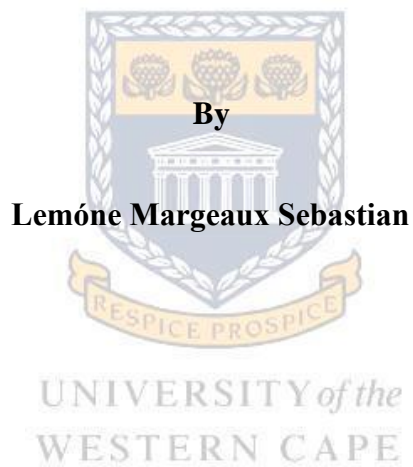


Tonic immobility in two species of southern African catsharks

Department of Biodiversity & Conservation Biology

University of the Western Cape

Private Bag X17, Bellville, 7535, South Africa



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Supervisor: Dr Vanessa Couldridge

Co-supervisor: Dr Enrico Gennari

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Abstract

Tonic immobility (TI) can be defined as an unlearned behavioural response described by a physical state of immobility. This behaviour can last from a few seconds up to several hours. Tonic immobility in sharks has not been investigated extensively, despite being observed and used widely. Due to this limited research, there is still uncertainty about the significance of this response, especially in smaller shark species. Anecdotal evidence suggests that females may enter a tonic state during courtship, but this has never been scientifically investigated. Alternatively, tonic immobility may function as an anti-predator response. The aim of this study was thus to examine the behaviour and physiology associated with tonic immobility in two closely related species of catshark, *Haploblepharus edwardsii* and *Haploblepharus pictus*, and to test for differences between males and females (if the evolutionary significance of TI was related to courtship), and between the two species (if TI evolved in response to species-specific drivers). This was done by manually inducing the catsharks into a state of tonic immobility. The induction time and duration time of TI, as well as respiration rate and heart rate of the sharks were recorded. The study demonstrated that both *Haploblepharus pictus* and *Haploblepharus edwardsii* do exhibit a tonic immobility response following human-induced inversion and it was evident that the way tonic immobility was expressed in these species is similar to other shark species. The time taken to induce TI differed significantly between species, taking longer in *H. pictus*, but the duration of TI did not differ. Results also showed that both sexes could be induced into a tonic state and there was no significant difference in either induction or duration times of TI between sexes. The study found that the responses of individuals did vary with repeated trials, but that there was no clear pattern of habituation to tonic immobility. In addition, there was no significant difference in the respiration rate before, during, and after TI between species or between sexes. The heart rate during TI was significantly higher in *H. pictus* but did not differ between sexes. There is still some uncertainty about the evolutionary significance of TI in these two species of catshark, but these results provide no evidence that TI is a sex-specific response, thus suggesting that TI has evolved primarily as a defense mechanism. This study has increased our understanding about the variability of TI in two ecologically similar species.

Keywords

Tonic immobility, catsharks, duration, induction, respiration, heart rate

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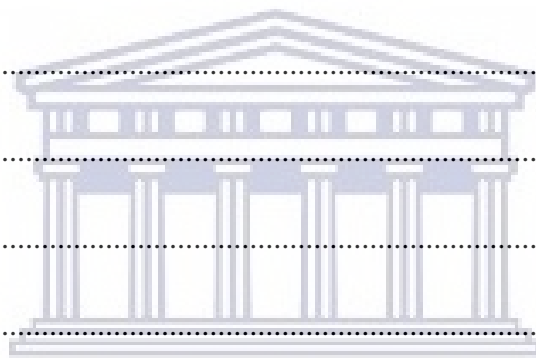
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Chapter 1: Introduction

Tonic immobility

Tonic immobility (TI) can be defined as an unlearned behavioural response described by a state of immobility (Watsky & Gruber 1990). It is a distinctive behaviour which is observed in a variety of animal taxa and described as a partial paralysis of the body in response to external stimuli (Rusli *et al.* 2016). Tonic immobility is characterized by a state of physical immobility, a “limp” body, trembling of the extremities, decreased vocalizations, eye closure, and changed physiological parameters such as the core body temperature, heart and respiration rate (Henning & Dunlap 1978). The behaviour of tonic immobility differs between animals, it may last from a few seconds in some and up to a several hours in others (Rusli *et al.* 2016). When an animal is in a state of tonic immobility, it is capable of the extinction as well as the retrieval of previously known associations (Watsky & Gruber 1990). The natural state of immobility that an animal displays acts as an anti-predator mechanism that allows an animal to escape detection or deter an attacking predator (Dodd 1989). These natural immobility techniques that animals adopt include death feigning, hypnosis, thanatosis and catatonia (Dodd 1989). Tonic immobility on the other hand is a behaviour that needs to be induced by briefly restraining an animal (Henning & Dunlap 1978).

The label, tonic immobility, has evolved over the centuries and there are a wide variety of common names given to tonic immobility that span across various languages such as English, Spanish, German, etc. (Gallup & Maser 1977). The phenomenon of tonic immobility has very often been blended or confused with other immobility behaviours such as death feigning, freezing, catalepsy, and hypnosis (Gallup & Maser 1977).

Catalepsy

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Catalepsy refers to limp muscle weakness which is triggered by emotions such as anger or humour (Overeem *et al.* 2002). This behaviour is characterized by the partial expression of rapid eye movement sleep, known as REM, which occurs at an inappropriate time (Overeem *et al.* 2002). Catalepsy could possibly be related to an ancestral trait of tonic immobility (Overeem *et al.* 2002). This term describes a complex exchange between systems that involve their emotions and motor controls. This behaviour is also observed during thanatosis (Nishino 1996).

Thanatosis/ Death feigning

Thanatosis is a type of animal hypnosis which is observed throughout the animal kingdom, ranging from invertebrates to humans. This behaviour is characterized as a startle response to mechanical stimuli (Nishino 1996). This is a well-known behaviour aimed to reduce an individual's mortality against predators (Franq 1969). It is known as a very stereotypical behaviour pattern whereby the animal would "freeze" momentarily with its head facing forward, and then flex ventrally by bending its head towards its chest. The abdominal and ventral muscles are contracted, and the tail would sometimes curl between the legs (Franq 1969).

Catatonia

Catatonia is a very puzzling psychiatric syndrome because it occurs for no apparent reason. The subject would mute, 'freeze' for a few minutes/hours without any awareness of the external environment (Moskowitz 2004). The subject would appear oblivious to pain and allow their limbs to be in all sorts of unusual and awkward positions (Moskowitz 2004).

Hypnosis

Hypnosis dates back to the 1600s and this behaviour is described by placing an animal into an uncomfortable position, whereby the animal would be induced into a state of

consciousness (Gilman *et al.* 1949). This would result in the animal experiencing some sort of entrancement (Gilman *et al.* 1949). The term “animal hypnosis” applies to a broad class of behaviours that animals display, such as immobility and decreased responsiveness that is brought about by various stimuli (Crawford 1977).

Immobility reflex

Immobility reflex is described by a reversible cataleptic immobility that is frequently prompted by manual restraint or a visual complex (Smith & Klemm 1977).

The above terms have been created by past and present scientists (Gallup & Maser 1977) and these terms often only touch on the source or purpose of the behaviour (Erhard *et al.* 1999). These terms do have a few things in common, where there is a form of physical restraint and a reversible physical immobility where the animal is able to make an instant transition from an immobile to a mobile state (Erhard *et al.* 1999). Tonic immobility is the preferred term as it best describes the behaviour pattern which is most common to and most noticeable in all animals (Prestrude & Crawford 1970). Over the years, it was established that tonic immobility is a more neutral term that describes a very complex phenomenon (Erhard *et al.* 1999; Crawford 1977). Previous studies have however used the term immobility when describing tonic immobility.

Ratner (1967) designed four measures of immobility that include the induction time, animals exhibiting an immobile posture, duration time and the type of stimulation that would be required to terminate immobility. A few dynamic characteristics were also identified to be associated with these measures, such as struggling after the animal was initially grasped. The struggle would then subside, and a tonic posture would be assumed. The animal would become less responsive and changes in physiological factors would be observed, and then the termination of the tonic state would be sudden and abrupt (Ratner 1967). Tonic immobility has been discussed for over a century in

previous literature, but these early studies were more observational and qualitative (Humphreys & Ruxton 2018).

Tonic immobility has been observed across a variety of animals, such as fish, amphibians, bird, reptiles, and mammals (Watsky & Gruber 1990; Rusli *et al.* 2016). In earlier years, the majority of studies on tonic immobility concentrated on avian species and these studies reported that tonic immobility was used as a fear response and that it would help reduce further predatory attacks (Ratner & Thompson 1960; Eyer & Ratner 1975; Gentle *et al.* 1989). Studies have shown that various animals have adopted this behaviour as a type of defense mechanism, where tonic immobility was seen as an indication of fear to challenging situations (Erhard & Mendl 1999). Immobility and fight or flight are seen as opposing defense strategies to threatening situations (Erhard & Mendl 1999). The fight or flight strategy represents an active response, whereas immobility is seen as a more passive response (Erhard & Mendl 1999).

Four fundamental mammalian defenses were listed by Cantor (2009) and these included withdrawal, aggressive defense, appeasement, and immobility (where two types of immobility were discussed, attentive and tonic). The withdrawal defense is when an animal uses the flight response to seek refuge in places that are inaccessible to predators (Cantor 2009). A rise in the risk of predation would lead to an increase in animals seeking refuge when under attack (Cantor 2009). An aggressive defense is separated into a confrontational response and a more threatening “fight” response. An example of an effective technique would be retaliation and if that fails, then an animal would initiate a more energy demanding fight response that would be riskier (Cantor 2009).

Appeasement has been detailed by Cantor and Price (2007) and both animal and human findings have suggested that appeasement forms the basis of complex PTSD (post-traumatic stress disorder). The definition of tonic immobility has already been stated, but the attentive type has been described as a temporary state of increased stimulation

and hypervigilance that occurs after the identification of a potential threat (Cantor 2009). Attentive immobility provides crypsis and helps prepare an individual for the fight or flight response. The only things that both attentive immobility and tonic immobility have in common are the fact that a state of immobility is assumed (Cantor 2009). Cantor (2009) describes tonic immobility as a last resort response or last desperate measure that an animal can display. The state of tonic immobility is an involuntary state of motor impediment that occurs despite the perseverance of awareness which activates during intense fear. Tonic immobility promotes survival through the repression of predator killing reflexes, confusing predators, deterring them by appearing as diseased, dead meat and reducing blood pressure, which helps reduce blood loss when wounded (Cantor 2009).

