynamic Body Acceleration) and Metabolic Rates

Daily overall dynamic body acceleration was significantly affected by the interaction between Period and Temperature ($wAIC_c = 1.00$; Table 2, Appendix Table A4). Although it increased with temperature during the baseline and feeding months, the slope was very small (Fig. 4, Table 2). It remained consistent during

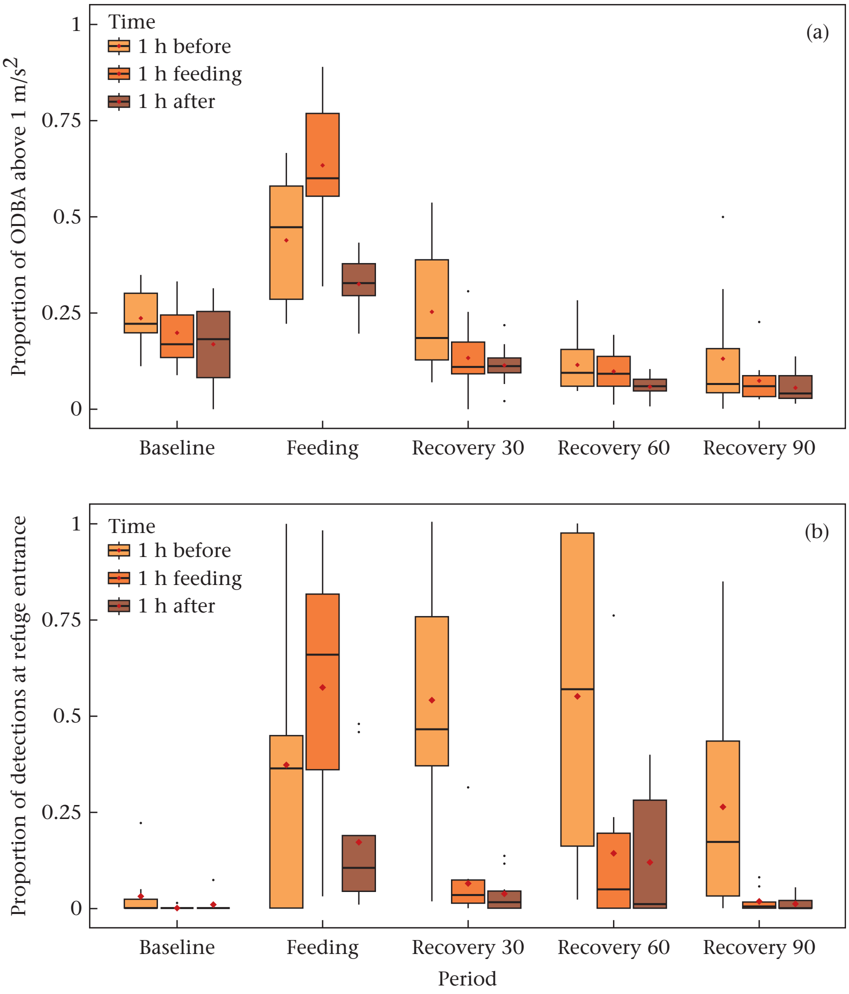

Figure 2. Box plot illustrating (a) the proportion of overall dynamic body acceleration (ODBA, m/s^2) above 0.9 m/s^2 (Burst Activity), and (b) the proportion of detections made at the refuge entrance (number of detections at refuge entrance/(number of detections at refuge entrance + number of detections inside refuge)) (Spatial Attraction) plotted against the Period. The horizontal lines inside the boxes mark the median and the red diamonds the mean for 1 h before, during and after feeding events. The upper and lower boundaries of the box represent the third and first quartiles. The whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers.

Table 1
Summary table for the models investigating the Period and Time effects on Burst Activity and Spatial Attraction

Effect	β	SE	*z*	*P*
Burst Activity				
(Intercept)	−1.390	0.235	−5.920	<0.001
Period (feeding)	0.885	0.245	3.609	<0.001
Period (recovery 30)	−0.166	0.264	−0.630	0.529
Period (recovery 60)	−0.888	0.241	−3.684	<0.001
Period (recovery 90)	−1.010	0.276	−3.661	<0.001
Time (during)	0.051	0.164	0.309	0.757
Time (after)	0.056	0.176	0.318	0.750
Period (feeding) * Time (during)	0.873	0.238	3.663	<0.001
Period (recovery 30) * Time (during)	−0.429	0.277	−1.549	0.121
Period (recovery 60) * Time (during)	−0.286	0.255	−1.120	0.263
Period (recovery 90) * Time (during)	−0.418	0.299	−1.396	0.163
Period (feeding) * Time (after)	−0.464	0.249	−1.866	0.062
Period (recovery 30) * Time (after)	−0.609	0.276	−2.203	0.028
Period (recovery 60) * Time (after)	−0.750	0.265	−2.827	0.005
Period (recovery 90) * Time (after)	−0.731	0.309	−2.362	0.018
Conditional R^2				0.30
Spatial Attraction				
(Intercept)	−6.789	0.965	−7.037	<0.001
Period (feeding)	5.568	1.113	5.003	<0.001
Period (recovery 30)	7.440	1.103	6.746	<0.001
Period (recovery 60)	8.000	1.090	7.342	<0.001
Period (recovery 90)	3.885	1.117	3.477	<0.001
Time (during)	−4.016	1.134	−3.541	<0.001
Time (after)	−3.305	0.773	−4.277	<0.001
Period (feeding) * Time (during)	5.689	1.160	4.904	<0.001
Period (recovery 30) * Time (during)	−0.522	1.187	−0.439	0.660
Period (recovery 60) * Time (during)	−1.450	1.177	−1.232	0.218
Period (recovery 90) * Time (during)	−0.365	1.242	−0.294	0.769
Period (feeding) * Time (after)	1.140	0.812	1.404	0.160
Period (Recovery 30) * Time (after)	−3.372	0.869	−3.880	<0.001
Period (recovery 60) * Time (after)	−2.398	0.829	−2.892	0.004
Period (recovery 90) * Time (after)	−1.735	0.904	−1.919	0.055
Conditional R^2				0.88

Estimated Period and Time effect coefficients (β) and their standard errors (SE), *z* values and the individual coefficient type I error estimate (*P*). Time (during) = 1 h of feeding, Time (after) = 1 h postfeeding, Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month. For the factor Time, the baseline level was the 1 h prefeeding.

the recovery month (Fig. 4, Table 2). Metabolic rates were significantly affected by the interaction between Period and Temperature ($wAIC_c = 1.00$; Table 2, Appendix Table A4). Across all periods, the metabolic rates increased significantly with temperature. However, they increased at a slower rate during the feeding period than the baseline period. In contrast, they increased significantly faster with temperature during recovery 60 and recovery 90 compared to the baseline period. Despite the significant effect of Period and of the interaction between Period and Temperature, variations were small compared to changes with temperature (Fig. 4).