Some previous studies focused solely on the response to tonic immobility. For example, Erhard and Mendl (1999) detailed the phenomenon of tonic immobility in pigs and their sensitivity to and/or duration of tonic immobility. They looked at whether the phenomenon was possibly related to the fear response experienced by the pigs. They discovered that the sensitivity and duration of tonic immobility would vary from one individual to the next (Erhard *et al.* 1999). Nakasi *et al.* (2013) used tonic immobility to test for the detection of fear within Japanese chickens at different stages of development. They found that a tonic immobility response was present during the early developmental stages but became more enhanced during the later stages of development. The study noted that genetic differences and sex had an impact or influence on the development of fear and tonic immobility response. A study by Gallup (1973) used the presence of a predator (hawk) to determine whether lizards remained in a state of tonic immobility for an extended period of time. This study used the common form of restraint that is used during tonic immobility, which involves restraining the animal until it becomes “limp”. This study used a “false” predator, and it was discovered that the lizard would remain in a tonic state longer when in the presence of

this “false” predator thus suggesting that tonic immobility was related to a fear response.

Studies done by Brodie *et al.* (1974) and Dodd (1989) tested the phenomenon of immobility in salamanders, where forms of tactile stimuli were used. Individuals (salamanders) in the study were subjected to tapping with a probe, pinching, touching and gentle restraint. The experimenters used this form of approach to mimic the predator attacking the salamander. The duration of the immobile state was recorded and the expression of immobility in the body was noted. Brodie *et al.* (1974) discovered that the salamanders exhibited an immobile response and that they could be moved around without any response. The salamanders would also secrete a toxin through the skin. The immobility and toxin secretion would allow the salamander to escape detection through crypsis, or if detected, to lessen the attack, or deter the predator or cause the predator to lose interest. In a later study, Dodd (1989) exposed the salamanders to different environmental temperatures and observed whether that would affect the duration of their immobile state. Dodd (1989) found that the immobility durations were longer at lower temperatures than at higher temperatures. This showed that environmental factors do impact the immobile state of salamanders.

Miyatake *et al.* (2009) conducted an interesting study on a beetle, which looked at these beetles using tonic immobility as a means to survive predator attacks, which results in the sacrifice of their neighbours. Immobility was seen as an effective survival tactic, but at the expense of more mobile prey. When the beetle remained in an immobile state it was found that the predator (spider) would lose interest. This demonstration of the predator trait (losing interest) and prey action (remaining in an immobile state) could have a significant variation in the predation frequency between mobile and immobile individuals, which could favour the evolution of tonic immobility behaviour.

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Previous studies have also compared tonic immobility between wild and captive individuals. Henning & Dunlap (1978) conducted various experiments on the lizard, as earlier studies had shown that this lizard responds well to tonic immobility. The first phase of the experiment 1 was to observe the effects of repeated testing of tonic immobility on the lizards. This first phase was carried out at 24-hour intervals during the first seven days in captivity. This was followed by a second phase where daily tonic immobility tests were done on another group that were adapted to captivity before testing and these results were compared with the first phase. The second experiment determined whether there was an increase in tonic immobility for the phase 1 lizard, on Day 3 of the experiment, and this depended on the repeated stimulation and if the stress of crowding had an impact. The third experiment tested whether the increased variability of tonic immobility durations in captivity was a hindered response to the original capture or whether another aspect of captivity had an impact.

In addition to this, the result of repeated capture and handling of tonic immobility was examined. The fourth experiment determined whether the presence of foliage had an effect, because previous studies done by Henning *et al.* (1976) noted that foliage could shorten the duration of tonic immobility under certain conditions. Lastly experiment 5 compared the results of experiments 1 and 4. They expected a decrease in tonic immobility that was found in experiment 1 in the presence of foliage. They found that after the lizards' third day in captivity, the duration and variability of tonic immobility had increased. However, they also found that when lizards had been released and then recaptured there was a slight decrease (non-significant) in tonic immobility duration, which suggests that environmental conditions were having an effect on the length of tonic immobility events. It was also found that in the presence of foliage the lizards became habituated to tonic immobility.

Habituation is when an organism passively refrains from further testing, which results in a reduction in duration strength (Henning & Dunlap 1978).

Another study by Passos *et al.* (2017) compared the tonic immobility responses between captive and wild frogs. This study found that wild frogs would remain in a tonic state longer than captive frogs. Similar to the Henning & Dunlap (1978) study, the captive frogs became habituated to handling and human/experimenter interaction. These studies revealed that wild species in captivity react differently to domesticated species. These experiments also showed that it is important to consider the initial stress in acclimatising species to the conditions of captivity (Passos *et al.* 2017).

Some studies have shown that habituation to tonic immobility occurs through repeated stimulation, thus decreasing the animal's tonic immobility response over time. For example, Nash & Gallup (1976) conducted a study on domestic chickens. This study defined various experiments which were run to look at the relationship between habituation and tonic immobility. The study found that the repeated stimulation of tonic immobility, along with human handling led to decreased durations of tonic immobility. They also found that the chickens became less susceptible to being induced into tonic immobility after repeated stimulation. In another study, Lefebvre & Sabourin (1977) tested the response of the goldfish, to repeated massed and spaced inductions into tonic immobility. Massed inductions refer to inducing the goldfish more than once during the course of the day, whereas spaced inductions refer to inducing the goldfish once a day. The study found that habituation followed an initial exposure to repeated tonic immobility. Smith & Klemm (1977) tested habituation of the immobility reflex in rabbits and the effect of fear inducing stimulation on the rabbits. It was evident that habituation did occur for immobility durations, although some rabbits did not habituate, demonstrating individual variation (Smith & Klemm 1977).

Physiological changes have also been observed to be associated with tonic immobility. Prestrude & Crawford (1970) conducted a study on lizards, where they recorded the duration time of the tonic state and observed any physiological changes that occurred.

They found that this species was an ideal candidate for tonic immobility tests and they also noted that there were changes in the lizards' respiration rate during tonic immobility. Prestrude & Crawford (1970) discovered that prolonged tonic immobility did not necessarily correspond with the fear response, but rather that it could be related to increased brain stem activity. Gentle *et al.* (1989) studied the domestic fowl, where they observed the characteristic changes that occur during tonic immobility. They discovered that changes do occur in physiological factors, such as heart rate and brain activity, during a tonic episode. The physiological changes suggested that the induction into tonic was stressful, but the stress would decline as a state of immobility was assumed. The changes in physiological factors during a tonic state would decrease the fowls' state of fear and therefore increase its survival by avoiding further predatory attack.

Although the above studies have focused on various aspects of tonic immobility, a recurring interpretation that can be seen is that the behaviour has evolved as a predatory defense mechanism (Gallup 1973). Tonic immobility is thought to be a defensive tactic that is initiated when a predator attack (Brooks *et al.* 2011). The voluntary immobility is a conscious decision to help decrease the chance of being detected by a predator, where heightened responsiveness transitions to a 'flight or fight' response (Brooks *et al.* 2011). Tonic immobility, as an anti-predatory response, is adopted by the prey to reduce death and thus increases the animal's chance of survival (Passo *et al.* 2017). These studies mention that environmental (wild and captive) factors could have an impact on tonic immobility. In the wild, factors such as stimulus intensity, predation pressure, stress levels and environmental disturbances can impact TI duration (Passo *et al.* 2017). For animals in a captive setting, stimuli such as sight of predators, loud noises, and extreme temperatures, can affect the duration of the TI response (Passo *et al.* 2017).

The majority of the research on tonic immobility within the animal kingdom has solely focused on the predator avoidance hypothesis (Rusli *et al.* 2016). Studies have mentioned that tonic immobility has evolved as a way to increase the prey's chance of survival. Studies have shown that there are benefits associated with using TI as an anti-predatory mechanism but there are also related costs involved (Humphreys & Ruxton 2018). These costs would have an impact on evolutionary trade-offs in species which would influence the way that animals express tonic immobility and the frequency of tonic immobility (Humphreys & Ruxton 2018). Some other traits that influence TI include the conditions that occur during rearing, sexual history, size and sex (Humphreys & Ruxton 2018). Some alternative last effort anti-predator behaviours that animals exhibit include the animal struggling with the predator, but eventually escaping or confronting the predator and attacking. These last effort behaviours would result in the animal experiencing a gain or loss and this would ultimately impact the expression of tonic immobility (Humphreys & Ruxton 2018). Some studies have shown that certain environmental aspects would influence the adaptive significance of tonic immobility.

Tonic immobility in sharks

Tonic immobility in sharks has been a topic that has not been researched extensively. Furthermore, the few studies that have looked at tonic immobility have concentrated on larger – bodied species of shark, such as nurse sharks (Klimley 1980), lemon sharks (Watsky & Gruber 1990; Edward *et al.* 2011), smooth dogfish sharks (Whiteman *et al.* 2013), black tipped reef sharks (Davie *et al.* 1993), whitetip reef sharks (Whitney *et al.* 2004), Caribbean reef sharks (Brooks *et al.* 2012) and zebra sharks (Williamson *et al.* 2018).

A review of elasmobranch literature (Heist 2004) revealed a few interesting points: firstly, when it comes to elasmobranchs, there are not many studies on the ecological or foraging roles. The majority of studies focus more on their role as a predator, but

this is an important oversight as most elasmobranchs are seen as both predator and prey at some point in their life history. Secondly, studies have shown that there are many ways that elasmobranchs avoid falling prey to predators. One of these many tactics includes a defense or a deterrence mechanism (Heist 2004). Lastly, studies have suggested that some elasmobranchs have evolved a number of defensive morphological characters to protect themselves from predators (Heist 2004).