Changes in Spatial Distribution

The top-ranked model for the comparison of the core area was the Null model ($wAIC_c = 0.93$; Appendix Table A5). The top-ranked model investigating the extents of the home range included Period as a fixed factor ($wAIC_c = 0.93$; Appendix Table A5). While there was no significant difference in core areas between periods (Period–RVI = 0.07; Table 3), home range was significantly larger during the first 30 days of recovery compared to the baseline period as well as the last 30 days of recovery (Period–RVI = 0.93; post hoc Tukey: baseline–recovery 30: $\beta = -309718$, $t_{21} = -3.508$, $P = 0.016$; Fig. 5, Table 3).

DISCUSSION

We investigated the impact of daily feeding events on the spatial distribution, activity (as a proxy for energy expenditure) and metabolic rates of juvenile lemon sharks at a site previously unfrequented by humans. Data collection started prior to any feeding events taking place providing the opportunity to collect baseline data, which is often missing in studies assessing the effects of wildlife tourism. Using data collected daily during the 1 h prefeeding, feeding and postfeeding events, we have shown evidence for anticipatory behaviour during prefeeding. Sharks exhibited elevated levels of Burst Activity and spent more time at the feeding site within 27 days of daily feeding events. However, overall daily activity, energy expenditure and broadscale spatial distribution were not affected. Burst Activity returned to baseline levels after the first 30-day recovery period during which feeding events had ceased. In contrast, the spatial association of the feeding site remained for at least 90 days postfeeding. Our findings reveal shark feeding operations may not affect the daily activity and metabolic rates of focal species or their broad distribution but can alter fine-scale spatial distribution patterns. We further found evidence for intraspecific variability with one individual, while being frequently detected on both refuge receivers, never participating in the feeding events. This variability is possibly due to differences in personality (Dhellemmes et al., 2020; Finger, 2019). Similar intraspecific variability with regard to wildlife feeding operations has previously been demonstrated in bull sharks in Fiji (Brunnschweiler & Barnett, 2013).

Anticipatory Behaviour

Anticipation is often used to investigate time–place learning capabilities of organisms by assessing behavioural changes preceding the introduction of a reward or punishment (Gómez-Laplaza

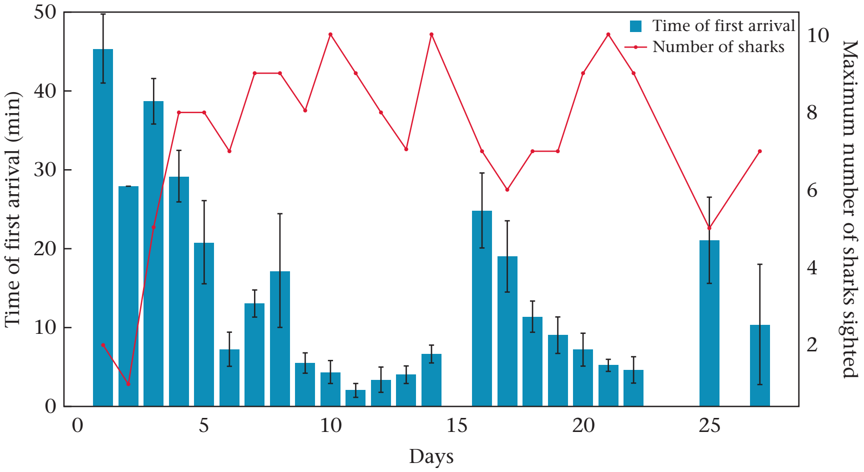

Figure 3. Illustration of the daily mean time of first arrival ± SE (min) following the introduction of the bait box at the feeding site, and the daily maximum number of sharks present during the feeding events.

& Morgan, 2005; Reebs, 1999). While juvenile lemon sharks are known to naturally use the shallow water inlet at Aya's Spot during high tides (Guttridge et al., 2012), we observed a shift in space use inside Aya's Spot as a result of daily feeding events. In the 1 h preceding the feeding event, sharks changed from being predominantly inside Aya's Spot to spending more time at its entrance where feeding events took place. This anticipatory behaviour usually indicates spatial learning linked to a temporal component

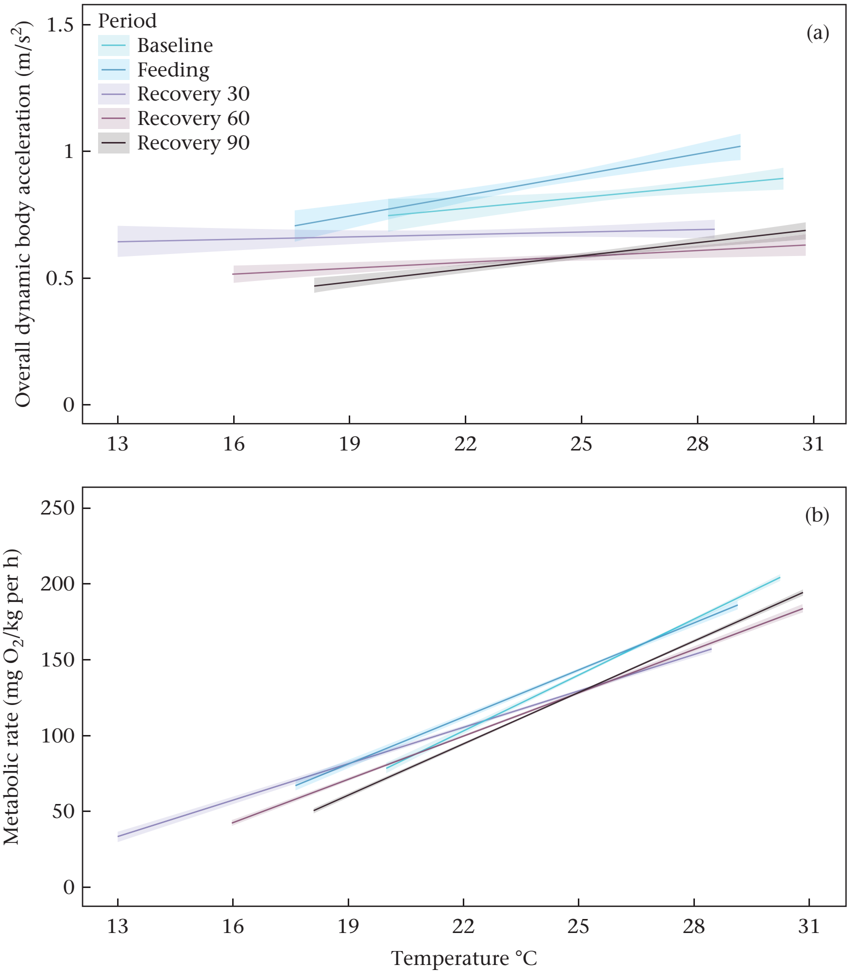

Figure 4. Relationship between (a) the mean daily overall dynamic body acceleration (m/s^2) and (b) the mean daily metabolic rate (mg O_2/kg per h) and water temperature. Lines of best fit were applied with 95% confidence intervals for each of the five periods.

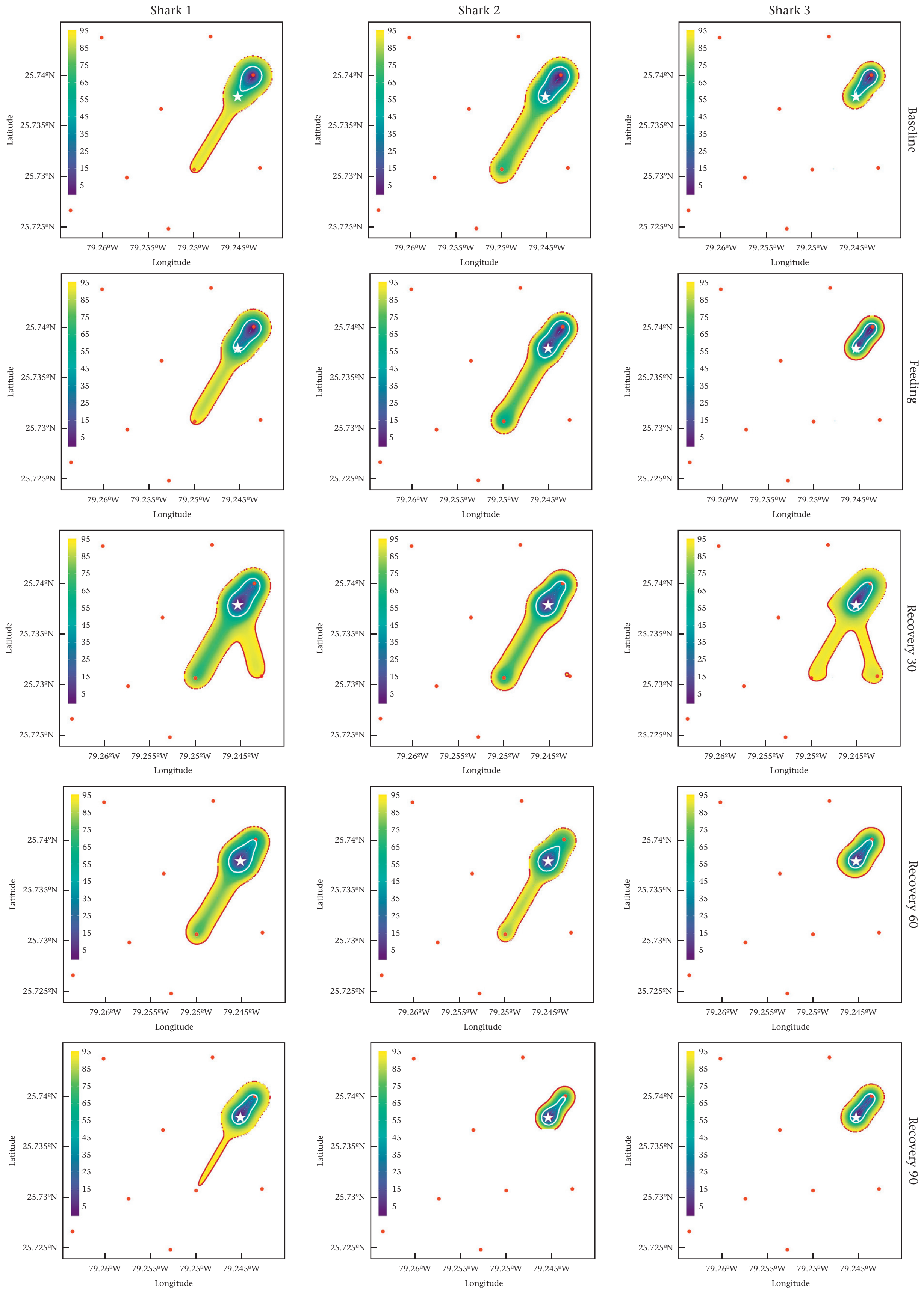

Figure 5. Brownian Bridge kernel utilization density estimates for each individual (sharks 1–6) and each period (baseline, feeding, recovery 30, recovery 60 and recovery 90). The white star marks the receiver located at the feeding site and the red dots mark the positions of the remaining nine receivers. The contours illustrate the core area (white) and the home range (red).