When looking at the research done on sharks, the studies lean more to examining the use of tonic immobility as a tool to help immobilise the shark (Henningesen 1994). This is done for a variety of procedures that require handling of the animal (Henningesen 1994). Tonic immobility has been used as a physical anaesthesia technique that involves the rapid lowering of the animal's body temperature and electroanaesthesia (uses electric current to immobilise the animal). There are four stages of anaesthesia and tonic immobility equates to a stage IIb (deeper anaesthesia), where there is a reduction of muscle tone and stability, and lack of ventilation (Kessel & Hussey 2015). However, because of the unresponsiveness to painful stimulation associated with this technique, tonic immobility could arguably induce stage III anaesthesia (surgical), which is similar to stage IIb, but there is no reaction to major stimulation (Kessel & Hussey 2015). Tonic immobility has widely been used as an anaesthetic technique for surgical implantation for elasmobranchs since the first acoustic telemetry study (Kessel & Hussey 2015). However, the technique largely has been overlooked in guideline materials and it has been put into question as to whether it is an acceptable technique for ethical anaesthesia by some ethics committee and regulatory bodies (Kessel & Hussey 2015). The advantage of using tonic immobility as a technique is that it provides immediate recovery when the individual is righted and there is minimal disruption to the animal's respiration (Kessel & Hussey 2015). Caution has to be taken however as some species experience adverse effects when they are under a tonic state for too long (Reebs 2007).

Studies have also shown that tonic immobility can be used to help safely hold and handle sharks, after being captured, for scientific investigation and/or aquarium husbandry (Brooks *et al.* 2011). Tonic immobility is sometimes used as an aid to assist with the handling of animals during different procedures (Henningsen 1994). The goal for this is to minimize any struggling that could be displayed by the animal (Henningsen 1994). Tonic immobility can thus be used as a form of anesthesia in the field and/or in an aquarium set up, when conducting minor surgery, or an overall examination of the animal (Reebs 2007). Some of these surgical procedures include the implanting of acoustic transmitters in sharks (Kessel & Hussey 2015). Another scenario in which TI is used is when anglers have to remove the hook from fish that are later released (Davie *et al.* 1993). They carefully remove the hook when the animal is immobilised, and this helps to improve the survival chances of the animal after release (Davie *et al.* 1993).

During tonic events, there are physiological and behavioural effects that occur. A study by Brooks *et al.* (2011) investigated these effects on juvenile lemon sharks. This study looked at the effect of prolonged tonic immobility, where the sharks were exposed to four, three-hour treatments which differed by the method of keeping the sharks. The sharks were either held in a state of tonic immobility, or the sharks were allowed to swim freely between blood tests (Brooks *et al.* 2011). Both sharks (individuals kept in state of tonic immobility or individuals allowed to swim freely) were either incorporated with the presence or absence of an exercise period (Brooks *et al.* 2011).

The exercise period replicates the physiological pressure that leads up to tonic immobility in the wild (Brooks *et al.* 2011). Results from this study suggested that tonic immobility can be seen as a stressful event and that there are short term disruptions in the blood parameters and ventilation efficiency of the lemon sharks (Brooks *et al.* 2011).

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Larger sharks are not typically considered prey, but they do feed on young sharks as well as species of shark categorised as small to medium, some cetaceans, pinnipeds and marine birds (Garla *et al.* 2015). Smaller species of shark would either hide in crevices or holes; or use camouflage to protect themselves (Garla *et al.* 2015). Although smaller shark species do have to protect themselves from larger predators, there is still uncertainty about the significance of this response in not only smaller species, due to limited research on the topic of tonic immobility, but larger species as well (Whiteman & Marshall 1986). Currently the role of tonic immobility in sharks unknown, but some studies have reported tonic immobility in some shark species as a courtship technique (Williamson *et al.* 2018).

When it comes to fishes, internal fertilization is a rather unusual form of reproduction, but for elasmobranchs it is the only form of reproduction (Tricas & Feuvre 1985). Most sharks have a wide roaming range in the ocean, it is often difficult to observe and document their mating behaviour in the wild (Tricas & Feuvre 1985). Very few courtship events have been documented from the wild. These records comprise of verbal explanations and illustrations of courtship, partner formations and mating scars (Tricas & Feuvre 1985). The only detailed accounts of shark copulation have been documented in aquariums (Tricas & Feuvre 1985). Courtship and copulation in sharks begin with the parallel swimming of sharks, which leads to both the male and female lying parallel on the substrate, where the male would then bite the female's pectoral fin. The female would then pivot and roll onto her back where the female would remain motionless; the male would then end up on top of the female where copulation would occur. The male would then roll over onto his back, lying parallel to the female. The male and female would remain in this position until copulation was completed. The above stages were recorded for nurse sharks by Klimley (1980). A study by Whitney

2010, had also revealed similar patterns of movement through the use of acceleration loggers. These loggers identified the changes in movement and orientation of the nurse sharks.

Some studies have made a possible connection between tonic immobility and reproduction. There have been reports of TI being used during courtship rituals by some species (Williamson *et al.* 2018). In addition, there have been recent studies that have observed tonic immobility in the wild (for example, Pratt & Carrier 2001; Kunze & Simmons 2004; Brunnschweiler & Pratt Jr 2008). It has been suggested that male sharks would subject females to tonic immobility during courtship rituals. After males have followed the females, they would then bite the females pectoral fin resulting in a pivot roll where the female would end up on her back, causing the females to be induced into a catatonic state (Williamson *et al.* 2018). The benefits of this would be to either maintain contact during courtship or to induce further cooperation of the female (Klimley 1980).



Background on species

The class Chondrichthyes consists of over 61 families, 200 genera and approximately 1200 species worldwide, which are among the oldest taxa of vertebrates (Camhi *et al.* 2008; Ebert and Hees 2015). The members of this class share a number of features, such as a) an endoskeleton, consisting of calcified cartilage instead of bone, b) four to seven separate internal and external gill openings, c) no lungs or swim bladders, d) paired copulatory organs (claspers) in males which assist with internal fertilization and e) a dermal skeleton of dermal denticles or placoid scales (Compagno 1999).

Cartilaginous fish are predominately marine, with a very small percentage found in freshwater environments (Compagno 1999).

The class Chondrichthyes is further divided into Elasmobranchii (sharks and rays) and Holocephalii (chimeras, ratfishes and elephant fishes) (Compagno 1999). The subclass Holocephalii consists of deep-sea animals that inhabit deep, cool waters (Nair & Zacharia 2004). In contrast, the subclass Elasmobranchii consists of very diverse taxa, which inhabit various types of environments that range from bodies of freshwater to bottom marine trenches and from polar regions to warmer inter-tropical waters (Nair & Zacharia 2004).

Sharks are found within this subclass and they occupy a wide variety of habitats that range from shallow coastal waters to deeper waters of up to 4000 m (Hemstra & Hemstra 2004).

Sharks comprise over 500 known species of elasmobranchs and the number of species continues to grow (Ebert *et al.* 2013). Sharks can be identified by the following features: cylindrical or flattened in shape, five to seven paired gill openings on both sides of the head, pectoral fins that are detached from the head and are found above the gill openings, one large caudal fin, and one or two dorsal fins (Ebert *et al.* 2013).

Southern Africa has the third richest, and most diverse, chondrichthyes fauna in the world, following Australia and Japan (Ebert & Hees 2015). Southern Africa is known to have one of the most diversified cartilaginous fish faunas in the world (Compagno 1999), containing 49 families, 111 genera and over 200 species (Ebert & Hees 2015). The Chondrichthyes within southern Africa are zoogeographically complex; the fauna includes endemics, near endemics and wide-ranging species (Compagno 1999). Southern Africa is a center of endemism for a variety of taxa in families such as Scyliorhinidae, Squaliformes, Chimaeriformes, Porscylliidae, and Triakidae (Compagno 1999). However, despite this rich and diverse fauna, larger and more charismatic sharks overshadow the other shark species (Ebert & Hees 2015). This has led to the 'lost sharks' of southern Africa remaining predominately unknown, to both the public and scientific communities (Ebert & Hees 2015).

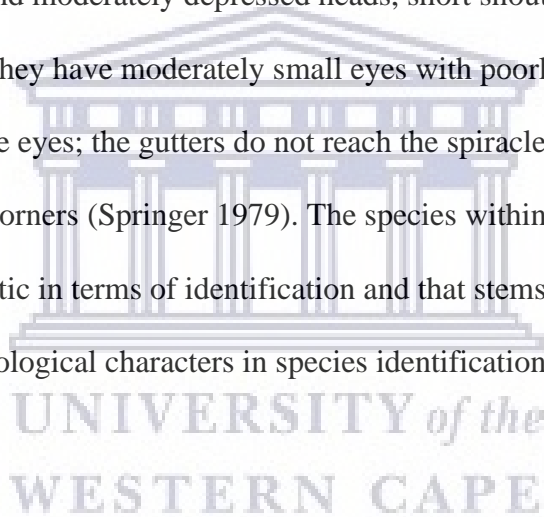
In southern Africa, there is a diverse group of at least eleven endemic species within the Scyliorhinidae family. However, there is very little information known about their biology, distribution and population dynamics (Human 2006). The species within this family are known to be weak swimmers due to their unique fin positioning, which occurs during the fin-to-limb transition during early development (Tanaka 2016); because of this, and their shape, these sharks do not migrate over long distances (Springer 1979). In South Africa they are found in temperate, shallow, benthic areas along the south and southwest coasts (Hemstra & Hemstra 2004).

Many species from the Scyliorhinidae family are relatively abundant in southern Africa; these include sharks from the genera *Halaelurus*, *Haploblepharus*, *Poroderma* and *Scyliorhinus*. Southern Africa has an abnormally large percentage of endemics from this family with a total of two endemic genera, 13 endemic species and a few near endemics (Compagno 1999; Human 2003; Compagno & Human 2003). The genus that formed the basis of this study is *Haploblepharus*, where three species of *Haploblepharus* are

currently recognised (Human, 2003), *Haploblepharus edwardsii* (Schinz 1822), *Haploblepharus pictus* (Müller & Henle 1838), and *Haploblepharus fuscus* (Smith 1950).

Haploblepharus has large confluent nasal flaps that extend over the upper lip and prominent labial furrows that widen around the corners of the mouth to form a continuous groove (Springer 1979). In addition, the connection between the nostrils and the mouth are formed by depressed areas of the upper lip and they lack a caudal crest of modified and enlarged denticles (Springer 1979). The three species mentioned are robust sharks, with wide and moderately depressed heads, short snouts, and wide mouths (Springer 1979). They have moderately small eyes with poorly developed subocular gutters below the eyes; the gutters do not reach the spiracles, which are very close to the posterior eye corners (Springer 1979). The species within this group have been historically problematic in terms of identification and that stems from the use of colour patterns and morphological characters in species identification keys (Springer 1979).