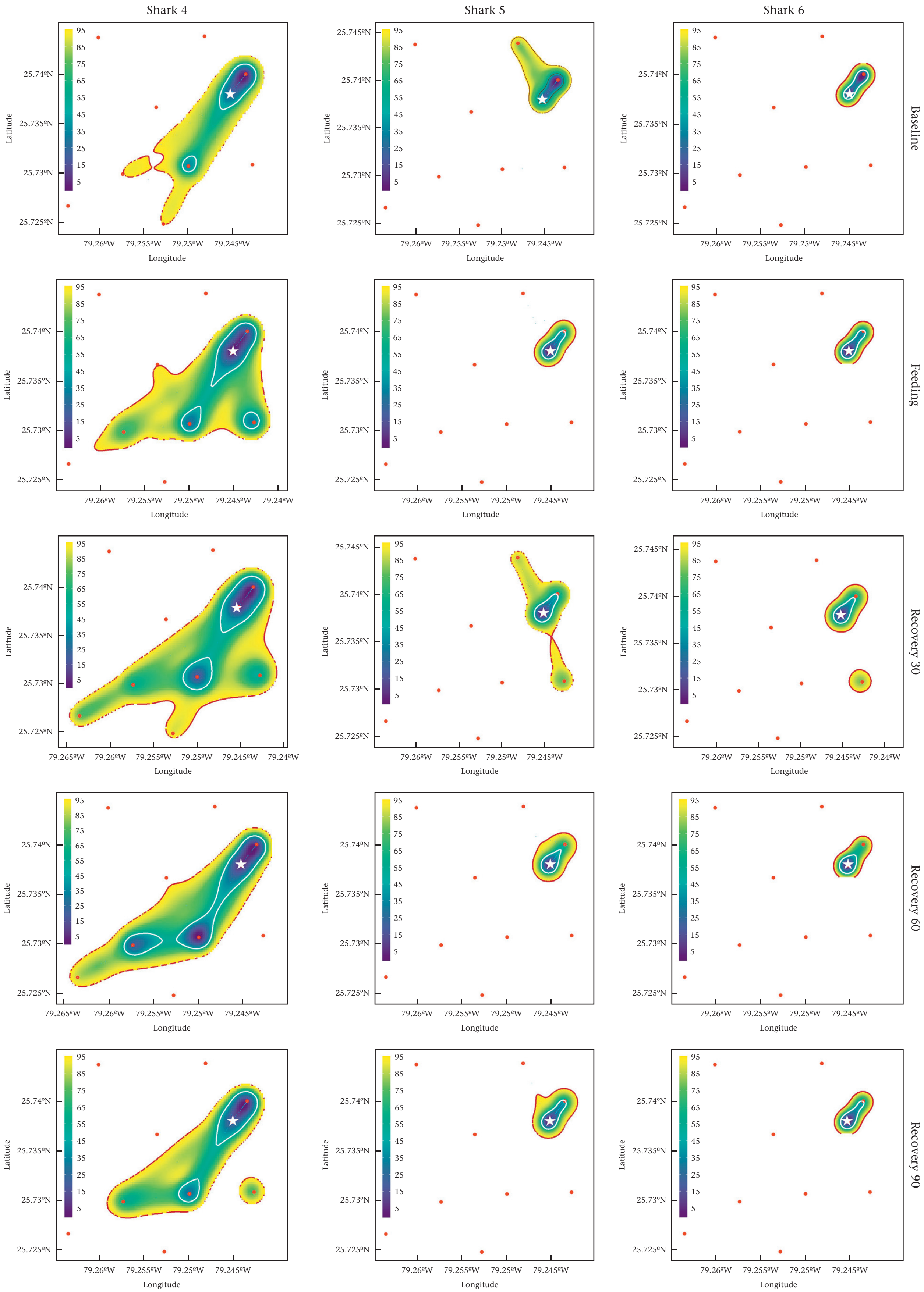

Figure 5. (*continued*).

Table 2
Summary table for the models investigating the Period and Time effects on the mean daily activity and the mean daily metabolic rates

Effect	β	SE	*Z*	*P*
Mean daily activity				
(Intercept)	−0.271	0.224	−1.212	0.226
Period (feeding)	0.371	0.282	1.316	0.188
Period (recovery 30)	0.416	0.247	1.690	0.091
Period (recovery 60)	−0.608	0.238	−2.555	0.011
Period (recovery 90)	−0.660	0.239	−2.766	0.006
Temperature	−0.006	0.008	−0.729	0.466
Period (feeding) * Temperature	−0.015	0.011	−1.396	0.162
Period (recovery 30) * Temperature	−0.036	0.009	−3.823	<0.001
Period (recovery 60) * Temperature	0.00002	0.009	0.003	0.998
Period (recovery 90) * Temperature	0.008	0.009	0.883	0.377
Conditional R^2				0.16
Mean daily metabolic rate				
(Intercept)	2.303	0.026	88.79	<0.001
Period (feeding)	0.075	0.033	2.28	0.022
Period (recovery 30)	0.008	0.028	0.28	0.778
Period (recovery 60)	−0.146	0.027	−5.34	<0.001
Period (recovery 90)	−0.132	0.027	−4.83	<0.001
Temperature	0.033	0.001	35.14	<0.001
Period (feeding) * Temperature	−0.003	0.001	−2.20	0.028
Period (recovery 30) * Temperature	−0.002	0.001	−1.45	0.147
Period (recovery 60) * Temperature	0.004	0.001	3.74	<0.001
Period (recovery 90) * Temperature	0.004	0.001	3.45	<0.001
Conditional R^2				0.50

Estimated Period and Temperature effect coefficients (β) and their standard errors (SE), z values of factors included in the model averaging (Appendix Table A4), and the individual coefficient type I error estimate (*P*). Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month.