This study concentrated on two species of catshark, *Haploblepharus edwardsii* and *Haploblepharus pictus*, from the catshark family Scyliorhinidae. These species occur between Cape Agulhas and False Bay, where they are commonly found inshore off the coast of South Africa. They are known to be abundant in shallow, coastal waters over sandy or rocky bottoms and kelp beds and are important predators in that environment (Dainty 2002). Studies that have been conducted on the *Haploblepharus* species and on a few topics such as laying patterns, systematics, egg-laying patterns, biogeography, and parasitism. There is still however very little known about the behaviour of tonic immobility within these two species.



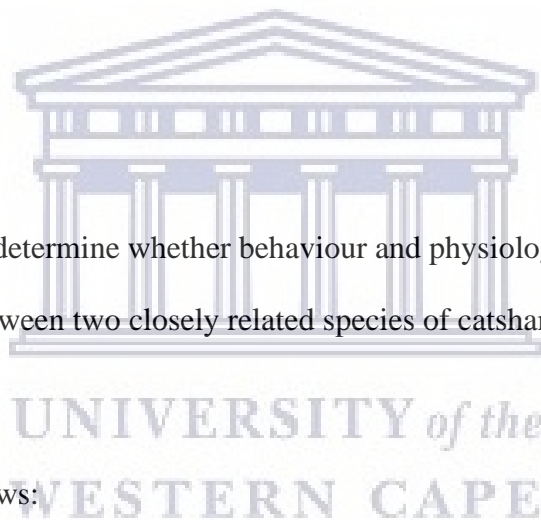
There is limited information on tonic immobility in smaller shark species. Very few studies have focused on tonic immobility as behaviour on its own, but rather on tonic immobility involved in surgical procedures, capturing, and tagging techniques. This study will test the hypothesis of tonic immobility in mating and whether there is a difference or not between sexes. The role of tonic immobility in the copulatory behaviour of smaller benthic sharks does however need more extensive investigating. There is a gap in the literature when it comes to the function of tonic immobility in sharks. In addition, the physiological changes associated with tonic immobility in sharks are poorly documented.

Aims and Objectives

The aim of the study is to determine whether behaviour and physiology associated with tonic immobility differ between two closely related species of catshark and between sexes.

The objectives are as follows:

1. To measure the induction time (time taken for shark to enter tonic immobility) and determine whether it is species and/or sex specific.
2. To measure the duration of tonic immobility and determine whether it is species and/or sex specific.
3. To determine whether the induction and duration times habituate across the repeated trials.
4. To measure physiological parameters (respiration and heart rate) during tonic immobility and to determine whether they are species and/or sex specific.



Chapter 2: General Methods and Materials

Study species

The two species selected for this study were *Haploblepharus pictus* and *Haploblepharus edwardsii*. These two species have previously been confused with one another and often misidentified due to their similar physical appearance (Human 2007). It would thus be interesting to see whether these species have behavioural and physiological similarities or differences.

Haploblepharus edwardsii, also known as the puffadder shyshark, can be identified by its slender body with pale to dark brown/grey-brown dorsal surface, golden brown/reddish saddles and a white underside (Ebert *et al.* 2013) (See Figure 1). The saddles have brown margins and there are white spots that are either found on or between saddles (Ebert *et al.* 2013). The puffadder shyshark has a broad head with large nostrils, frontal nasal flaps that extend to the mouth, and gill slits on the upper sides of the body (Ebert *et al.* 2013). These sharks hatch at about 10 cm and mature at 37-56 cm (males) and 37-55 cm (females) and can grow to a maximum length of 60 cm (Ebert *et al.* 2013). Puffadder shysharks are found along the Eastern and Western Cape coast of South Africa, on the continental shelf, on or near sandy or rocky bottoms. These sharks are very gregarious animals and even in captivity they can be found resting in groups. They feed on small bony fish, polychaetes, crustaceans, cephalopods and fish offal (Ebert *et al.* 2013).



Figure 1: Picture of *Haploblepharus edwardsii* (Murch 2013)

Haploblepharus pictus is commonly known as the dark shyshark and can be identified by its dorsal saddle markings without obvious dark edges, saddles with sparse dots, and the absence of larger white spots between saddles (Ebert *et al.* 2013) (See Figure 2).

Like the puffadder shyshark, this species also has a broad head, large nostrils, frontal nasal flaps that extend to the mouth, and gill slits on the upper side of the body (Ebert *et al.* 2013). They hatch at about 11 cm, mature at 40-57 cm (males) and 36-60 cm (females) and grow to a maximum size of 60 cm (Ebert *et al.* 2013). Dark shysharks are found from central Namibia to East London in South Africa, on the continental shelf, in kelp forests, rocky inshore reefs and sandy areas (Ebert *et al.* 2013). They feed on bony fish, polychaetes and cephalopods in addition to sea snails, echinoderms and occasionally algae (Ebert *et al.* 2013).



Figure 2: Picture of *Haploblepharus pictus* (Verhoog 2018).

Collection Site

The sharks used in this study were collected from two sites, the False Bay Yacht Club (34°11.541'S 18°26.047'E) and Granger Bay (33.9044° S, 18.4149° E) (See Figure 3), both located in Cape Town, South Africa. The False Bay Yacht Club is in Simon's Town and Granger Bay is found along the western coastline. Both sites consist of Atlantic (cooler) waters. The two species were collected from different sites, *Haploblepharus edwardsii* was collected from the False Bay Yacht Club and *Haploblepharus pictus* was collected from Granger Bay. These sites were selected as the study species are densely populated in the respective areas.

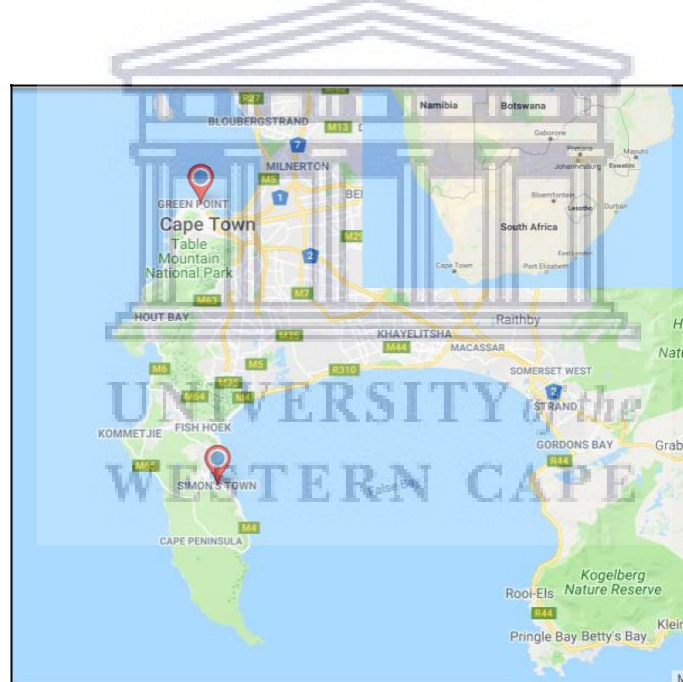


Figure 3: Map showing the locations where sharks were collected. Google Earth 2020

Field Collection and Animal Housing

The animals were caught using a net, whereby divers entered the water to collect the sharks by hand. The species, sex and approximate size of the sharks were confirmed at the collection sites, to ensure the required sample sizes were collected. A total of 10

males and 10 females of *Haploblepharus edwardsii* were collected from False Bay during August 2017 and a total of 9 males and 10 females of *Haploblepharus pictus* were collected from Granger Bay during September 2017. The sharks were between 36 cm and 51 cm in size for females and between 37 cm and 53 cm for males (total length). Males were checked for the presence of calcified claspers which is an indication of sexual maturity (Ebert *et al.* 2006).

Captured sharks were transported to the Two Oceans Aquarium in Cape Town, where all experimental trials were conducted. Sharks were transferred to a vehicle fitted with an 800L oval tank to hold the sharks for transportation. Measures were put in place to ensure the sharks received 100% oxygen through a diffuser. These high levels of oxygen were used to ensure the sharks had enough oxygen for the duration of the journey. Due to the possibility that sharks would excrete in the tank, the high levels of oxygen also helped to counteract the effects of ammonia and to assist in calming the sharks. The temperature of the water was 15°C during transportation for both species. The tanks were white and constructed of fiberglass and wood to ensure the temperature of the water in the tank did not rise by more than 1 degree Celsius during transportation.

Once at the Two Oceans Aquarium, sharks were measured by placing the shark in a tray that had a tape measure on the side where the shark was measured from the tip of its nose to the tip of the tail to the nearest centimeter and weighed on a scale to the nearest gram. They were also PIT (implantable passive integrated transponder) tagged underneath the skin on their dorsal fin for identification purposes. The sharks were then transferred into a holding tank (202 cm long x 71 cm wide x 83 cm high) that was kept at 15°C. The holding tank was located in the quarantine section of the aquarium. The holding tank had PVC (Polyvinyl Chloride) piping for the sharks to escape from harsh light levels. The pipes were there to mimic the cracks and crevices in which sharks hide in, in their natural environment. The sharks were left in these tanks to allow them to

acclimatize for 3 to 4 days to their new surroundings. The two species of shark were kept separate, but individuals of the same species were housed together.

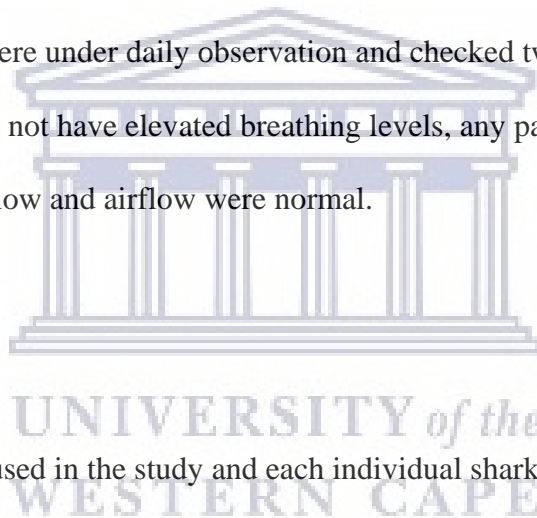
For the feeding regime, the sharks were purged to ensure that they consumed no food before trials, as they could regurgitate their food during the human-induced tonic immobility. Also, if they eat before the trials, it could affect the respiration rates of the sharks, as they would be digesting their food. Sharks were thus fed after the trials, on that same day. A mixture of pilchards, squid and white mussel was given to them. The day between trials allowed them to digest their food and to recover for the next trial.