marked by the rising of the tide. Fish and other organisms are capable of tracking time by reference to an internal clock, which is often set by light cycles (i.e. circadian rhythm) and is maintained even in the absence of external cues (Amaral et al., 2014; Bell-Pedersen et al., 2005; de Almeida Moura, da Silva Lima, Silveira, Miguel, & Luchiari, 2017). Marine organisms, including sharks, are, however, also predisposed to linking behaviour to tidal cycles (Gibson, 2003; Shepard et al., 2006); as a result, associating this natural cycle with feeding for our experiment potentially made it easier for sharks to anticipate the timing of feeding events. Regarding the spatial component of the learning process, juvenile lemon sharks possess an innate homing ability and can successfully return to their home range after being displaced by up to 16 km (Edrén & Gruber, 2005). Many shark species are known to return to a resided-in area after making long-distance movements (e.g. Port Jackson sharks, *Heterodontus portusjacksoni*: Bass et al., 2017), display site fidelity (Bond et al., 2012; Vianna, Meekan, Meeuwig, & Speed, 2013) or return to their exact birth place (i.e. natal philopatry; e.g. lemon sharks: Chapman, Feldheim, Papastamatiou, & Hueter, 2015; Feldheim et al., 2014). Spatial learning has also been demonstrated on a much smaller spatial scale, with grey bamboo sharks, *Chiloscyllium griseum*, being able to locate a goal position inside a maze using a directional strategy or spatial learning based on visual landmarks (Fuss, Bleckmann, & Schluessel, 2014). Given the visual landmarks used in our study, juvenile lemon sharks might have also used a combination of direction strategy and spatial learning to locate the feeding site.

Table 3
Summary table for the models investigating the Period and Time effects on the core areas and home ranges

Effect	β	SE	*t*	*P*
Core area				
(Intercept)	88782.69	39492.24	2.248	0.035
Period (feeding)	15955.50	21183.56	0.753	0.460
Period (recovery 30)	32208.30	21183.56	1.520	0.143
Period (recovery 60)	37656.63	20775.68	1.813	0.084
Period (recovery 90)	−1869.02	20775.68	−0.0900	0.929
Conditional R^2				0.15
Home range				
(Intercept)	572388.7	252480.50	2.267	0.034
Period (feeding)	62213.9	88286.02	0.705	0.489
Period (recovery 30)	309717.7	88286.02	3.508	0.002
Period (recovery 60)	81309.6	86651.35	0.938	0.359
Period (recovery 90)	−57849.9	86651.35	−0.668	0.512
Conditional R^2				0.32

Estimated Period effect coefficients (β) and their standard errors (SE), t values of factors and the individual coefficient type I error estimate (P). Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month.

The shift in space use inside Aya's Spot during the 1 h of prefeeding shows that sharks moved close to the feeding site in anticipation of the upcoming feeding event. This is further supported by the simultaneous increase in Burst Activity. Based on time at first arrival and number of sharks sighted, juvenile lemon sharks required 5–10 days to start anticipating feeding events and ca. 11 days to reach the learning criterion. This learning period is similar to that of bamboo sharks trained to locate a goal inside a maze (ca. 8 days; Fuss et al., 2014) and Port Jackson sharks trained in a spatial task (ca. 15 days; Heinrich, Vila Pouca, Brown, & Huveneers, 2020). Anticipation of a feeding event and subsequent spatial learning have also been demonstrated in bull sharks (Brunnschweiler & Barnett, 2013) and short-tail stingrays, *Bathytoshia brevicaudata* (Pini-Fitzsimmons, Knott, & Brown, 2018). Following ca. 30 years of fish frames being frequently discarded at a boat ramp cleaning station, short-tail stingrays consistently visited the boat ramp in late afternoon when fish cleaning typically occurred, regardless of whether fish cleaning was taking place. This suggests that these rays anticipate afternoon feeds as a result of a learnt time–place association (Pini-Fitzsimmons et al., 2018).

Despite small declines in anticipation following periods of inclement weather when we were unable to feed sharks, some anticipation was still observed well after the feeding events had fully ceased. For instance, Burst Activity was still significantly higher than baseline levels during the first 30 days of recovery. Spatial anticipation lasted even longer, with sharks still spending more time at the feeding site during the 1 h feeding event after 60–90 days of recovery compared to the baseline period. Our results suggest that a learnt spatial association can be retained for extended periods in wild populations of elasmobranchs. Indeed, laboratory studies on memory retention capacities demonstrated that sharks can memorize learnt behaviours for long periods of time. Juvenile grey bamboo sharks remembered a learnt visual discrimination task for up to 50 weeks in the absence of reinforcement (Fuss & Schluessel, 2015). Similarly, Port Jackson sharks retained a learnt association with air bubbles for up to 40 days (Guttridge & Brown, 2013). However, the extent of memory retention and extinction rates in elasmobranchs requires further studies in more controlled environments.

Daily Activity and Metabolic Rates

The increased activity prior to and during the feeding period was not sufficient to affect sharks' daily overall dynamic body acceleration and metabolic rate. These results contrast with a previous study showing that wildlife tourism targeting reef sharks increased the amount of time whitetip reef sharks spent swimming and subsequently their daily overall dynamic body acceleration (Barnett et al., 2016). The discrepancy between Barnett et al.'s

(2016) study and ours is probably because whitetip reef sharks typically spend most of the day resting on the sea floor (Barnett et al., 2016; Fitzpatrick et al., 2011), similar to other benthic sharks like the Port Jackson shark (Kadar, Ladds, Mourier, Day, & Brown, 2019). The increased activity of whitetip reef sharks due to tourism occurs during the day when these sharks would normally be resting. If they had been fed in the evening when they are naturally active, feeding events would probably have had little perceivable impact on their activity levels and hence energy budget. Although lemon sharks are able to rest on the benthos, they spend most of their time swimming (Bouyoucos, Suski, Mandelman, & Brooks, 2018), and the slight increase in activity during periods when sharks are normally active and swimming was not sufficient to substantially affect routine metabolic rates. This suggests that species that are naturally more active, such as pelagic species, are less likely to be affected by wildlife tourism events at the level of their metabolic rates than benthic species that would naturally rest during the day.

In the absence of a significant effect on shark activity, the observed differences in metabolic rates were driven primarily by changes in ambient water temperatures. This was expected, as temperature is the most important external factor determining energy expenditure in ectotherms like sharks, with basal or standard metabolic rates of ectotherms doubling to tripling with every 10 °C increase in temperature (Clarke & Johnston, 1999; Gillooly, Brown, West, Savage, & Charnov, 2001). While the interaction between temperature and period seemed important in the model, the effect size was relatively small and did not exceed the variation in metabolic rate that occurs throughout the day in lemon sharks (Bouyoucos et al., 2018). Given the extent of the temperature effect on the metabolic rates of sharks, it appears unlikely that short, daily feeding events (ca. 1 h) is substantial relative to the temperature effect. This line of reasoning leads us to conclude that the effect of feeding events will be most apparent at times of stable ambient temperatures.

Spatial Distribution and Fine-Scale Movement Patterns

The broad space use (core area and home range) of juvenile lemon sharks was not influenced by 1 h daily feeding events. Within Aya's Spot, however, there was a clear shift in space use towards the feeding site. The shift lasted for at least 90 days after feeding cessation, further illustrating an extended learning extinction period in these sharks. It also demonstrates that a feeding period as short as 27 days can have significant, long-lasting effects on the fine-scale space use of juvenile sharks. Previous studies support our results, even though most were carried out at well-established feeding sites (Brunnschweiler & Baensch, 2011; Clua, Buray, Legendre, Mourier, & Planes, 2010; Corcoran et al., 2013). For instance, Brunnschweiler and Barnett (2013) demonstrated a change in the fine-scale movements of bull sharks at a feeding site in Fiji with sharks visiting the feeding site for more extended periods on feeding days than on nonfeeding days (Brunnschweiler & Barnett, 2013). The extended home range during the first 30 days of recovery could indicate that sharks were searching for the former feeding site landmarks in the area surrounding Aya's Spot in case the feeding site had moved. Once they were satisfied that the feeding location was nowhere to be found their home ranges returned to baseline levels.

The number of sharks present at the feeding site increased from only three at the start of the daily feeding events (two tagged, one untagged) to ca. 10 within 10 days of feeding. Of the maximum number of 10 sharks we observed during feeding, six were tagged and four were untagged. The recruitment we observed at Aya's Spot during the feeding events might be linked to social learning, which has previously been demonstrated in elasmobranchs (Pouca, Heinrich, Huveneers, & Brown, 2020; Thonhauser et al., 2013), including juvenile lemon sharks (Guttridge et al., 2013). Increases in shark abundance are commonly reported at feeding sites, such as with bull sharks in Fiji (Brunnschweiler, Abrantes, & Barnett, 2014) and southern stingrays, *Dasyatis americana*, in the Grand Cayman Islands (Corcoran et al., 2013; Semeniuk & Rothley, 2008). These changes in abundance can have severe, detrimental effects on the health of the animals, including increased parasite loads, increased conspecific bites and a reduced overall condition (Corcoran et al., 2013; Semeniuk & Rothley, 2008). However, the southern stingray is the only species of elasmobranchs that has so far been reported to suffer such severe detrimental effects from wildlife tourism operations (Trave, Brunnschweiler, Sheaves, Diedrich, & Barnett, 2017). Furthermore, given the natural group-living conditions that juvenile lemon sharks are exposed to daily inside Aya's Spot, it is unlikely they would suffer from the same effects, at least not to the extent witnessed in southern stingrays.

Conclusion

The short feeding period of only 27 days had a significant impact on the fine-scale movement pattern and the Burst Activity of juvenile lemon sharks, indicating that sharks are able to learn associations at a similar rate in natural and laboratory conditions (Fuss & Schluessel, 2015; e.g. ; Guttridge & Brown, 2013). Daily feeding events may result in quick behavioural changes via conditioning, which may be retained for at least 90 days in the absence of any further reinforcement. These responses, in particular the changes in fine-scale spatial distribution, appear to be similar across different species (Barnett et al., 2016; Brunnschweiler & Barnett, 2013; Corcoran et al., 2013). In contrast, the physiological response, metabolic rate, was not substantially affected, and this was probably due to feeding events occurring at times when lemon sharks are naturally active. The ability to collect baseline data prior to the initiation of daily feeding events replicating standard tourism operations provided a unique opportunity to assess how wildlife tourism affects shark behaviour. Our study shows that, while changes in shark behaviour were observed, these changes were spatially restricted and only occurred close to the feeding event. Some of the observed local effects were, however, long lasting. In conclusion, our experiment had minimal effects on the metabolic rate of juvenile lemon sharks, but managers of wildlife tourism industries should consider the potential impacts of long-lasting changes in shark behaviour, even if they are likely to be localized to the feeding site and time. However, the small sample size of our study needs to be taken into consideration. While our results indicate possible effects of feeding activities on the behavioural ecology and spatial distribution of sharks they do not necesseraily apply to all species and indiviuals equally. This is shown by the individual we tagged that, despite being present inside Aya's Spot on feeding days, never participated in any of the feeding events. We therefore recommend to run small-scale pilot studies on target species/populations before establishing new feeding operations to ensure the most sustainable strategies are applied and the negative impacts are minimized.

Author Contributions

All authors contributed to the study design. D.H., F.D., M.S. and T.G. set up the experiments. D.H. collected the data with the help of the station's volunteers, ran the statistical analysis and drafted the manuscript. All authors revised the manuscript, gave final approval and are accountable for accuracy.

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Appendix

Table A1
Ranking of all models assessing the Burst Activity and Spatial Attraction

Model	*df*	AIC_c	ΔAIC_c	wAIC_c
Burst Activity				
Period * Time	17	6784.22	0.00	1.00
Period + Time	9	6837.05	52.83	0.00
Period	7	6901.02	116.79	0.00
Time	5	6973.35	189.13	0.00
(Null)	3	7040.76	256.53	0.00
Spatial Attraction				
Period * Time	17	3201.46	0.00	1.00
Period + Time	9	3734.01	532.54	0.00
Time	5	3803.25	601.79	0.00
Period	7	5236.55	2035.09	0.00
(Null)	3	5298.49	2097.03	0.00

AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top-ranked model, wAIC_c = model probability. Shark ID and Day were included as random factors in all models. For models that contained an interaction, single factors were also included, but not listed in the table.