Because these species of shark do not move around very much, their energy expenditure is fairly low. The sharks were under daily observation and checked twice a day to ensure that the animals did not have elevated breathing levels, any parasites, or any abrasions, and that water flow and airflow were normal.

Trialing Protocol

A total of 39 sharks were used in the study and each individual shark was trialed five times, giving a combined total of 195 trials that were conducted. Following the acclimatization period (3-4 days) the sharks were trialed one by one. The sharks were transferred from the holding tank to a separate trial tank (82 cm long x 49 cm wide x 53cm high). The shark was allowed to acclimatize to the trial tank for about a half an hour. Individual sharks were identified using a Virbac scanner, which scanned the PIT tag.

Tonic immobility was induced by turning the shark onto its dorsal surface and then waiting for the shark to become “limp”, as this indicates the onset of tonic immobility.



The induction time and the duration time of tonic immobility were recorded. The induction time represented the time taken for the shark to enter a tonic state (become “limp”) after turning. The duration time represents the duration of the TI event, from the onset of TI to the moment the shark terminated TI. Once the shark righted itself, that would indicate the end of the tonic state. It was found that the sharks were able to come out of their tonic state on their own. The shark was then returned to the holding tank.

The respiration rate was recorded by counting the number of gill movements. However, because the duration of the sharks’ tonic state was unpredictable, the gill movement was only observed for 20 seconds. The 20 seconds was then multiplied by three to get gill movement per minute (60 seconds). A pre-TI, TI and post-TI respiration rate was recorded for each individual. The pre-TI respiration rate was the gill movement that was recorded while the shark was resting. The TI respiration rate was recorded while the shark was in a tonic state and the post respiration rate was recorded after the shark righted itself after terminating TI. Heart rate was also recorded during TI using a portable ultrasonic diagnostic system (model: A6V, SN 26271226), where an ultrasound was taken of the heart beating. The shark heart beats were counted for 20 seconds and then multiplied by three to get heart beats per minute (60 seconds).

After a shark completed its first trial, the process was then repeated another four times for each individual. Trials occurred every second day after the initial trial, so that the experimental procedure took a total of nine days per individual (one trial every second day, for 9 days). The two days in between trials allowed the shark to recover from the previous trial and to digest their food effectively. Once the sharks completed all five trials, they were released back into the ocean at the site from which they were caught.

Data Analysis

Data were analysed using Statistical Package for the Social Sciences (SPSS version 23). As the data did not fit a normal distribution, they were transformed following the method by Templeton (2011) before conducting statistical analyses. A significance of 0.05 was used for all tests.

Repeated measure ANOVAs were done to test for a difference in the induction and duration time of tonic immobility between species and sex. The repeated measures ANOVAs were also used to determine whether individuals became habituated to tonic immobility over time, by testing for a change in induction and duration times over successive trials. A decrease in induction and duration response times of tonic immobility would be an indication of habituation. Repeated measures ANOVAs were also performed on the physiological data (pre-TI respiration rate, TI respiration rate, post-TI respiration rate and heart rate), to test for a difference between species and sexes. Pearson's correlations were used to determine whether the sharks' pre-TI respiration rate was related to induction time, and whether the sharks' TI respiration rate was related to duration of TI, for each species. The heart rates of each species were also correlated with the duration time of tonic immobility. Before performing each repeated measures ANOVA, the condition of sphericity was tested and if not met, then a Greenhouse-Geisser correction was applied.

Chapter 3: Results

Descriptive Statistics

Table 1: Means and standard deviations for the lengths, weights, induction and duration times, and physiological parameters recorded during tonic immobility for two *Haploblepharus* species.

		Length in centimeter's	Weight in kilograms	TI Induction in seconds	TI Duration in seconds	Pre-TI respiration count - gill movement per Minute	TI respiration count - gill movement per minute	Post-TI respiration count - gill movement per minute	Heart rate count - heart beats per minute
<i>H. edwardsii</i>	Female	37.7 ± 2.9	0.25 ± 0.05	20.81 ± 5.73	70.45 ± 38.89	63.37 ± 15.23	59.41 ± 13.95	66.97 ± 13.73	57.49 ± 14.14
	Male	40.2 ± 1.7	0.28 ± 0.03	22.47 ± 9.62	46.73 ± 17.66	58.99 ± 15.93	64.57 ± 14.21	63.01 ± 18.33	55.81 ± 15.06
	AVG		0.27 ± 0.02	21.64 ± 7.76	58.59 ± 31.81	61.18 ± 15.33	61.99 ± 13.96	64.99 ± 15.89	56.65 ± 14.24
<i>H. pictus</i>	Female	45.9 ± 4.8	0.45 ± 0.15	28.52 ± 9.92	61.90 ± 37.77	69.78 ± 10.86	68.76 ± 10.30	70.32 ± 8.77	68.28 ± 6.93
	Male	47.0 ± 4.4	0.49 ± 0.11	31 ± 12.3	56 ± 28.6	69.60 ± 9.35	68.01 ± 14.43	66.27 ± 12.20	65.20 ± 11.29
	AVG	46.5 ± 0.78	0.48 ± 0.03	29.82 ± 10.89	59.01 ± 32.97	69.70 ± 9.89	68.40 ± 12.07	68.40 ± 10.44	66.82 ± 9.11

From the descriptive statistics above (Table 1) it can be noted that *H. edwardsii* is smaller in size ($t(37) = -5.700, p < 0.001$) and weight ($t(37) = -6.343, p < 0.001$) in comparison to *H. pictus*, but within each species males and females did not differ. Table 1 also shows that TI induction times are lower than the TI duration times for both species, although duration times were highly variable. The mean values of all physiological measurements were higher in *H. pictus*, but not necessarily significantly so (see statistical analyses below).

TI induction and duration times

Objective 1: To determine whether the time taken to induce tonic immobility is species and/or sex specific.

The results showed that there was a significant difference in induction time between *H. pictus* and *H. edwardsii* ($F_{1, 35} = 4.703; p = 0.037$), with *H. pictus* taking longer to induce into a tonic state than *H. edwardsii* (Table 1). However, both species had no significant difference in induction time between males and females (Table 2). The results also show that there is no significant interaction between sexes and the induction time for both species (Table 3). When looking at whether there was a correlation between the induction time and the length and weight of the two species, the results showed no significant correlations for both *H. edwardsii* [Length: $r = -0.024, r^2 = 5.768E-4, (p = 0.920), n = 20$, Weight: $r = 0.112, r^2 = 0.013, (p = 0.637), n = 20$] and *H. pictus* [Length: $r = -0.033, r^2 = 0.001, (p = 0.898), n = 18$; Weight: $r = 0.014, r^2 = 2.102E-4, (p = 0.954), n = 18$].

Table 2: Between subject's effects test for the induction time between the sexes of both species

	Df	F	Sig. (p-value)
<i>H. edwardsii</i>			
Sex	1	0.036	0.851
Error	18		
<i>H. pictus</i>			
Sex	1	0.033	0.857
Error	18		

Table 3: Within-subjects effects test for an interaction between induction time and sex for *H. edwardsii* and *H. pictus*

		Df	F	Sig. (p-value)
<i>H. edwardsii</i>				
Induction Time*sex	Sphericity assumed	4	0.075	0.990
Error		72		
<i>H. pictus</i>				
Induction Time*sex	Sphericity assumed	4	0.746	0.564
Error		72		

Objective 2: To determine whether the duration time of tonic immobility is species. and/or sex specific

No significant difference in the duration of tonic immobility was seen between males and females of both species (Table 4). There was also no significant difference in duration time between *H. pictus* and *H. edwardsii* ($F_{1, 35} = 0.051$; $p = 0.823$). No significant interaction was seen between the duration time and sex, for both *H. edwardsii* and *H. pictus* (Table 5). When looking at whether there was a correlation between the duration time and the length and weight of the two species, the results showed no significant correlations for *H. edwardsii* [Length: $r = 0.042$, $r^2 = 0.002$, ($p = 0.859$), $n = 20$, Weight: $r = 0.072$, $r^2 = 0.005$, ($p = 0.763$), $n = 20$] but significant

correlation was seen in *H. pictus* for their weight [Length: $r = 0.220$, $r^2 = 0.048$, ($p = 0.381$), $n = 18$, Weight: $r = 0.470$, $r^2 = 0.221$, ($p = 0.049$), $n = 18$].

Table 4: Between subject's effects test for the duration time between the sexes of both species

	Df	F	Sig. (p-value)
<i>H. edwardsii</i>			
Sex	1	2.366	0.141
Error	18		
<i>H. pictus</i>			
Sex	1	0.056	0.816
Error	17		

Table 5: Within-subjects effects test for the duration time between *H. edwardsii* and *H. pictus*

		Df	F	Sig. (p-value)
<i>H. edwardsii</i>				
Duration Time*Sex	Sphericity assumed	4	0.407	0.803
Error		72		
<i>H. pictus</i>				
Duration Time*Sex	Sphericity assumed	4	1.004	0.412
Error		68		

Objective 3: To determine whether induction and duration times of tonic immobility habituate over repeated trials

The results show that there is a significant difference in the induction times across the trials conducted for both *H. edwardsii* ($F_{4, 72} = 3.799$, $p = 0.007$ and *H. pictus* ($F_{4, 72} = 3.624$, $p = 0.010$). Although there is a significant difference across the trials, the species do not necessarily become habituated to being induced into tonic immobility, but rather.

respond differently across repeated trials (Figure 4). Trials 1 and 4 differ significantly for *H. edwardsii* for both sexes. Whereas *H. pictus* differs significantly between trial 2 and trial 4 between females

No significance was shown across the duration time trials for *H. edwardsii* ($F_{4, 72} = 0.865, p = 0.489$), but there was a significant difference shown across trials for *H. pictus* ($F_{4, 68} = 5.232, p = 0.001$). But once again it can be seen that the species respond differently across trials, with no clear pattern of habituation (Figure 5). Trials 1 and 3 differ between *H. edwardsii* females and trials 1 and 5 differ between the males. For *H. pictus*, females differ between trials 3 and 5 and the males differ between trials 1 and 3.



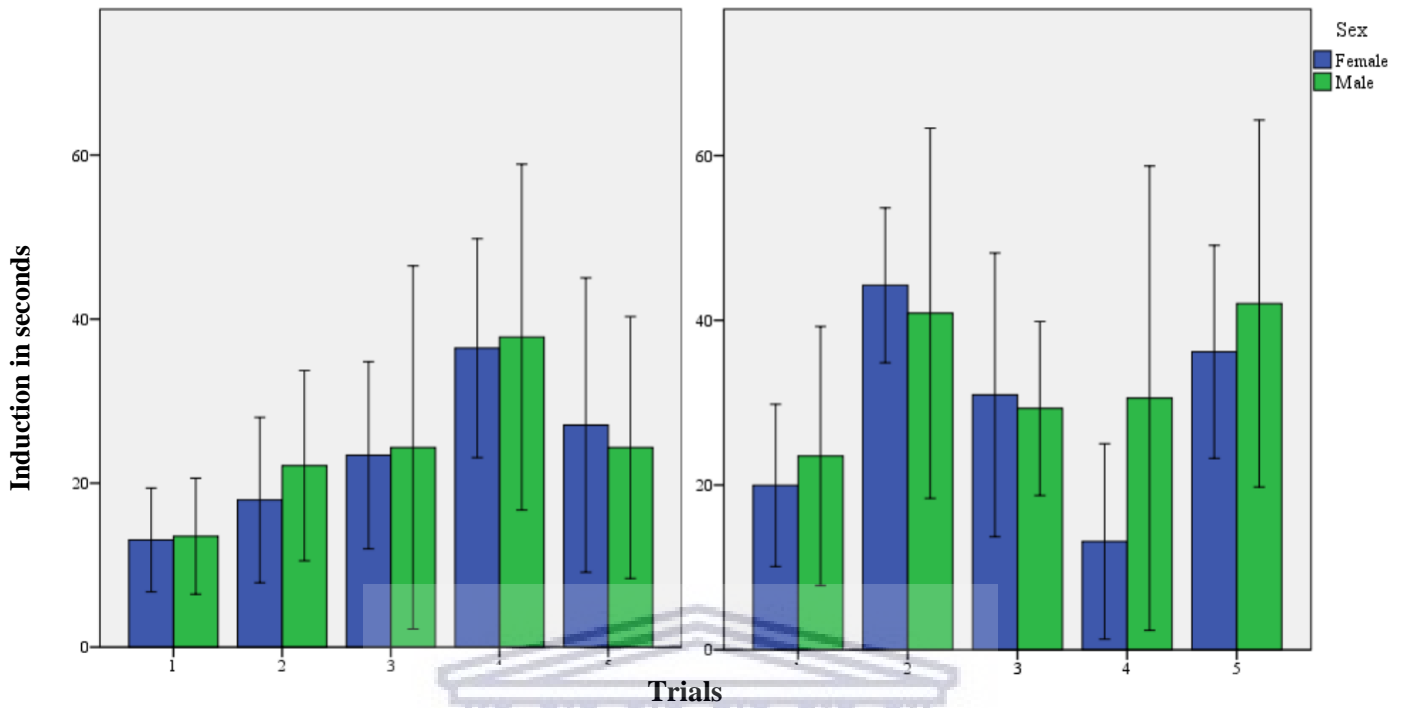


Figure 4: The average TI induction time across trials for *H. edwardsii* (Left) and *H. pictus* (Right) with 95% CI error bars

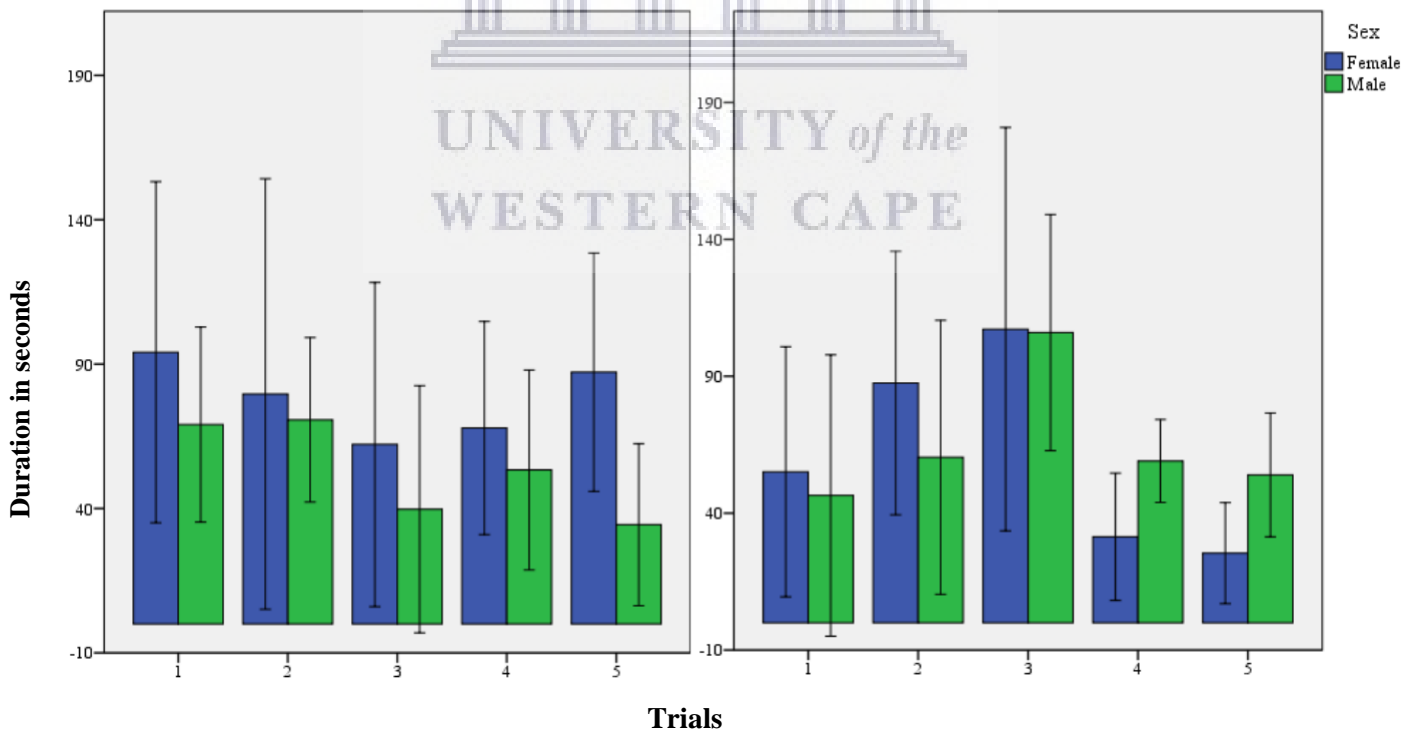


Figure 5: The average TI duration time across trials for *H. edwardsii* (Left) and *H. pictus* (Right) with 95% CI error bars.

Objective 4a: To determine whether there is a difference in pre-TI respiration rate between species and/or sexes

There was no statistically significant difference in pre-TI respiration rate between *H. pictus* and *H. edwardsii* ($F_{1, 35} = 3.176$; $p = 0.083$). There were also no significant differences in pre-TI respiration rate between males and females of both species (Table 6), and no significant interaction between pre-TI respiration rate and sexes for both species (Table 7).

Table 6: Between subject's effects test for the pre-TI respiration rate for both species

	Df	F	Sig. (p-value)
<i>H. edwardsii</i>			
Sex	1	1.287	0.272
Error	18		
<i>H. pictus</i>			
Sex	1	0.357	0.558
Error	17		

Table 7: Within subject's effects test for the pre-TI respiration rate between *H. edwardsii* and *H. pictus*

		Df	F	Sig. (p-value)
<i>H. edwardsii</i>				
Pre-TI respiration*Sex	Sphericity assumed	4	1.022	0.402
Error		72		
<i>H. pictus</i>				
Pre-TI respiration *Sex	Sphericity assumed	4	2.081	0.093
Error		68		

Objective 4b: To determine whether there is a difference in TI respiration rate between species and/or sexes

There was no significant difference in TI respiration rate between *H. pictus* and *H. edwardsii* ($F_{1, 35} = 0.580, p = 0.451$). There was also no difference between males and females of the two species of catshark (Table 8). The TI respiration rate and sex interaction was not significant for both species (Table 9).

Table 8: Between subject's effects TI respiration rate result for *H. edwardsii* and *H. pictus*

	Df	F	Sig. (p-value)
<i>H. edwardsii</i>			
Sex	1	1.338	0.262
Error	18		
<i>H. pictus</i>			
Sex	1	0.072	0.791
Error	17		

Table 9: Within subject's effects test results looking at the interaction between TI respiration rate and sex in *H. edwardsii* and *H. pictus*.

		Df	F	Sig. (p-value)
<i>H. edwardsii</i>				
TI respiration*Sex	Sphericity assumed	4	1.118	0.355
Error		72		
<i>H. pictus</i>				
TI respiration *Sex	Sphericity assumed	4	0.655	0.625
Error		68		

Objective 4c: To determine whether there is a difference in post-TI respiration rate between species and/or sexes

There was no significant difference in post-TI respiration rate between *H. pictus* and *H. edwardsii* ($F_{1, 35} = 0.008$, $p = 0.928$). There was also no significant difference in post-TI respiration rate between sexes (Table 10). The interaction between post-TI respiration rate and sex also showed no statistical significance for *H. edwardsii* and *H. pictus* (Table 11).

Table 10: Between subject's effects test for the post-TI respiration rate between the sexes of both species

	Df	F	Sig. (p-value)
<i>H. edwardsii</i>			
Sex	1	1.151	0.298
Error	18		
<i>H. pictus</i>			
Sex	1	0.583	0.456
Error	17		

Table 11: Within subject's effects test for the post-TI respiration rate between *H. edwardsii* and *H. pictus*

		Df	F	Sig. (p-value)
<i>H. edwardsii</i>				
Post-TI respiration*Sex	Sphericity assumed	4	1.141	0.344
Error		72		
<i>H. pictus</i>				
Post-TI respiration*Sex	Greenhouse-Geisser	4	2.768	0.060
Error		68		

Objective 4d: To determine whether there is a relationship between (average) respiration and heart rates, and time taken for the sharks to be induced and to recover from a tonic state.

The correlation between pre-TI respiration rate and induction time for *H. edwardsii* was not statistically significant, both for females [$r = -0.001$, $r^2 = 1.193E-6$, ($p = 0.998$), $n = 10$] and males [$r = 0.34$, $r^2 = 0.117$, ($p = 0.33$), $n = 10$]. Both females [$r = 0.22$, $r^2 = 0.056$, ($p = 0.54$), $n = 10$] and males [$r = 0.07$, $r^2 = 0.458$, ($p = 0.86$), $n = 9$] of *H. pictus* likewise show no significant correlation between pre-TI respiration rate and induction time.

When looking at the correlation between the TI respiration rate and duration time for *H. edwardsii*, there was no significant correlation for females [$r = 0.10$, $r^2 = 0.010$, ($p = 0.78$), $n = 10$] or males [$r = 0.47$, $r^2 = 0.221$, ($p = 0.17$), $n = 10$]. The same trend can be seen for *H. pictus*, where there is no significant correlation between the TI respiration rate and duration for both females [$r = -0.32$, $r^2 = 0.049$, ($p = 0.37$), $n = 10$] and males [$r = 0.01$, $r^2 = 0.005$, ($p = 0.98$), $n = 9$]. The correlation between the heart rate and TI duration time for *H. edwardsii* was marginally non-significant for females [$r = 0.576$, $r^2 = 0.331$, ($p = 0.082$), $n = 10$] and was significant for males [$r = 0.681$, $r^2 = 0.464$, ($p = 0.030$), $n = 10$]. In contrast, both females [$r = 0.096$, $r^2 = 0.009$, ($p = 0.792$), $n = 10$] and males [$r = 0.069$, $r^2 = 0.005$, ($p = 0.860$), $n = 9$] of *H. pictus* showed no significant correlation between the heart rate and TI duration time.

Objective 4e: To determine whether heart rate varies between species and/or sexes.

There was a significant difference in heart rate during tonic immobility between the two species ($F_{1, 35} = 11.900, p = 0.001$), with *H. pictus* having a higher mean heart rate (Table 1). The variation in heart rate across trials can be seen below in Figure 6. However, no significant difference in heart rate was observed between males and females from both species (Table 12) and there was no significant interaction between heart rate and sexes for both *H. edwardsii* and *H. pictus* (Table 13).

Table 12: Between subject's effects test for heart rate between sexes for both species

	Df	F	Sig. (p-value)
<i>H. edwardsii</i>			
Sex	1	0.705	0.412
Error	18		
<i>H. pictus</i>			
Sex	1	0.104	0.751
Error	17		

Table 13: Within subject's effects test for an interaction between heart rate and sex for each species

		Df	F	Sig. (p-value)
<i>H. edwardsii</i>				
Heart rate*Sex	Sphericity assumed	4	0.809	0.523
Error		72		
<i>H. pictus</i>				
Heart rate*Sex	Greenhouse-Geisser	4	0.626	0.645
Error		68		

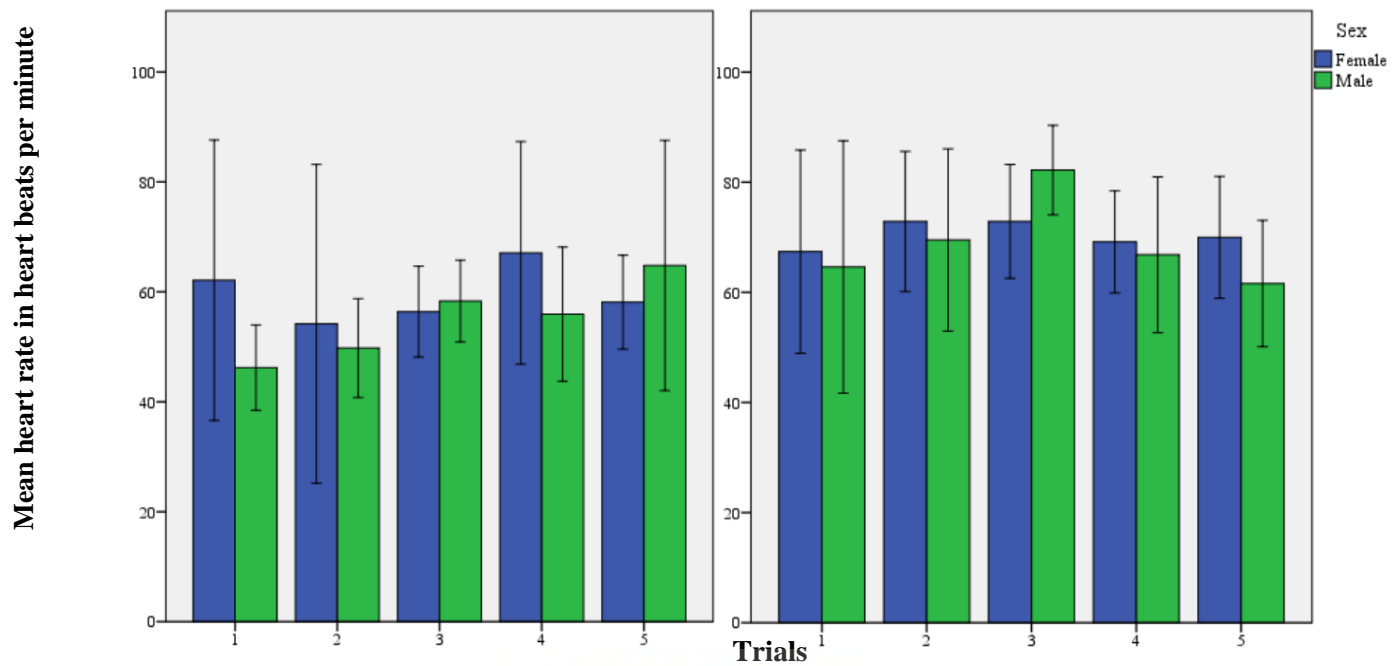
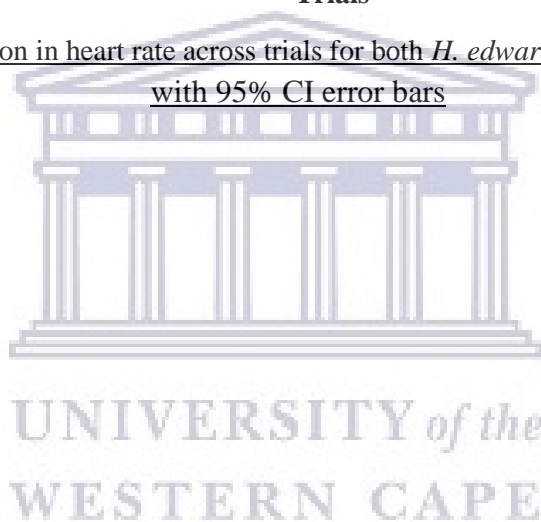


Figure 6: Shows the variation in heart rate across trials for both *H. edwardsii* (Left) and *H. pictus* (Right) with 95% CI error bars



Chapter 4: Discussion and Conclusion

The intention of this study was to determine whether the behaviour (induction and duration) and physiology (respiration and heart rate) associated with tonic immobility differed between two species of catshark, *Haploblepharus edwardsii* and *Haploblepharus pictus*, and/or between sexes (male and female). The study found that the time taken to induce tonic immobility differed significantly between the two species, but not between sexes. This agrees with previous studies by Henningsen (1994) and Brooks *et al.* (2011) which demonstrated that the induction into tonic immobility and the onset of tonic immobility would vary from one species to the next. Henningsen (1994) recorded these induction times from larger species of shark such as the *Triakis semifasciata* (leopard shark), *Trianodon obesus* (whitetip reef shark), *Carcharhinus melanopterus* (blacktip reef shark) and *Carcharhinus perezii* (Caribbean reef shark). A study by Williamson (2011) on zebra sharks also found that there was a significant variation between species. Although these previous studies reported on the difference amongst species, there is no clear indication whether there is a difference between sexes. This is because their induction time values were combined for both sexes, with no separation between males and females. The *Haploblepharus pictus* species was larger in size in comparison to *Haploblepharus edwardsii* and this could have been the reason for the difference in induction times. This however was not the case because there was no significant correlation between the heart rate and the two species length and weight.

When having a look at the duration times of tonic immobility for the two *Haploblepharus* species, very similar average values were found for the two species. However, the duration of TI was highly variable, with a high standard deviation. Thus,

although the average duration of TI was noticeably higher in females than in males for both species (Table 1), these differences were not significant. Whiteman and Marshall (1986) recorded duration times in smooth dogfish, *Mustelus canis*, similar to what was recorded for the *Haploblepharus* species. Watsky & Gruber (1990) also found that there was no significant difference in duration times for tonic immobility for the lemon shark, *Negaprion brevirostris*. The study by Watsky and Gruber (1990) did, however, differ in that trials were conducted once a day for 24 days, whereas our trials were once every two days for nine days. Another difference is that a Watsky & Gruber (1990) placed the lemon shark in an acrylic V-shaped trough to induce TI, which differed to the technique used in this study on the *Haploblepharus* species. But once again, these values recorded by Whiteman and Marshall (1986) and Watsky and Gruber (1990) were from a combination of both males and females. A positive correlation was seen between *H. pictus* duration time of tonic immobility and their weight. This is very interesting because even though there was this significant correlation, there was no significant difference between the overall duration times for both species.

A possible explanation for the similar duration times for *H. edwardsii* and *H. pictus* could be because these two species are closely related and also ecologically similar. However, it is interesting that they differ in induction times, and this suggests that there may be some behavioural divergence between these two species. The lack of a significant difference between sexes in both induction and duration times for both species indicates that males and females have similar behavioural responses to tonic immobility and that the evolutionary roots of tonic immobility may be as an anti-predator mechanism rather than as courtship behaviour.

The study also tested whether the shark species in question, *Haploblepharus edwardsii* and *Haploblepharus pictus*, became habituated to being inducted into tonic immobility.

The results revealed that the sharks responded differently across the repeated trials, with response times either increasing or decreasing, and no clear pattern of habituation.

Unlike this study, previous studies found that some shark species did experience habituation during tonic immobility. The study by Watsky and Gruber (1990) found that even though there was a significant difference between massed and spaced trials, the lemon sharks from the different treatments demonstrated a decrease in tonic immobility duration response. Henningsen (1994) also stated that when conducting repeated trials using tonic immobility, care needs to be taken because it decreases the duration time and thus could impact the results, unless testing for habituation. Studies have shown that repeated introduction to stimuli would result in the shark incorporating a specific reaction to the stimuli as a fixed behavioural component (Bres 1993). It becomes apparent that in previous studies on sharks and tonic immobility, habituation has not been discussed extensively. A possible reason as to why the *Haploblepharus* species did not experience any clear habituation could be because the sharks had two days between trials to “recuperate” before the next trial commenced. This period may have been too long.

The physiological component of this study looked at respiration rate and heart rate of the two species of shark. The respiration rate was recorded during the resting stages, as a stress response following the onset of TI, as well as during the immediate recovery from TI.

There were, however, no consistent trends for changes in respiration between species and sexes, nor did the respiration rate change significantly when sharks entered a state of tonic immobility. A study by Davie *et al.* (1993) examined changes in respiration rate during tonic immobility. The respiration rates of *Carcharhinus melanopterus* (black tipped reef shark) were recorded before and after tonic immobility.

Similar to our study, Davie *et al.* (1993) found that there was no significant difference in the respiration rates for *Carcharhinus melanopterus* (black tipped reef shark).

The heart rate was recorded only during tonic immobility. This is because measuring heart rate while the shark was conscious would have been stressful and led to an inaccurate result. The heart rate was higher in *H. pictus*, possibly due to their larger size, but showed no significant difference between sexes. Davie *et al.* (1993) found that the cardiac response remained stable during trials conducted on black tipped reef sharks, *Carcharhinus melanopterus*. Although there was no significant change across trials in the heart rate of the two *Haplobleparus* species tested here, it did not appear to remain stable, but rather showed some fluctuation across trials. The variation that was observed could be a result of a change in stroke volume, which is the volume of blood that is pumped into the heart per heartbeat (Bruss & Raja, 2019). There is also a possibility that because the heart rate was measured as soon as the shark was induced into tonic immobility, the stress of inverting the shark could have caused an elevated heart rate.

This study also found no correlation between the induction time and the pre-respiration rate recorded. A similar result was found between the duration time of tonic immobility and the TI respiration rate recorded. The heart rate and duration time of tonic immobility were, however, positively correlated, but only in *H. edwardsii*. Overall, the study demonstrated that tonic immobility had no adverse effect on the respiration and heart rate, which remained fairly constant. This is contradictory to studies such as Brooks *et al.* (2011) that found the opposite of this, where the respiratory system was temporarily compromised during tonic immobility. Davie *et al.* (1993) also noted changes in the respiratory rate, where this decrease also resulted in changes in the sharks' blood pressure and heart rate. The studies by Davie *et al.* (1993) and Brooks *et al.* (2011) however incorporated manipulations such as brachial irrigation, irrigation

flow, an injection of atropine and a ventilatory tube which possibly impacted their results.

Overall, we could argue that the environment of captivity could have had an impact on the sharks. Studies have shown how animals adjust or modify their behaviours to a specific environmental stimulus (Bres 1993). Captivity can present different stresses which the animal would not experience in its natural habitat and this could have an effect on the outcome of tonic immobility responses. This is another aspect that needs more investigation, whereby future studies could test the response to tonic immobility between captive and non-captive animals and whether a difference can be noted between the different environments.

Another notion that could be put forth is that perhaps tonic immobility may not be a stressful experience for these catshark species. Our results showed that tonic immobility has little or no adverse effect on the sharks when looking at respiration and heart rate. Previous studies on larger species of shark have found that tonic immobility does have an effect on the physiological parameters that accompany tonic immobility. These previous studies, however, looked at parameters such as ventilation rate, buccal pumping and blood parameters (Brooks *et al.* 2011). It is possible that different species respond differently to similar stimuli. So, it is possible that these species experience a non-stressful tonic immobility event.

One of the aims of this study was to determine whether these species of catshark utilize tonic immobility as a defensive mechanism and/or as a mating technique. Previous studies on sharks (Whiteman *et al.* 1986; Davie *et al.* 1993; Reeb 2007; Brunnschweiler & Pratt 2008; Humphreys & Ruxton 2018), have put forth two hypotheses; 1) looking at the evolutionary significance of tonic immobility as a

defensive mechanism or 2) a mating technique. When looking at the evolutionary use as a defensive mechanism, the majority of the previous literature on sharks does not discuss this use in much detail. Studies have found it unlikely that larger sharks would employ tonic immobility as a defensive mechanism as they are seen as meso and apex predators (Garla *et al.* 2015). Studies on small to medium species of shark have found that more benthic and sedentary sharks tend to employ alternative ways to avoid or prevent predation by using holes and crevices to remain undetected. Garla *et al.* (2015) discovered that younger nurse sharks use “hiding behaviour” as a way to protect themselves from larger species of sharks and carnivorous teleost. By remaining within crevices during daylight, these sharks would be able to minimize their exposure time to these larger predators. This is a possible technique that *Haploblepharus* could be adopting, as these species are also benthic and sedentary sharks.

Sharks only use defensive tactics when they are related to combative displays. Sharks use these displays as a way to showcase their fighting ability or hierarchical status (Garla *et al.* 2015). Sharks have notoriously been known to attack when “feeling” threatened, rather than fleeing or playing dead. Studies have shown that the sharks’ attack is motivated by hunger, but there are studies that have hypothesized an alternative explanation that could be related to a defensive mechanism (Bres 1993). These studies have been on larger sharks; however, and therefore it could be possible that small to medium sized sharks would use tonic immobility as a defensive mechanism due to the fact that they are incorporated in the diet of larger sharks, carnivorous teleost’s, cetaceans, pinnipeds and even some marine birds (Garla *et al.* 2015).

Tonic immobility in sharks has been documented in a few studies and has been reported to play a role in mating techniques. However, studies such as that by Afonso *et al.* (2016), have noted that mating interactions amongst sharks are very elusive and

therefore, difficult to observe and report on. The studies were able to observe a sort of tonic immobility that came into play during shark courtship techniques, which lead to reproduction. Very few of these interactions have been observed in a natural environment or in the wild, and the majority of the studies occurred in captivity. A study done by Brunnschweiler and Pratt (2008) documented same sex mating rituals between male zebra sharks and the study shows how one male would invert another male zebra shark onto its back. The study, however, put forth a compelling explanation that this encounter was not sexually related behaviour, but rather two male sharks expressing or establishing dominance. There is a possibility that both males and females can be induced into a tonic state and can experience a tonic event when individuals seek to fight for hierarchical status.

This study demonstrated that the species *Haploblepharus pictus* and *Haploblepharus edwardsii* do exhibit a tonic immobility response following inversion and it is evident that the way tonic immobility was expressed in this study is similar to the way tonic immobility has been expressed in previous studies (Whiteman *et al.* 1986; Watsky & Gruber 1990), where the animals exhibit a “limp” response during tonic immobility. However, if our expectation is for tonic immobility to be related to mating, then there should be a difference between sexes, which appears to not be the case. This could suggest that there could be a possibility of an additional function of tonic immobility, at least in these particular shark species, which could be related to an anti-predator technique. The majority of previous studies examining tonic immobility were conducted on larger species of shark and not smaller to medium sized benthic sharks. Due to the fact that these are smaller species of shark in comparison to the previous studies, a defensive mechanism or use could be plausible. Smaller sharks do have larger predators that they need to protect themselves from in addition to using holes and crevices as a refuge from predators.

The literature on *Haploblepharus* species and how they respond to predators or fear is limited, but there is some mention of how these catsharks respond to threatening situations. It has been reported by SCUBA divers that these catsharks assume a doughnut shape where they cover their eyes with their tail when feeling threatened or when responding to a threatening situation. The likelihood that these species of catshark use tonic immobility as a defensive mechanism is quite possible. Tonic immobility could be a way in which the *Haploblepharus* species calm themselves to help reduce stress when faced with the stress of human interaction or interference. However, this study used respiration and heart rate as stress proxies and the results showed that tonic immobility had no effects on these. The use of tonic immobility as a defense mechanism in *Haploblepharus* requires further investigation.

Tonic immobility has been defined by a “lack of behaviour” displayed by animals and does not involve any special anatomical adaptations, which would suggest that tonic immobility is a relatively simple defense mechanism to evolve (Humphreys & Ruxton 2018). Tonic immobility is partly under genetic control and is found to be consistent within individuals but can vary within and between populations in how it is used and how frequently it occurs (Humphreys & Ruxton 2018). Tonic immobility thus differs in the way individuals within a species exhibit the behaviour.

Tonic immobility has not been researched extensively in the past, but recently there are a growing number of studies that are providing a better understanding about tonic immobility. There is a need for biologists to create opportunities in which tonic immobility can be observed and studied in the wild. Studies that have been done in a

laboratory setting has highlighted the behaviour and physiology of tonic immobility and it would be highly beneficial to explore whether similar relationships occur in a natural environment (Humphreys & Ruxton 2018).

This study helps provide more insight into tonic immobility and how benthic sharks respond to it. These results also provide information on induction and duration times that may assist in using tonic immobility as a capture method or for surgical procedures as a suitable alternative to anesthesia. Tonic immobility has been used as a non-lethal capture method to help ensure the welfare of the animal in comparison to other non-lethal capture methods.

In conclusion, the majority of previous literature on tonic immobility has focused on its use as a predator avoidance response, with few studies focusing on the potential reproductive significance of tonic immobility. Tonic immobility has been adopted by animals to decrease predator detection and increase survival. In terms of reproduction, the sharks would incorporate tonic immobility in their mating strategies. This study represents the first investigation into the behavioural and physiological response to tonic immobility in small to medium species of shark. This study has demonstrated that these two species of catshark, *H. edwardsii* and *H. pictus*, do exhibit tonic immobility, and that there are some differences between the two species, notably in induction times. However, we found no clear evidence to suggest that tonic immobility is used as a reproductive technique. If tonic immobility were related to reproduction, we would have expected to observe differences between males and females. These results thus suggest that tonic immobility has not evolved as a mating behaviour in these species of shark, but more likely arose for anti-predatory purposes. However, it is still possible that tonic immobility functions in courtship and mating, but that this is not its primary or original function.

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