Table A2
Ranking of all possible models assessing the Burst Activity and Spatial Attraction around the night-time high tides

Model	*df*	AIC_c	ΔAIC_c	wAIC_c
Burst Activity				
Period + Time	9	6737.80	0.00	0.9
Period * Time	17	6742.23	4.43	0.1
Period	7	6753.85	16.05	0.0
Time	5	6833.71	95.91	0.0
(Null)	3	6849.95	112.15	0.0
Spatial Attraction				
Period * Time	17	3292.33	0.00	1.0
Period + Time	9	3335.21	42.88	0.0
Time	5	3374.58	82.25	0.0
Period	7	4525.02	1232.69	0.0
(Null)	3	4561.03	1268.70	0.0

AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top-ranked model, wAIC_c = model probability. Shark ID and Day were included as random factors in all models. For models that contained an interaction, single factors were also included, but not listed separately in the table.

Table A3
Summary table for the models investigating the Period and Time effects on the Burst Activity and Spatial Attraction around night-time high tides

Effect	β	SE	*Z*	*P*
Burst Activity				
(Intercept)	−0.923	0.227	−4.072	<0.001
Period (feeding)	0.130	0.243	0.534	0.593
Period (recovery 30)	−0.765	0.227	−3.368	<0.001
Period (recovery 60)	−1.006	0.226	−4.449	<0.001
Period (recovery 90)	−1.401	0.253	−5.542	<0.001
Time (during)	−0.165	0.176	−0.942	0.346
Time (after)	−0.232	0.180	−1.288	0.198
Period (feeding) * Time (during)	0.0549	0.263	0.209	0.834
Period (recovery 30) * Time (during)	0.0423	0.268	0.158	0.875
Period (Recovery 60) * Time (during)	−0.174	0.272	−0.640	0.522
Period (recovery 90) * Time (during)	−0.00973	0.285	−0.034	0.973
Period (feeding) * Time (after)	−0.499	0.270	−1.850	0.064
Period (recovery 30) * Time (after)	0.0127	0.256	0.050	0.960
Period (recovery 60) * Time (after)	−0.176	0.261	−0.676	0.499
Period (recovery 90) * Time (after)	−0.0272	0.284	−0.096	0.924
Conditional R^2				0.16
Spatial Attraction				
(Intercept)	−2.437	0.854	−2.853	0.004
Period (feeding)	0.768	1.012	0.759	0.448
Period (recovery 30)	4.044	0.953	4.243	<0.001
Period (recovery 60)	3.781	0.940	4.023	<0.001
Period (recovery 90)	−0.531	0.982	−0.541	0.589
Time (during)	−4.101	0.546	−7.516	<0.001
Time (after)	−5.029	0.572	−8.798	<0.001
Period (feeding) * Time (during)	0.137	0.713	0.193	0.847
Period (recovery 30) * Time (during)	−0.637	0.675	−0.943	0.345
Period (recovery 60) * Time (during)	1.348	0.590	2.284	0.022
Period (recovery 90) * Time (during)	0.502	0.672	0.746	0.456
Period (feeding) * Time (after)	−0.858	0.871	−0.984	0.325
Period (recovery 30) * Time (after)	−0.644	0.694	−0.927	0.354
Period (recovery 60) * Time (after)	1.977	0.603	3.280	0.001
Period (recovery 90) * Time (after)	0.924	0.656	1.408	0.159
Conditional R^2				0.86

Estimated effect coefficients (β) and their standard errors (SE), *z* values and the individual coefficient type I error estimate (*P*). Time (during) = 1 h of feeding, Time (after) = 1 h postfeeding, Period (feeding) = feeding month, Period (recovery 30) = first 30 days of recovery, Period (recovery 60) = second 30 days of recovery, Period (recovery 90) = third 30 days of recovery. The baseline level for the factor Period was the baseline month. For the factor Time, the baseline level was the 1 h prefeeding before the feeding event.

Table A4
Ranking of all possible models assessing the mean daily activity and mean daily metabolic rates

Model	*df*	AIC_c	ΔAIC_c	wAIC_c
Mean daily activity				
Period * Temperature	27	111285.3	0.00	1.00
Period + Temperature	23	111326.4	41.19	0.00
Temperature	19	111342.5	57.22	0.00
Period	22	111350.9	65.65	0.00
(Null)	18	111366.9	81.67	0.00
Mean daily metabolic rates				
Period * Temperature	13	−80668.84	0.00	1.00
Period + Temperature	9	−80584.77	84.07	0.00
Temperature	5	−80473.31	195.53	0.00
Period	8	−68614.08	12054.76	0.00
(Null)	4	−68534.66	12134.18	0.00

AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top-ranked model, wAIC_c = model probability. Shark ID was included as a random factor in all models. For models that contained an interaction, single factors were also included, but not listed separately in the table.

Table A5
Ranking of all possible models assessing the core areas and home ranges

Model	*df*	AIC_c	ΔAIC_c	$wAIC_c$
Core area				
Null	3	795.68	0.00	0.93
Period	7	800.78	5.1	0.07
Home range				
Period	7	898.66	0.00	0.93
Null	3	903.77	5.1	0.07

AIC_c = Akaike's information criterion corrected for small sample size, ΔAIC_c = the difference in AIC_c between the current and the top-ranked model, $wAIC_c$ = model probability. Shark ID was included as a random factor in all models.

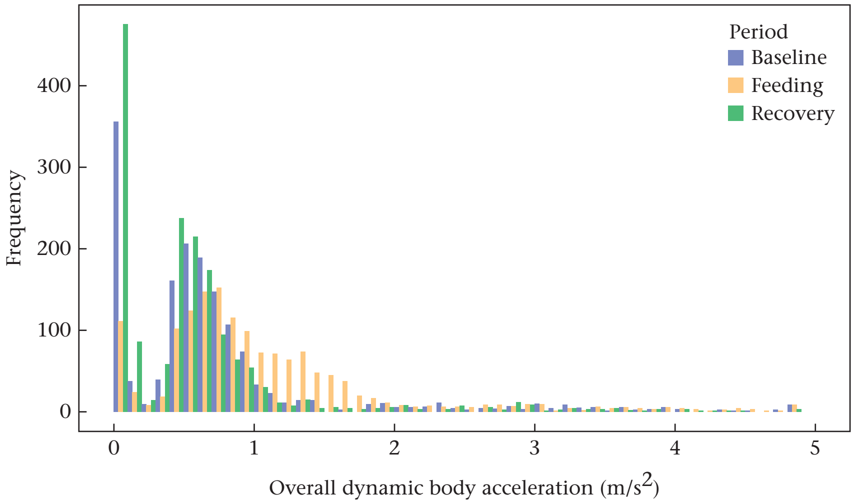

Figure A1.Frequency distribution of shark overall dynamic body acceleration across the baseline, feeding and combined recovery periods. The data used only included the three 1 h windows of the daytime high tides we focused on during our investigation of the anticipatory behaviour of the sharks.

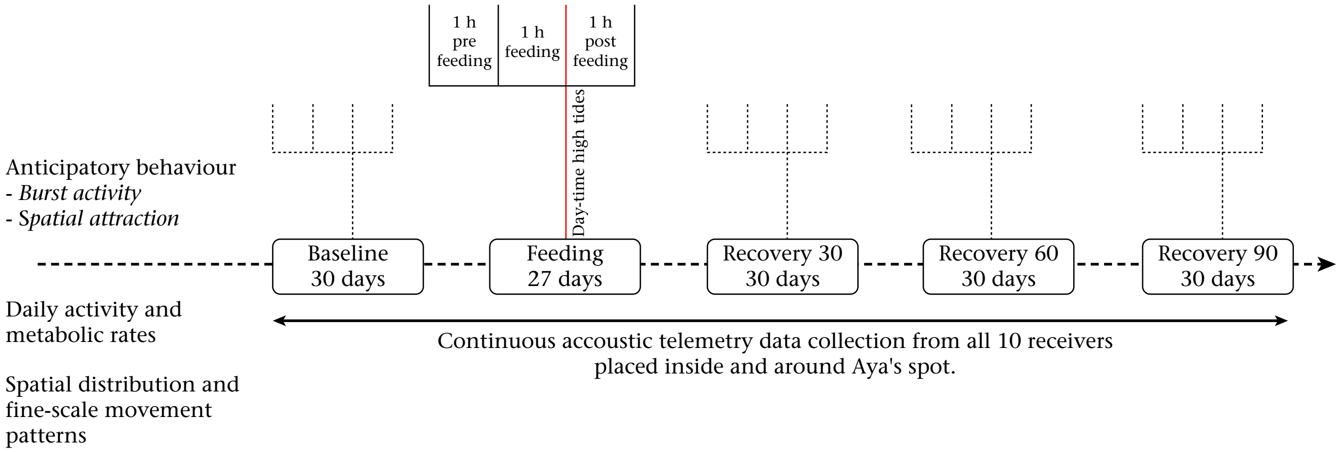

Figure A2. Experimental timeline, showing the length of each of the five periods and illustrating the 3 h window around the high tides.

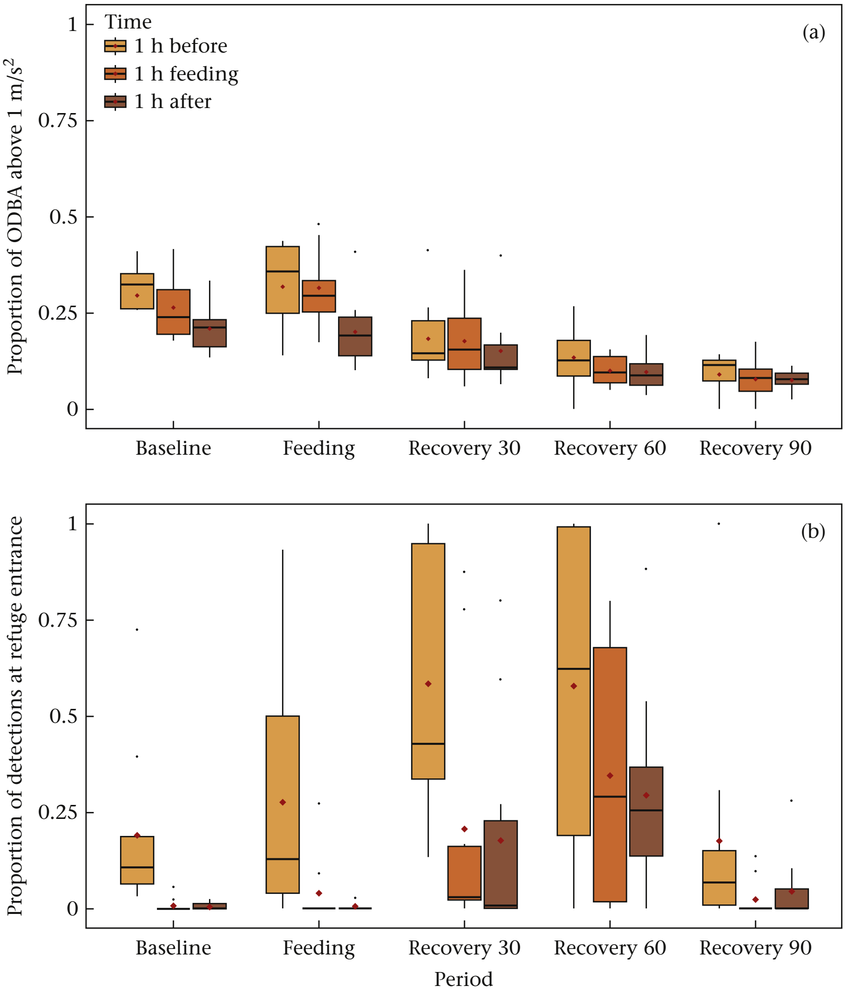

Figure A3. Box plot illustrating (a) the proportion of overall dynamic body acceleration (ODBA, m/s^2) above 1 m/s^2, and (b) the proportion of detections made at the refuge entrance during prefeeding, feeding and postfeeding around the night-time high tides plotted against the Period. The horizontal lines inside the boxes mark the median and the red diamonds the mean for 1 h before, during and after feeding events. The upper and lower boundaries of the box represent the third and first quartiles. The whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers.