THE EFFECTS OF CYCLIC THERMAL STRESS ON OVIPAROUS SHARK DEVELOPMENT

by

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ABSTRACT

Globally, shark species have undergone a dramatic decrease in abundance due to the environmental degradation caused by anthropogenic activities and global warming. There are limited studies on the effects of global warming on embryological development of threatened tropical sharks. This study investigated the impact of cyclic thermal stress on the embryonic development of the grey carpet shark (*Chiloscyllium punctatum*), as well as the ability of grey carpet shark neonates to tolerate a hypoxic challenge following exposure to thermal stress.

Detailed characterisation of all developmental stages observable through the egg case to the naked eye formed the basis for the investigation of thermal stress. The data clearly showed that embryos exposed to 28 °C in a cyclic manner, displayed a delay in the appearance of three developmental milestones: (i) the time pigmentation took to reach the pectoral fins; (ii) widening of the trunk region; and (iii) the internalization of yolk. The time to hatching was increased by 10 days and neonates were significantly shorter in total body length in response to cyclic thermal exposure (28 °C) compared to control egg cases only exposed to 24 °C.

No morphometric changes were observed on a separate experiment on neonates reared at 24°C, then exposed for five days either to intermittent thermal stress at 32°C or control conditions 24°C. This short exposure to cyclic thermal stress did not alter their ability to withstand a hypoxic challenge at 30 days post-hatching. During the hypoxic challenge, neonates were observed to use ventilation depression and diminished their use of buccal musculature. The strongest morphometric predictor of the time to loss of righting reflex in response to the hypoxic challenge was the ratio of the span of the first dorsal fin to the total length of the neonate, which could reduce the amount of energy needed during escape attempts and exploratory behaviours observed during the hypoxia exposure. My research sheds light on the potential survival of this near threatened tropical shark. Future studies should explore the impact of higher cyclic temperatures on grey carpet shark embryo development to expand upon the understanding of the impact of cyclic thermal stress on shark embryos.

Keywords

Global warming, tropical shark, grey carpet sharks, *Chiloscyllium puncatum*, embryo development, developmental stages, thermal stress, hypoxia.

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CHAPTER 1

GENERAL INTRODUCTION

Global warming imposes challenges to the survival of organisms across the globe, as surface water temperatures are elevated, the extent and intensity of hypoxic events worldwide is exacerbated (Rabalais *et al.,* 2010). In combination, global warming and hypoxia can cause the loss of habitat for several fish species (Budnik *et al.,* 2020). Coral reef bleaching has also been linked to global warming and ocean acidification (Burt *et al.,* 2019; DeCarlo *et al.,* 2017; Green *et al.,* 2019). Coral bleaching has a direct effect on the health of its inhabitants, and as such, the effect on the overall health of marine species is exacerbated (Bonin *et al.,* 2009; Coker *et al.,* 2009). Fish and elasmobranchs responses to increased temperature and hypoxia have been well described and include changes at the behavioural, physiological and biochemical level (Hochaka, 1980; Nikinmaa and Rees, 2005; Nilsson *et al.,* 2010; Pistevos *et al.,* 2015; Rosa *et al.,* 2014; Rummer and Munday, 2017). For instance, in the Coco Islands, a total of 11 different fish species including a reef shark, *Carcharhinus melanopterus*, died due to the combined effect of increased temperature (from 33 to 35 ºC) and hypoxia (Hobbs and McDonald, 2010). Several studies have also shown that severe temperatures can impact the ability of fish to successfully reproduce. When *Acanthochromis polyacanthus* individuals were exposed to 31.5 ºC and 30 ºC in combination with reduced food intake, there was a dramatic decrease in egg production (Donelson *et al.*, 2010). Despite the bulk of research on the effects of global warming and hypoxia on freshwater fish and specifically teleosts, the response of marine species and in particular the responses of elasmobranchs is still not fully understood. Presently global temperatures are approximately 0.7 ºC above temperatures experienced within the past 420,000 years (Hoegh-Guldberg *et al.*, 2007), and recent predictions suggest that between the years 2050 to 2100, global water temperatures will rise by 2 ºC (Davis *et al.*, 2010; Hobday and Lough, 2011; Hoegh-Guldberg *et al.,* 2007). In marine environments, global warming will continue to affect the levels of water oxygen and temperature in the open ocean

and the coastal waters, alongside water eutrophication due to anthropogenic activities in these environments (Fennel and Testa, 2019). Hence, it is of extreme importance to explore the possible responses of different marine teleosts and particularly chondrichthyan species, which are arguably more vulnerable due to their extremely long gestation period, high level of maternal investment, late sexual maturity, low population growth rates and weak density-dependent compensation in juvenile survival (Dulvy *et al.,* 2014). While some previous literature on sharks has identified species that are surprisingly resilient when exposed to thermal stress and anoxia (Chapman and Renshaw, 2009; Chapman *et al.,* 2011; Routley *et al.,* 2002), different life stages do not respond necessarily in the same way; hence, the mechanisms that are used to cope with the stressors require further research. Based upon the trends observed in different fish species under present-day temperatures, and the projected temperature changes, it could be expected that sharks would be more severely impacted due to their life-history traits, which can render them more vulnerable to the effects of increased temperature and concomitant hypoxia (DeCarlo *et al.,* 2017; Hoegh-Guldberg *et al.,* 2007; Neilson *et al.,* 2017; Sternberg *et al.,* 2015). Although the impact of global climate change seems to vary depending on the type of environment (e.g., marine, freshwater or terrestrial) (Donnelly *et al.,* 2011; Obryk *et al.,* 2016); this general introduction will focus on the effects of global warming on shallow marine environments, because the animal model used in this study lives on coral reef platforms and estuaries.

Temperature and Organismal Biology

Climate change can dramatically affect many biological aspects of organisms such as (i) distribution, (ii) metabolism, (iii) aerobic scope, iv) survival and v) reproduction (Chase *et al.,* 2018a; Chase *et al.,* 2018b; Cheung *et al.,* 2010; Loukos *et al.,* 2003; Lucey and Nye, 2010;

Nay *et al.*, 2018; Nilsson and Ostlund-Nilsson, 2004; Nilsson *et al.*, 2009; Rummer *et al.*, 2014; Zak *et al.*, 2018). Coral reef flat communities are vulnerable to climate change at low tides, when most organisms are exposed to shallow water levels, low dissolved oxygen levels (hypoxia), as well as increased temperatures. For instance, five coral reef fish species (*Acanthochromis polyacanthus*, *Pomacentrus moluccensis*, *Ostorhinchus cyanosoma*, *Ostorhinchus doedeleini*, and *Cheilodipterus quinquelineatus*), on the coral flats of Lizard Island elicited different gene expression in response to the rapid and intense heating of the water (Bernal *et al.*, 2020). The initial response to the heatwave occurring during the tide change was an upregulation of the expression of various genes across all five species, which allowed them to increases their mitochondrial activity, respiratory chain complex, synthesis of fatty acids, and oxidoreductase activity (Bernal *et al.*, 2020). Along the northeast coast of Queensland, Australia, the water of the reef flats is heated by infrared radiation from the sun during low tide, resulting in water temperatures reaching between 26 to 30.5 ºC at the Great Barrier Reef, extending from 9ºS to 22ºS (Zhu *et al.*, 2014). This increase in temperature is not unique to the Great Barrier Reef, as it has also been observed in Hawaii, where the reef flats are on average 1-2 ºC above the open ocean's surface temperature, which ranges between 28 and 29 ºC (Jokiel and Brown, 2004). However, this difference in surface temperatures across the reef flat and open ocean can vary due to coastline geometry, bathymetry, water types, tide, and wave mixing (Zhu *et al.*, 2014). As recorded by the CSIRO (Commonwealth Scientific and Industrial Research) on Heron Island, during January of 2010, temperature variation from the core of the reef flat to the surrounding water could differ by up to ~3 ºC (CSIRO, unpublished data). However, if there are no internal waves to influence water movement in the coral reefs, such as on the Dongsha Atoll in the Northern South China Sea, temperatures on reef flats could be 2.0 $\pm$ 0.2 ºC warmer (Reid *et al.*, 2019). These changes in

temperature, although rather subtle in appearance, can have detrimental effects on the corals that form the bulk of the reef, as well as other organisms that make use of the reef flats (Chase *et al.*, 2018b; Marques *et al.*, 2019).

In general, temperature increase can also affect organismal metabolism (Brijs *et al.*, 2018; Dietrich *et al.*, 2018; Eme *et al.*, 2018; Sandersfeld *et al.*, 2017; Sanhueza *et al.*, 2018). Temperature is a critical environmental factor for many organisms, and particularly for ectotherms (Breau *et al.*, 2011; Guderley and St-Pierre, 2002; Sandersfeld *et al.*, 2017). Ectotherms rely on the ambient temperature to regulate their body temperature (Zak *et al.*, 2018). When organisms are exposed to an environmental temperature that is outside their thermal optima, they become stressed, and use specialised protective mechanisms to cope with this stressor (Chase *et al.*, 2018a; Nay *et al.*, 2018; Wang and Lin, 2018). Specifically, changes in temperature can affect the standard metabolic rate (SMR), the minimum amount of energy required at rest and in a post-absorptive manner to keep vital functions at a specific temperature in ectotherms (Jeffries *et al.*, 2018; Le *et al.*, 2017; Nilsson *et al.*, 2009; Rosa *et al.*, 2016).

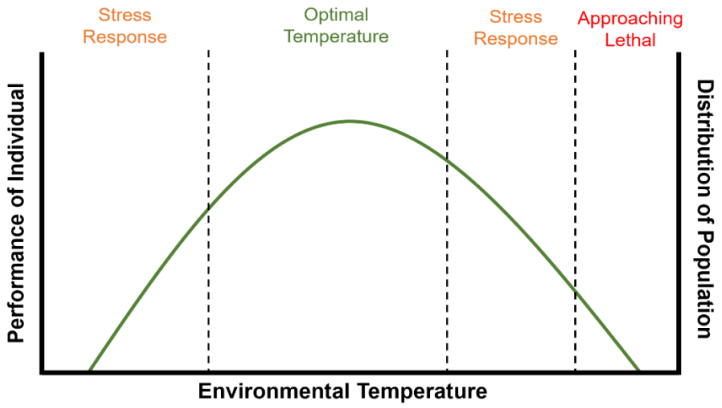

Figure 1. Thermal optima of organisms, the change in their distribution and performance (based on Jeffries *et al*, 2018). Individual performance improves as the environmental temperature approaches the optimal temperature of the organism. However, as it drifts away from that temperature, the performance of the individual starts to decline once more, and can elicit a stress response or even induce death.

In rainbow trout (*Oncorhynchus mykiss*), fish metabolic requirement for the functioning of the gut was increased by 3.7-fold (16.0 $\pm$ 3.3 ml $O_2H^{-1}Kg^{-1}$), when the temperature was increased from their optimal temperature of 10 ºC to 15 ºC (Brijs *et al.,* 2018). The gut epithelium did not show an increase in oxygen uptake capacity despite the increase in energy requirement with the 5 ºC increase (Brijs *et al.,* 2018). This result suggests that when rainbow trout are exposed to higher temperatures, the energy required to maintain their standard metabolic rate can become too costly (Brijs *et al.,* 2018). Metabolic rate plays a key role in the individual's aerobic scope as well as on their survival (Jeffries *et al.,* 2018; Le *et al.,* 2017; Nilsson *et al.,* 2009; Rummer *et al.,* 2014). The aerobic scope, the difference between minimum and maximum metabolic rate at which the organism could uptake the required oxygen to function without generating a severe deficit (Jeffries *et al.,* 2018), can also be greatly impacted by changes in temperature. For instance, in two cardinalfish species (*Ostorhinchus cyanosoma* and *Ostorhinchus doederleini*), a reduction of more than half of their aerobic capability was observed when subjected to temperatures only 2 ºC above their optimal temperature of 29 ºC (Harborne, 2013; Nilsson *et al.,* 2009). Interestingly, in three different species of damselfish (*Dascyllus anuarus*, *Chromis atripectoralis*, and *Accanthochromis olyacanthus*), with an optimal temperature between 28.5 and 29.5 ºC, a reduction of the aerobic scope was also observed when subjected to a slight increase in temperature; however, in a less extensive way than was observed in the cardinalfish (Nilsson *et al.,* 2009). These results strongly suggest that if an animal is in an environment where its aerobic scope cannot be maintained, a large consumption of body reserves could result, and if unable to maintain their metabolic requirements, they must move to an environment where their aerobic scope can be restored, to avoid mortality (Le *et al.,* 2017). These metabolic demands are not the same for all organisms (Clarke and Fraser, 2004; Rangel and Johnson, 2019), or all stages in life history.

Shifts in metabolic demands occur during embryo development due to the energetic demands of the rapid cell divisions supporting growth (Tullis and Peterson, 2000), and this may be a challenge to the progress of embryogenesis. In summary, as a result of climate change, elevated temperatures that are capable of inducing metabolic disruptions may contribute to developmental malformation that ultimately affects the survival of the embryo.

Environmental Stress and Embryo Development

In some cases, and depending on the developmental stages, many animals are unable to avoid environmental stressors such as increased temperature and low dissolved oxygen. These stressors become particularly important when the offspring develops in an external egg-case attached to the reef platform, such is the case of oviparous sharks (Kempster *et al.,* 2013). When oviparous sharks oviposit their eggs on reef platforms, the eggs are potentially exposed to several environmental stressors which include but are not limited to hypoxia (low dissolved oxygen), ocean acidification, and temperature variation (Dahlke *et al.,* 2017; Di Santo, 2015; Di Santo *et al.,* 2016; Gervais *et al.,* 2016; Gervais *et al.,* 2018; Leo *et al.,* 2018; Nay *et al.,* 2018).

When considering the impact of thermal stress on embryo development, various effects have been reported in different studies. For example, when faced with prolonged thermal stress during development, epaulette shark (*Hemiscyllium ocellatum*) hatchlings displayed significant changes in body pigmentation; however, the impact of this change on its physiology is unknown (Gervais *et al.,* 2016). Thermal stress during development can directly affect the physiology of elasmobranchs after hatching. Neonatal grey carpet sharks (*Chiloscyllium punctatum*), exposed to thermal stress (30 ºC), displayed dramatic increases in pancreatic trypsin digestive enzymes and alkaline phosphatase, both of which are key enzymes in

breaking down proteins (Rosa *et al.*, 2016). Alkaline phosphatase increased from approximately 7 U mg^{-1} to approximately 15 U mg^{-1}, while pancreatic trypsin increased from 3.25 U mg^{-1} to approximately 8 U mg^{-1} (Rosa *et al.*, 2016). However, when combined with ocean acidification, further disruptions were evident. The levels of pancreatic trypsin declined 44% and alkaline phosphatase levels declined 49% (Rosa *et al.*, 2016). This altered physiology in response to thermal stress could greatly determine the energy available to the sharks because these enzymes play a role in the breakdown of proteins (Rosa *et al.*, 2016). These trends observed in neonates suggest that enzyme abundance in developing embryos could be altered as a result of temperature increase, and this has the potential to affect their development.

Cyclic thermal stress has been noted to have contrasting effects on the survival and physiology of organisms when compared to exposure to constant temperatures (Eme *et al.*, 2018). In the lake whitefish (*Coregonus clupeaformis*) exposed to various temperatures, starting their exposure at 5 ºC on day one post-fertilization, 6 ºC on day two, 8 ºC on day three, 6 ºC on day four, 4 ºC on day five, 2 ºC on day six post-fertilization, 4 ºC on day seven, 6 ºC on day eight and 8 ºC on day nine, different changes were observed (Eme *et al.*, 2018). Across these thermal treatment groups, the survival from fertilization to hatching was significantly reduced in embryos exposed to the cyclic exposure and the continuous exposure of 8 ºC, when compared to embryos exposed to continuous temperatures of 2 and 5 ºC (Eme *et al.*, 2018). Thermal stress has been noted to increase the rate of development for embryos of numerous species, such as the lake whitefish, the grey carpet shark, Arctic cod, *Boreogadus saida*, and walleye Pollock, *Theragra chalcogramma* (Laurel *et al.*, 2018; Lee *et al.*, 2016; Rosa *et al.*, 2014). Most of the previous literature on the impact of thermal stress on embryo development has been focused on species of teleost; and as a result it is critical to explore impacts on other marine taxa.

Low dissolved oxygen can occur as a result of increased water temperature (Richards *et al.,* 2007), which can be detrimental in some species but not others. For instance, the Atlantic salmon, *Salmo salar* exposed to hypoxia displayed a decrease in growth and aerobic metabolism before hatching (Wood *et al.,* 2019a). While in the Utchee creek rainbowfish (*Melano-taenia utcheensis*), reproductive success was impaired in individuals exposed to fluctuating hypoxia levels, that dropped to as low as 10% dissolved oxygen (Flint *et al.,* 2018); nevertheless, no clear impacts were seen in the embryos produced (Flint *et al.,* 2018). Similarly, when embryos were exposed to similar conditions (diel 10% dissolved oxygen exposures), embryo development or survival did not seem to be affected (Flint *et al.,* 2018) Nevertheless, as indicated above, not all organisms respond physiologically in the same fashion. For instance, in the marine polychaete *Hydroides elegans*, hypoxia was found to increase the frequency of malformations in embryos, which were characterized by uneven cleavage or deformation of the embryo itself (Shin *et al.,* 2014). Because of the large variation in the impacts of hypoxic exposure on embryo development, it is critical to understand how different taxa and species can tolerate hypoxia, and how it influences their embryonic development and overall survival.

Elevations in temperature, also result in changes in water hydrogen potential or pH (Hoegh-Guldberg *et al.,* 2007; Mostofa *et al.,* 2016; Nagelkerken and Connell, 2015; Rummer and Munday, 2017). Changes in environmental pH can cause a wide variety of developmental malformation in aquatic animals. For instance, under low pH exposure, Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*), displayed an increased incidence of larval malformations (Dahlke *et al.,* 2017; Leo *et al.,* 2018). The observed malformations in both studies in response to ocean acidification have the potential to influence the fitness and survival of the organisms. While the specific type of malformations were not indicated by Dahlke and

colleagues (2017), or by Leo and colleagues (2018), the literature on other species of teleosts has exhibited various malformations as a result of ocean acidification. In the marine medaka (*Oryzias melastigma*) malformations were observed in the eyes, pigmentation of the head and brain when exposed to ocean acidification (Wang *et al.,* 2017). There was a reduction in eye size, combined with an altered position of the eye which can influence behaviours that require the binocular vision of the fish, potentially altering its response to visual cues of threats (Wang *et al.,* 2017). While in the brain, a variation in the asymmetry of the main three brain vesicles (forebrain, midbrain, and hindbrain) was observed (Wang *et al.,* 2017).

The environmental factors previously mentioned (elevated temperature, lowered dissolved oxygen, and pH) are directly associated with global climate change (Hoegh-Guldberg and Bruno, 2010; Hoegh-Guldberg *et al.,* 2007; McDonnell *et al.,* 2019; Rosa *et al.,* 2017). Most of the research on the effect of the above-mentioned parameters and their impact on the embryology of marine vertebrates have been performed mainly on teleosts (Eme *et al.,* 2018; Lugowska and Witeska, 2018; McDonnell *et al.,* 2019). There is little information regarding how global climate change and particularly elevated temperature can impact the development of sharks. While the environmental factors related to global climate change occur together, an understanding of how each stressor acts is needed. Hence, the focus of my research was to examine the influence of thermal stress on embryonic development, and the neonate's ability to cope with hypoxic stress. For this, I used an oviparous elasmobranch, the near-threatened grey carpet shark. Because previous research on this species looking at the embryo development that was done under control and constant temperature of 25 ºC (Onimaru *et al*., 2018), it was imperative for me to develop a new embryo development atlas that mimics their exposure under natural conditions. The creation of this atlas is highly valuable and can be used in the field with minimal invasive techniques. Specifically, my research aimed to quantify

embryo: (i) yolk consumption rate; (ii) time to hatching; (iii) time to appearance of key developmental milestones visible through the egg case; iv) size at hatching; and v) embryo survival under two temperature treatments. Furthermore, the second part of my study aimed at identifying the effect of thermal stress post-hatching on the ability of neonates to cope with a second stressor (hypoxia). The findings of this research have been separated into three distinct but complementary chapters as follows:

Chapter two, presents the novel, non-intrusive *in ovo* atlas of key developmental milestones to illustrate the milestones outlined in chapter two.

Chapter three, covers the impact of cyclic changes in temperature on embryo development.

Chapter four, explores the influence of a post-hatching exposure to thermal stress on the hypoxia tolerance of one–month-old hatchlings.

Chapter five, offers a general discussion based on the overall findings of my research.

CHAPTER 2

A novel, non- intrusive *in ovo* atlas of key developmental milestones for the near threatened grey carpet shark (*Chiloscyllium puncatum*)

INTRODUCTION

Sharks have two different modes of reproduction, oviparity, and viviparity (Hamlett and Hysell, 1998; Onimaru *et al.,* 2018; Tomita *et al.,* 2017). Species that use viviparity, give live birth and the embryos are more thoroughly protected from the environment as they are maintained in the relatively constant environment of their mother's uterus (Hamlett and Hysell, 1998). On the other hand, species that are oviparous, such as the grey carpet shark, and the lesser spotted dogfish shark, lay fertile eggs in which the embryos develop (Ballard *et al.,* 1993; Rosa *et al.,* 2014). These eggs are exposed to fluctuating environmental conditions, such as temperature, ocean acidification, hypoxia, as well as predation (Kempster *et al.,* 2013; Rosa *et al.,* 2014). The development of these embryos represents a series of critical times at which changes in environmental factors could potentially affect their survival. Life history theory dictates that animals with "slow life histories", like sharks, adult survivorship is the key to population persistence (Braccini *et al.,* 2006; Dulvy *et al.,* 2008; Dulvy *et al.,* 2014; Stevens *et al.,* 2000). However, if other life stages, such as embryo development and neonates decline or are reduced, shark recruitment could be compromised (Powter and Gladstone, 2008).

The oviparous elasmobranch, the grey carpet shark was used as a model because they are a species that has been classified as near threatened worldwide (Dudgeon *et al.,* 2016). Grey carpet sharks inhabit coral reef platforms, sheltered tidal pools, offshore bays, and seagrass beds ranging from Australia, north to Japan, and west to India (Dudgeon *et al.,* 2016; Harahush *et al.,* 2007). Very little is known about their reproductive behaviour in their natural habitat, while their reproductive behaviour has been more thoroughly examined in captivity (Harahush *et al.,* 2007). In captivity, grey carpet sharks are polygamous and females are capable of long term sperm storage of up to 45 months (Bernal *et al.,* 2015; Harahush *et al.,*

2007). However, the timing of reproduction cannot reliably be extrapolated to their natural habitat due to the manipulation of conditions in the aquaria. From this lack of literature in their natural habitat, it is clear that ecology and reproductive behaviour needs to be further explored for this species.

Due to the dramatic changes caused by climate change and the potential threat that this poses for normal embryo development, there is an increased need to develop protocols that allow us to monitor shark embryo development inside the egg cases (Gervais *et al.,* 2016; Johnson *et al.,* 2016; Rosa *et al.,* 2014). Of the few studies looking at the development of elasmobranchs, just a handful examine shark development of the lesser spotted dogfish, (Ballard *et al.,* 1993), the grey carpet shark (Onimaru *et al.,* 2018), the greater spotted catshark, (Musa *et al.,* 2018), and the white-spotted bamboo shark (Tullis and Peterson, 2000). Although the Ballard *et al.,* (1993) and Onimaru *et al.,* (2018) papers offer great embryological tools in the lab, it is not possible to track the described developmental stages in the field without sacrificing several embryos, and using specialized equipment. Furthermore, the sacrifice of threatened or near-threatened species like the grey carpet shark should be avoided.

The main purpose of this chapter begun in early 2018, and it was to create a simple and easy-to-use developmental atlas using only visible by eye milestones of shark embryos *in ovo* to be used to assess the impact of environmental factors in field situations. Recently, the temperate greater spotted catshark, from the family *Scyliorhinidae*, was used to follow the embryonic development inside the egg case and provide a simplified guide to embryo development for seven different developmental stages (Musa *et al.,* 2018), without the need to anesthetize or euthanize the organisms. The studies by Musa and colleagues (2018; 2020) provides a useful comparison to the development of a separate family of elasmobranchs under constant temperature. However, in my research, the development of the grey carpet shark was

studied for the first time under 24 °C, which is the regular temperature at which the egg cases of the grey carpet shark are found under natural conditions on the reef platforms.

The aim of my study was to develop a criteria-based atlas to describe the grey carpet shark embryo developmental stages that were clearly visible through the egg case, using the detailed milestones described in Onimaru *et al*., (2018) at the optimum rearing temperature of 25 °C. I was able to identify 11 criteria-based developmental milestones which could reliably be observed *in ovo*, as follow: i) appearance of the embryo ii) appearance of external gill filaments, iii) formation of fin buds, iv) formation of eye placodes, v) heterocercal shape of the caudal fin, vi) buccal pumping, vii) pigmentation banding on the tail, viii) resorption of the external gill filaments, ix) pigmentation up to the pectoral fins, x) dramatic widening of the trunk with the shrinkage of yolk, and finally xi) the internalization of the yolk sac prior to hatching. This field atlas will assist researchers in characterising the embryological stages of the grey carpet shark during its development while the embryos remain in the egg cases, without the need for anesthesia, preservation of the individuals, or specialized equipment. I believe that my study is of great importance because to my knowledge, it is the first study on embryo development under cyclic temperature stress to be made on a threatened tropical shark using non-invasive methods.

MATERIALS AND METHODS

This non- intrusive *in ovo* developmental atlas was created to illustrate the specific morphological and behavioural traits that characterize each individual developmental milestone for the near-threatened grey carpet shark. For its creation, I only used traits that were visible to the naked eye using the candling technique on sharks incubated at 24 ºC (control group). This atlas should be used as supplemental material for the developmental stages indicated in Chapter 2. For details regarding the experimental protocol used in the creation of this atlas, please refer to the materials and methods section in chapter 2.

RESULTS

Fertile egg cases

The mean yolk dimensions in fertile egg cases was 19.6 $\pm$ 1.94 mm^3 placed centrally, with no visible blastodisc or embryo at oviposition (Figure 2.1). The collagenous egg cases of the grey carpet shark were flat and ovoid, typically a dark brown colour with olive coloured tendrils which normally attach the egg case to the substrate or coral (Figure 2.1). The mean length of the egg cases was 10.1 $\pm$ 0.60 cm, with a width of 4.20 $\pm$ 0.21 cm.

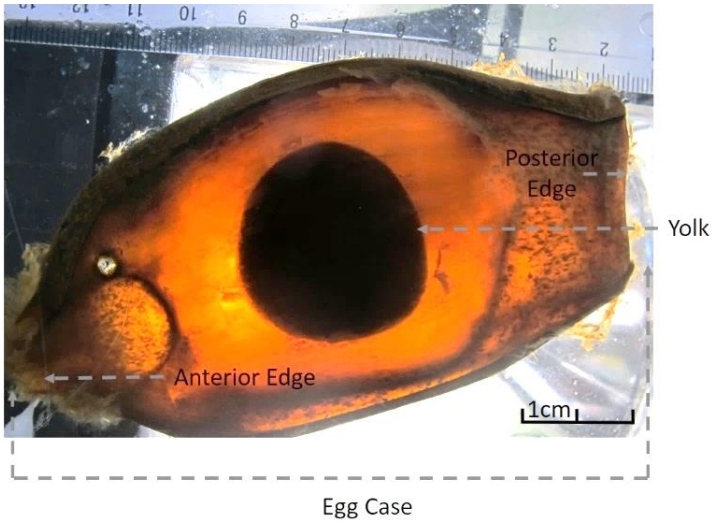

Figure 2.1. Grey carpet shark egg case with a large central yolk. The posterior edge of the egg case serves as the exit point for the embryo upon completion of development, and allows water influx and out flux upon the seal breaking. The anterior portion of the egg case is identifiable by the thumb shaped imprint which has excess tendrils to serve as the anchor for the egg case to connect it to substrate and/or corals.

Embryo Oviposition – Stage Zero

Stage zero was characterized by the presence of a blastodisc, a small swelling atop the yolk, indicating that the egg was fertile, and corresponds to stage pre-stage nine outlined by Onimaru *et al.*, (2018). Due to the colour of the egg case (amber), as well as the deep yellow colour of the yolk, sometimes it was difficult to locate the blastodisc on the animal hemisphere of the egg (Figure 2.2). This dark colouration also affected the observation of the rapid mitotic division, or cleavage, occurring at the level of the blastodisc. It is important to note that at arrival, some egg cases had already passed this stage zero because the egg cases retrieved by the divers could have been deposited some hours or a day prior to retrieval.

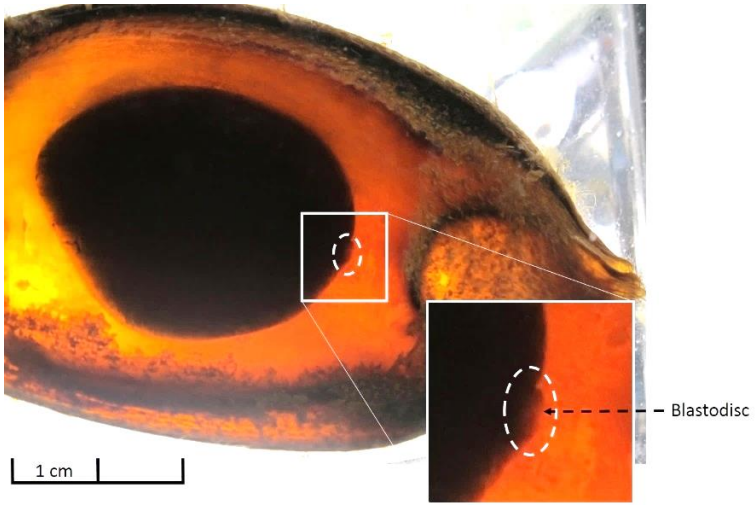

Figure 2.2. Grey carpet shark at stage zero in embryo development. A swelling blastodisc can be seen on the yolk surface (see enlarged photo, outlined by white box).

Stage One

In this stage, a small embryo was clearly observed connected to the egg yolk and the vitelline vein was visible as well, and corresponds to pre-stage nine outlined by Onimaru *et al.*, (2018). Gastrulation occurs during this stage, and the three germ layers find their placement within the embryo prior to the start of organogenesis. Unfortunately, due to the small size of the embryo at this stage, as well as the opacity of the egg case, it was impossible to obtain a high-resolution photo record of gastrulation.

Stage Two

This stage was defined by the appearance of the external gill filaments (Figure 2.3), and corresponds to stage 28 outlined by Onimaru *et al.*, (2018). Embryos displayed the external gill filaments on day 22 $\pm$ 7.6 post-oviposition. When the external gill filaments were first visible to the naked eye, the embryo's head was well defined and clearly distinct from the thoracic region (Figure 2.3). The thoracic region started to become less translucent; however,

most of the posterior end of the embryo was still translucent. Somites are normally visible at this stage, however, due to the opacity of the egg case, it was impossible to obtain a high-resolution photo record of somites.

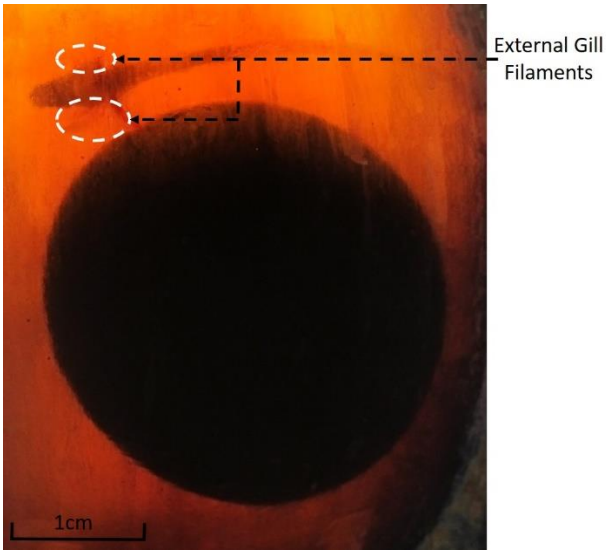

Figure 2.3. External gill filament formation in grey carpet shark embryo development, stage 2.

Stage Three

This stage was defined by the appearance of the embryo's fin buds, and corresponds to stage 29 outlined by Onimaru *et al.*, (2018). While previous studies reported that the pectoral and dorsal fins develop first (Onimaru *et al.*, 2018), their initial growth was not visible to the naked eye in my assessment. So, to prevent misidentification of early fin growth, stage 3 was identified by the presence of complete pectoral, pelvic, and dorsal fin buds. Upon appearance, the fin buds were highly translucent; however, as the embryo continued to develop, the fin buds became denser and less translucent (Figure 2.4). Embryos displayed their fin buds on day 25 $\pm$ 7.5. At the time that the fin buds appeared, the embryo had a clearly defined head region but with no apparent structures such as eyes, nares or mouth. While the cloaca region on the embryo was apparent, the caudal end of the embryo was still highly translucent.

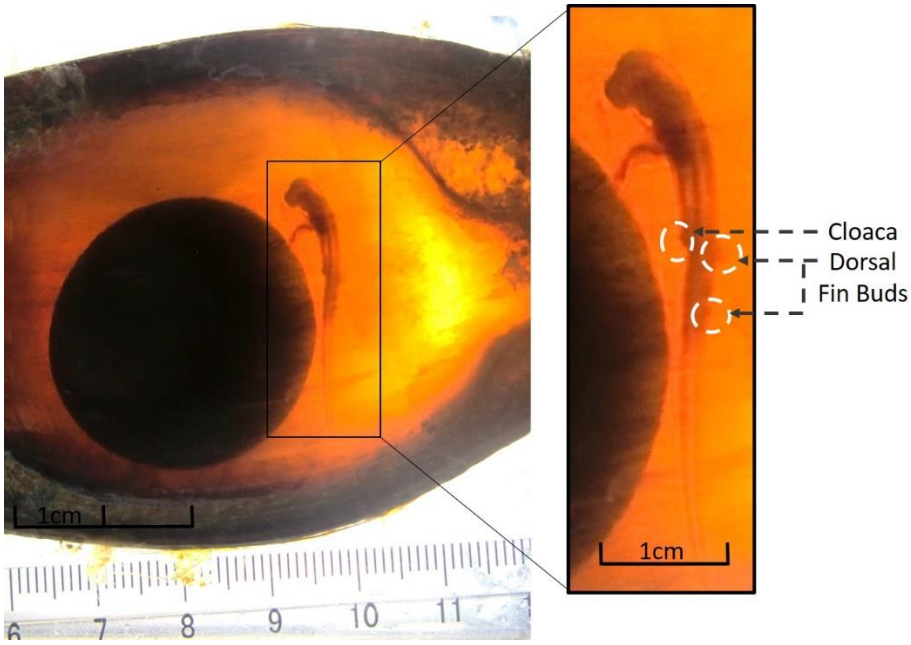

Figure 2.4. Fin bud formation in grey carpet shark embryo development, Stage 3. White circle encloses the first dorsal fin bud.

Stage Four

This stage was defined by the formation of the eye placodes, and corresponds to stage 29.5 outlined by Onimaru *et al.*, (2018). The formation of the eye placodes appeared on average on day 30 $\pm$ 7.9. At this stage of development, the embryo's head had started to swell, indicative of increased brain development and the cervical and thoracic regions were more easily distinguished. An increased growth of the external gill filaments was observed at this stage, and the caudal region of the embryos became less translucent (Figure 2.5). The pigmentation of the lens of the eye did not occur until much later in embryonic development (Figure 2.6), but it is shown here to provide a reference to the clear appearance of the pigmentation through the egg case at stage 6.

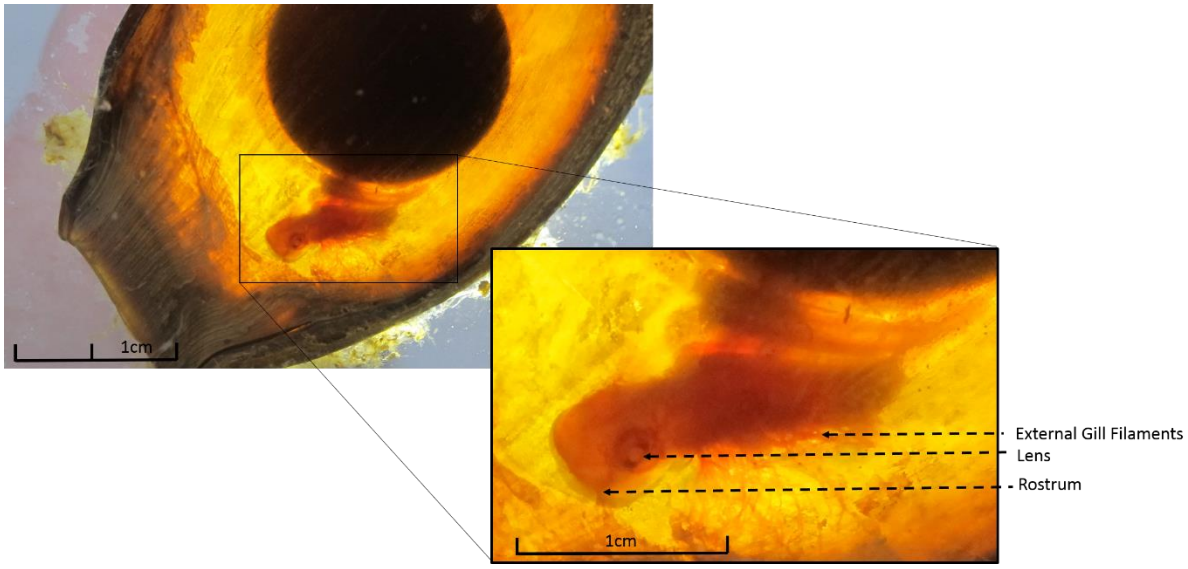

Figure 2.5. Eye placode formation in grey carpet shark embryo development, Stage 4.

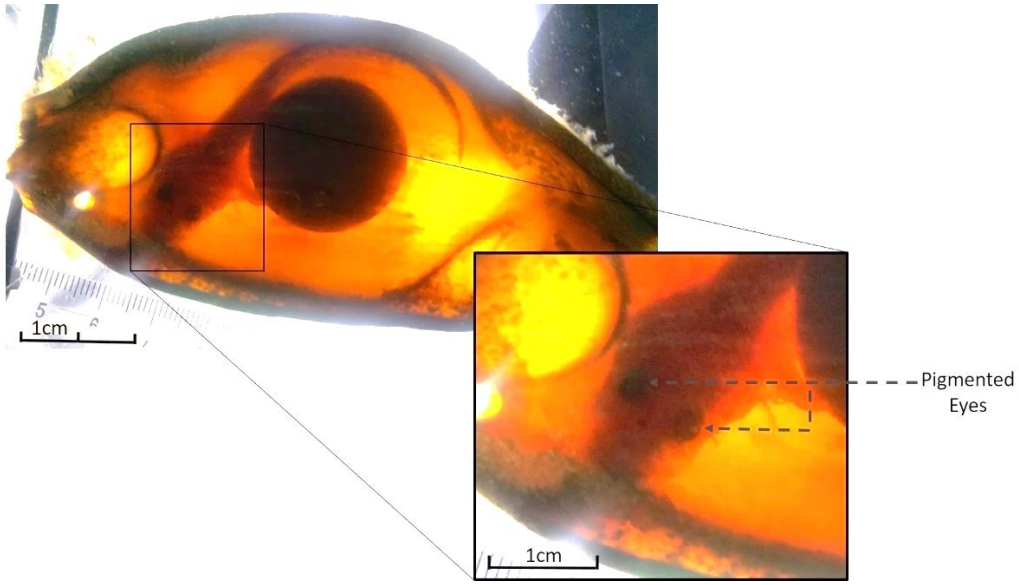

Figure 2.6. Eye pigmentation development in Grey carpet shark embryos.

Stage Five

This stage is defined by the first appearance of a heterocercal shaped caudal fin (Figure 2.7), and corresponds to stage 31 outlined by Onimaru *et al.*, (2018). The heterocercal tail appeared on average on day 36 $\pm$ 10.6. At this stage of development, the embryo was only translucent around the outer edges of the tail, where the heterocercal shape of the tail had begun to form, while all other regions of the body were simply translucent. The development of the heterocercal tail beyond the translucent appearance observed in stage five continued throughout development. The heterocercal tail became more opaque in the central region of the tail initially, then the opaque region gradually widened laterally to achieve a dense heterocercal caudal fin (Figure 2.8).

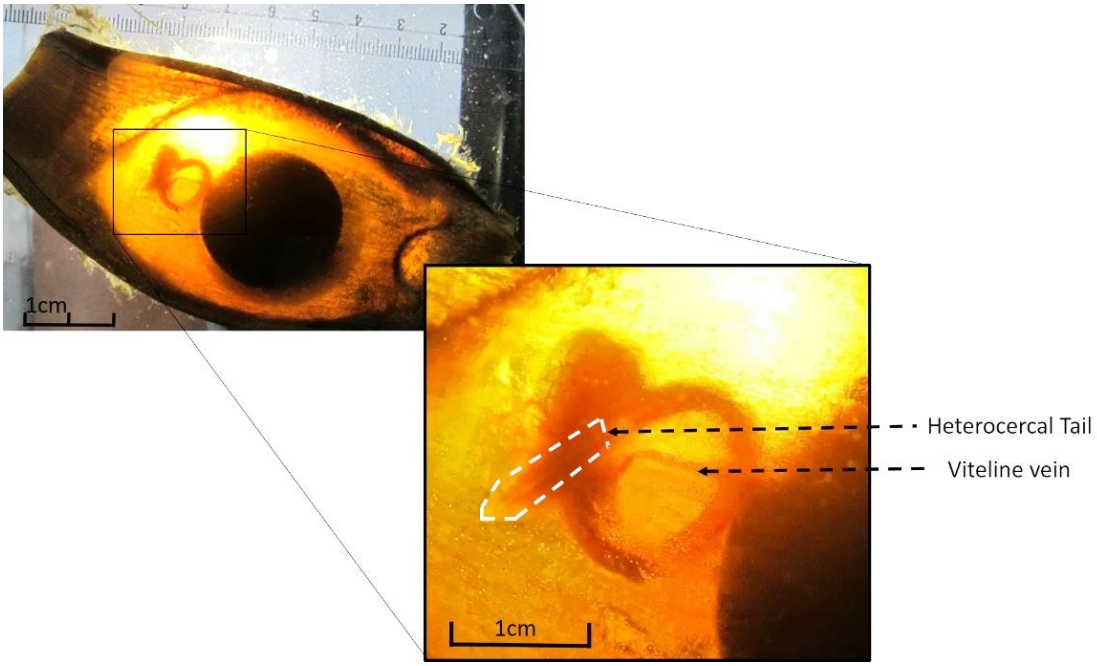

Figure 2.7. Formation of heterocercal shape in the caudal fin of grey carpet shark embryos, stage 5.

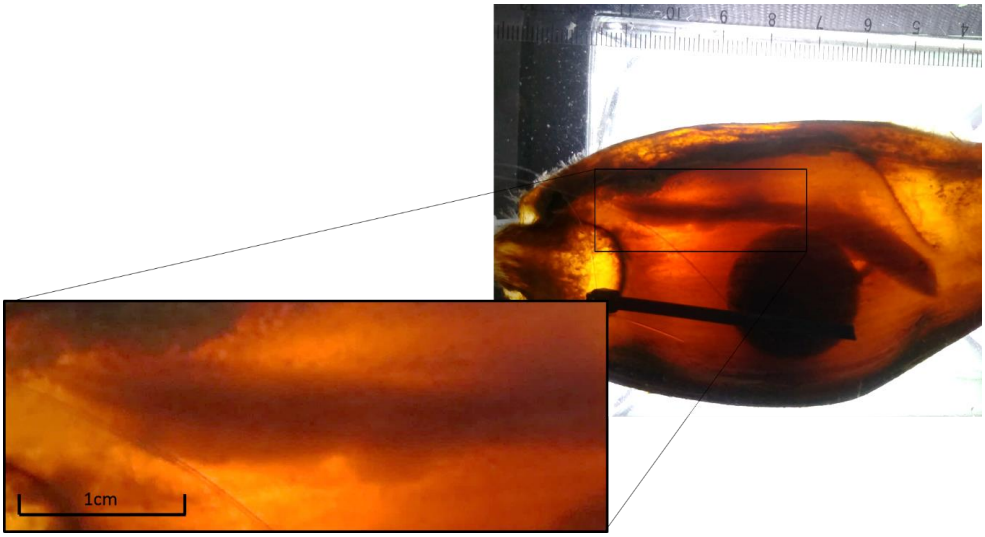

Figure 2.8. Late heterocercal caudal fin structure in Grey carpet shark embryos, dense core structure of tail while still semi-translucent outer edges.

Stage Six

This stage was defined by the appearance of the buccal pumping, a physiological mechanism used by teleost and shark embryos to increase oxygen uptake over the internal gills (Figure 2.9), and corresponds to stage six outlined by Musa *et al.*, (2018) in the lesser spotted dogfish. Specifically, the buccal pumping occurred on average on day 59 $\pm$ 7.8. Interestingly, at the onset of buccal pumping, the external gill filaments were not fully reabsorbed. The resorption of the external gill filaments occurred on average 5.40 $\pm$ 4.16 days after buccal pumping had been observed (stage eight). One individual resorbed the external gill filaments on the same day as the first observed case of the buccal pumping. Embryos sometimes had colour banding on their tail (stage seven) prior to the onset of buccal pumping. Pigmentation of the eyes was present in all individuals.

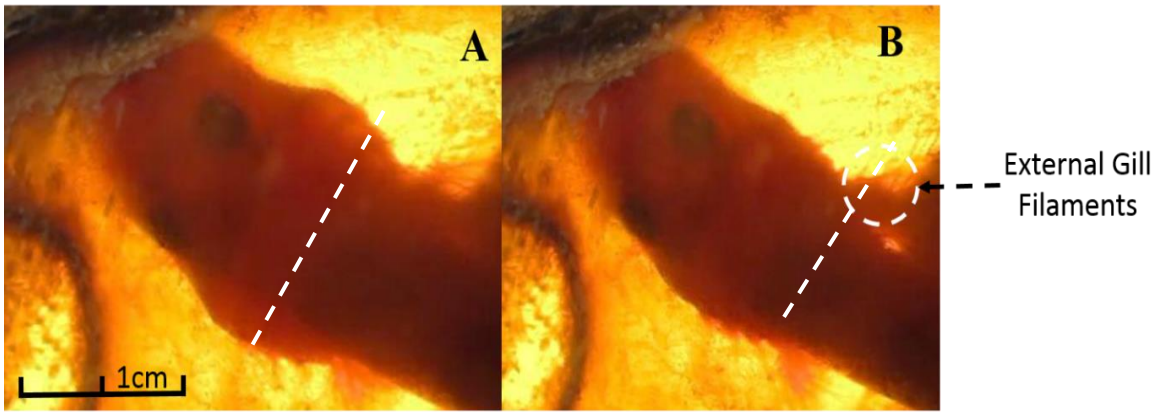

Figure 2.9. Resorption of external gill filaments. Full buccal cavity expansion in grey carpet sharks (**A**), followed by an individual with compressed buccal cavity after exhalation (**B**).

Stage Seven

This stage was defined by the appearance of the striped colour banding extended to the tail (Figure 2.10), and corresponds to stage 34 outlined by Onimaru *et al.*, (2018). Embryos completed their tail banding on day 59 $\pm$ 4.8. Some variation in the appearance of tail banding pattern was evident relative to the onset of buccal pumping. Two individuals displayed banding on the tail five and nine days prior to the onset of buccal pumping, respectively. All other individuals displayed banding on the tail on the same day that buccal pumping had started or subsequently.

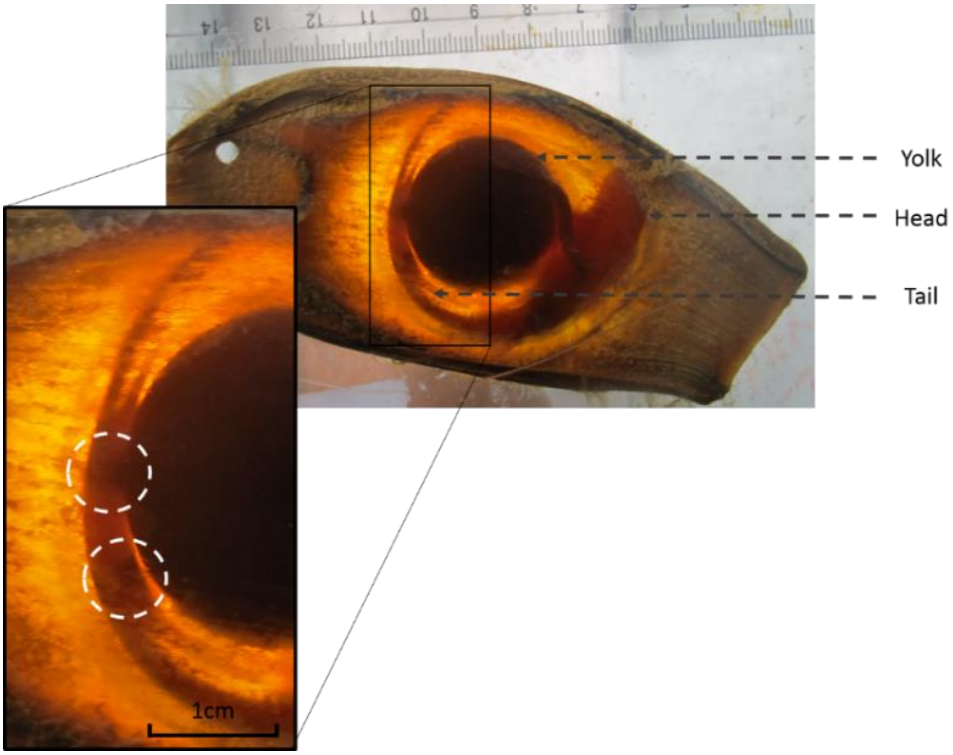

Figure 2.10. Tail banding in grey carpet shark embryo development, stage 7. White circles indicate areas of pigmentation on tail of embryo.

Stage Eight

This stage was defined by the complete resorption of the external gill filaments of the embryo (Figure 2.11), and corresponds to stage 36 outlined by Onimaru *et al.*, (2018). The external gill filaments were resorbed typically on day 65 $\pm$ 3.1.

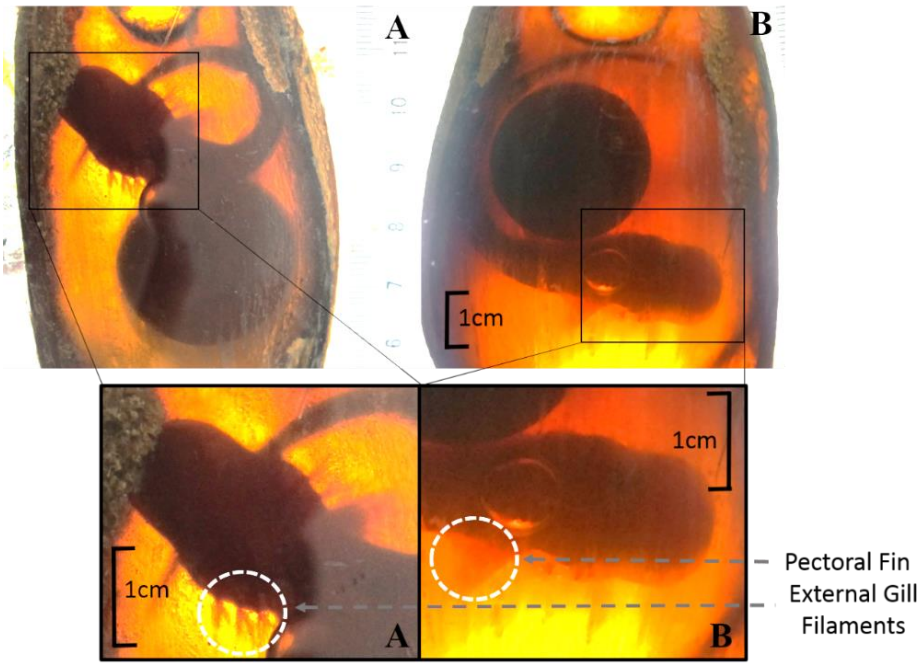

Figure 2.11. Early resorption of external gill filaments in grey carpet sharks (**A**), complete resorption of external gill filaments (**B**). The apparent projection in photo B, extending from the embryo over the yolk (deep orange line), exists on the external portion of the egg case, as some egg cases developed a layer of residue over time when they had been scraped so that the embryo could be viewed. This residue was thus periodically scraped off; however, small amounts were sometimes missed despite thoroughness of scraping the egg case.

Stage Nine

This stage was defined by presence of banded pigmentation up to the tip of the pectoral fins (Figure 2.12), and corresponds to stage 37 outlined by Onimaru *et al.*, (2018). This body banding pattern typically started out as a pale coloration and slowly started to darken until pigmentation spread completely along the length of the embryo, from the tail to the rostrum. The average time that the pigmentation took to reach the tips of the pectoral fins was by day 63 ± 5.1.

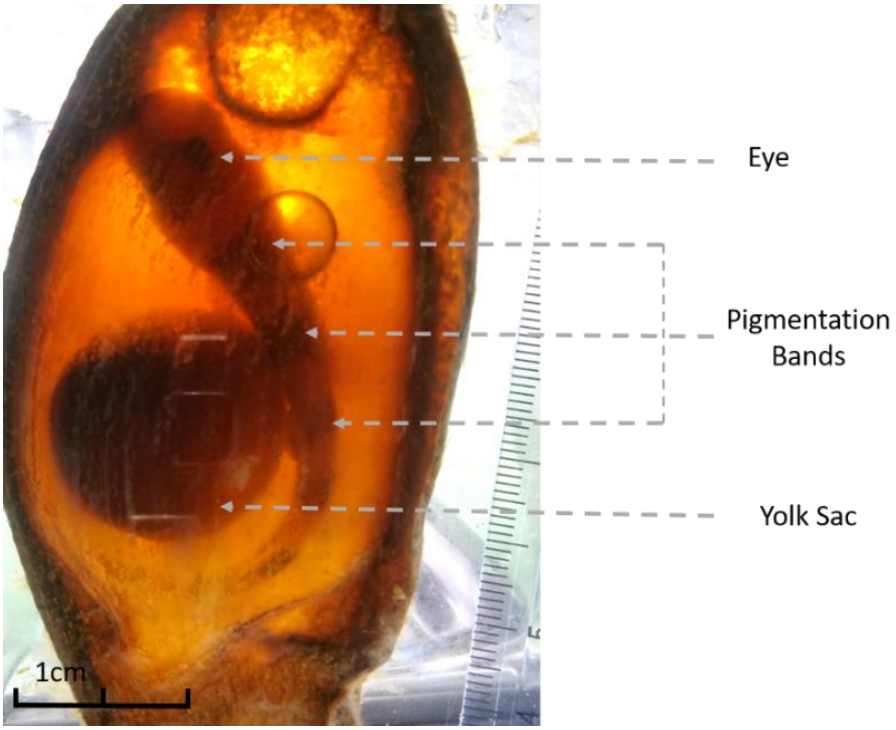

Figure 2.12. Pigmentation expanding to the pectoral fins in grey carpet shark embryo development, stage 9. The dashed lines indicate areas of pigmentation on embryo.

Stage 10

This developmental stage was defined by the observed widening of the trunk (Figure 2.13), and corresponds to stage 37.5 outlined by Onimaru *et al.*, (2018). The widening of the trunk was apparent on average on day 84 $\pm$ 2.6. There was a noted increase of the trunk width by 40% between stage nine and stage ten (0.89 $\pm$ 0.08 cm, which increased to 1.46 $\pm$ 0.13 cm). Furthermore, this stage was accompanied by an increase in the width of the head as the thoracic cavity widened. The width of the head was not measured due to the interference incurred during buccal pumping.

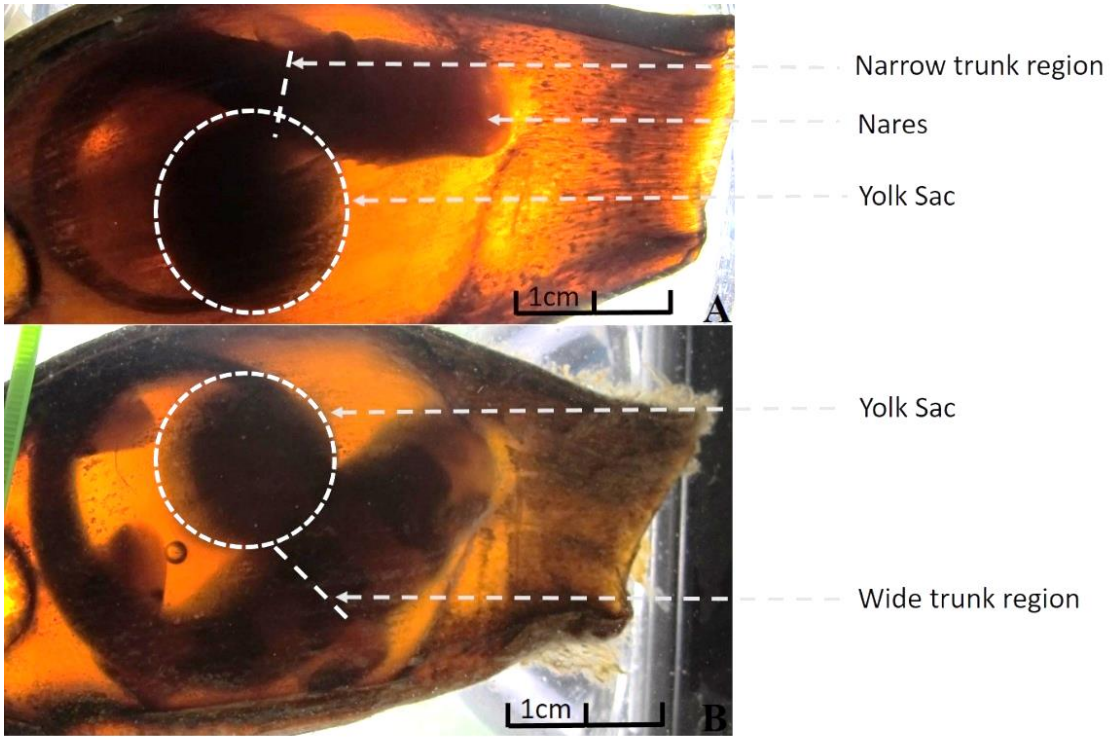

Figure 2.13. Widening of the grey carpet shark embryo trunk. **A** is a photograph of an embryo at stage 9, prior to widening of the trunk, illustrating the narrow head and trunk widening, as well as the size of yolk sac prior to yolk internalization. (**B**) is a photograph of an embryo at stage 10, in which the yolk was dramatically diminished in size and the trunk and head had widened

Stage 11

At this stage, body pigmentation as well as resorption of external gill filaments were completed, and buccal pumping was in use. This stage was defined by the complete internalization of the yolk (Figure 2.14), and corresponds to stage 39 outlined by Onimaru *et al.*, (2018). The yolk was internalized on average by day 105 $\pm$ 4.9. The overall time to hatching for the embryos was 120 $\pm$ 3.2 days.

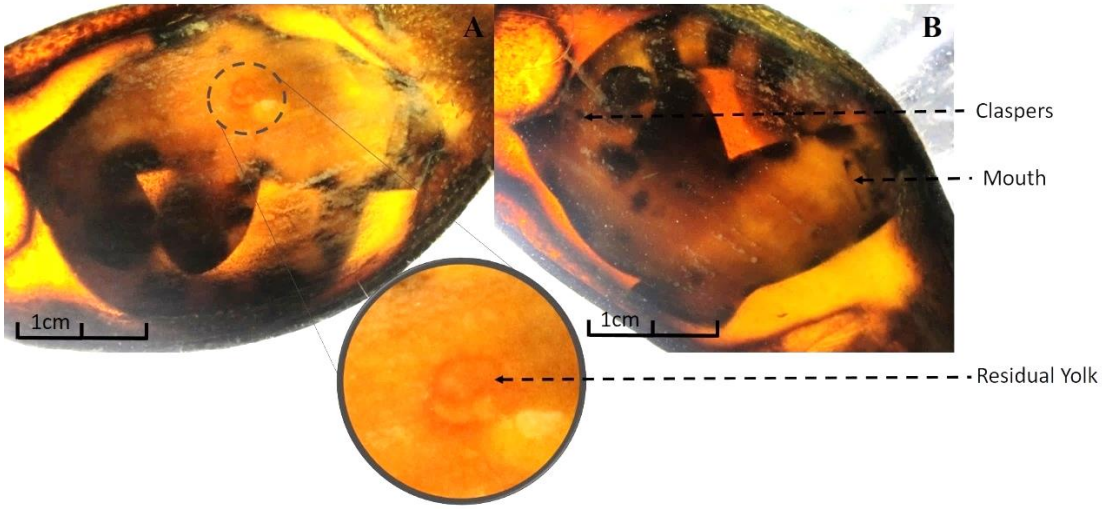

Figure 2.14. Internalization of yolk, at stage 11. **A** almost complete internalization of the yolk, the small remnant of the yolk sac can be seen in the zoomed in image (outlined by grey circle). **B** complete internalized the yolk sac.

DISCUSSION

It has been proposed that the phases of somitogenesis and organogenesis are the most vulnerable stages of development for embryos (Latif *et al.*, 1999; Shang and Wu, 2004). The beginning of organogenesis corresponds to the **stage one**, when the elongated form of the shark sitting on top of the yolk sac can be observed through the cleaned egg case with the naked eye. The appearance of the external gill filaments in **stage two** play a key role in providing oxygen to the embryos as they develop up until the point at which buccal pumping becomes the primary source of oxygen intake (Tomita *et al.*, 2014). The external gill filaments are threadlike tissue extending out of the forming gill slits in embryos, and contain blood vessels which are responsible for taking in oxygen and transporting the oxygen to the rest of the developing embryo (Pelster and Bemis, 1992). These external gill filaments are key to oxygen uptake during embryonic development as they function *via* passive diffusion, which means that they

do not require energy to take the oxygen into the tissue of the embryo (Tomita *et al.*, 2014). However, as the embryo grows and their metabolic requirement increases, the switch to buccal pumping commences, capable of providing increased oxygen to the embryos and allowing the embryo to regulate its oxygen intake by changing the rate of pumping to meet the metabolic demands (Tomita *et al.,* 2014).

In **stage three**, the fin buds became evident consisting of the first and second dorsal fin, the pectoral fins and the pelvic fins. Each fin plays an important role in the motility of the embryos upon hatching and into adulthood (Maia and Wilga, 2013a; Maia and Wilga, 2013b). While the function of these fins has not been directly explored in the grey carpet shark, their function can be extrapolated from other species. For example, in the close relative white-spotted bamboo shark, the dorsal fins generate thrust during steady swimming and provides stability (Maia and Wilga, 2013a, 2016; Maia *et al.*, 2017). The second dorsal fin provides thrust, as a result of the more posterior portion of the dorsal fin deviating from the center axis of the body of the shark, and moving from the right to the left as a result of the tail movement (Maia and Wilga, 2013b; Maia *et al.,* 2017). More specifically, it has been reported that the second dorsal fin had a stronger function in developing thrust, while the first dorsal fin (most anterior) is more important in stabilizing the shark in an upright position (Maia and Wilga, 2013b; Maia *et al.,* 2017). While the pectoral and pelvic fin function has not been explored in grey carpet sharks or any close relatives, some hypotheses on their function could be made from observations of neonatal behaviour. Neonate locomotion consists mainly of tetrapodal movements, resulting in sinuous waves of the body passing from the head to the tail, as if they were walking on the floor of the aquarium using their pectoral and pelvic fins as pseudo feet. In addition, steady swimming was observed in neonates that were around 70 days post-hatching (Personal Observation).

The eye placodes in **stage four** represent the precursors of eye formation (Fuhrmann, 2010; Gilbert and Barresi, 2016). The eyes of the embryo and neonate are critical for detecting predators (Kempster *et al.,* 2013). Even though sharks also rely on electroreception (detection of the bioelectric field of other organisms) to avoid predation (Gauthier *et al.,* 2019; Kempster *et al.,* 2013; Newton *et al.,* 2019), electroreception seems to appear later during development (Kempster *et al.,* 2013; Onimaru *et al.,* 2018). Interestingly, when sharks perceive the small bioelectric output of predators and prey alike, they ceased movement to limit their own bioelectric output, which was proposed to be a strategy to avoid detection (Kempster *et al.,* 2013).

The heterocercal tail present in **stage five** is largely responsible for the generation of thrust during steady swimming in adults, juveniles and neonates (Maia and Wilga, 2013a, 2016; Maia *et al.,* 2017). In grey carpet shark embryos, the heterocercal tail most likely serves the same purpose as observed in the little skate, to draw oxygen rich water into the egg case, ensuring that oxygen requirements are met for metabolic processes (Di Santo *et al.,* 2016). In the little skate exposed to hypoxia, an increased tail beat frequency was observed while its metabolism decreased to cope with low levels of oxygen (Di Santo *et al.,* 2016).

Buccal pumping observed in **stage six**, requires the use of muscles in the buccal cavity in order to expand and reduce the volume of water flowing over the gills (Ballard *et al.,* 1993). While performing buccal pumping, sharks are able to rhythmically draw in seawater through the mouth and over the gills, so that the oxygen extracted by the gills can provide oxygen to the organism (Ballard *et al.,* 1993; Thomason *et al.,* 1996). Up to the onset of buccal pumping, the embryos relied upon the passive diffusion of oxygen *via* their external gill filaments (Ballard *et al.,* 1993; Onimaru *et al.,* 2018). The commencement of buccal pumping signifies the shift of the embryo's major respiratory organ from the external gill filaments to the gill

lamellae of the internal gills (Tomita *et al.*, 2014). In the cloudy catshark (*Scyliorhinus torazame*), there were four developmental changes occurring prior to the commencement of buccal pumping (Tomita *et al.*, 2014). These four developmental changes were: i) regression of external gill filaments; ii) development of blood vessels within the "internal gills"; iii) completion of development of hyoid skeletal and muscular elements; and iv) development of the oral valve (Tomita *et al.*, 2014). The final three developmental changes were not visible to the naked eye in my study. However, the resorption of external gill filaments was easily observed and was not completed until after buccal pumping commenced in four of the five individuals (Personal Observation).

Pigmentation was first observed in the eyes of the embryo in **stage seven,** when the embryo's mouth becomes oval, followed by the appearance of a black dot on the second dorsal fin of the embryos, which occurred shortly after the head developed an obtuse angle along the top of the head; these two patterns of pigmentation have been previously reported by Onimaru *et al.*, (2018). This pigmentation is generated by melanocytes from the neural crest cells (Erickson and Goins, 1995; Sommer, 2011). Previous studies on the grey carpet shark neural crest cells migration showed that the migration of cells occurs in a similar pattern as in zebrafish and amphibians (Collazo *et al.*, 1993; Collazo *et al.*, 1994), and it occurs in two waves (Juarez *et al.*, 2013). The first migration of neural crest cells migrate dorsolaterally between somites and the ectoderm to generate the melanocytes, and populate the dorsal part of the integument (Juarez *et al.*, 2013). The cells migrating over the rostral neural tube, move rostrally first over the hind brain, while other cells migrate ventrally (Juarez *et al.*, 2013). A similar pattern of pigmentation migration was observed in the lesser spotted dogfish when developing at a constant temperature of 16 °C (Ballard *et al.*, 1993). The appearance of pigmentation in the eyes corresponds to the dense concentration of neural crest cells in the

rostral region first, and it is similar to the findings reported in the grey carpet shark (Juarez *et al.,* 2013). The discrepancy with the second wave of melanocytes appearing more caudal-dorsally in my study has previously been reported in other studies (Onimaru *et al.,* 2018). Because the embryos were observed within the egg case, it is plausible that the initial faint melanocyte migration was not always observed in the trunk area compared to the more pronounced banding on the thin tail (Onimaru *et al.,* 2018).

The complete resorption of external gill filaments in **stage eight** was previously thought to be a precursor to buccal pumping in shark embryos, as was found in the cloudy catshark (Tomita *et al.,* 2014). However, this was not the case in my study, where four out of five embryos resorbed their external gill filaments after buccal pumping had commenced.

The body pigmentation reaching the pectoral fins of the embryonic grey carpet sharks in **stage nine**, as suggested elsewhere, could serve as a form of Batesian mimicry to reduce the chances of predation (Kempster *et al.,* 2013). Newly hatched neonates can be very vulnerable to predation (Heupel and Simpfendorfer, 2011). Hence, by displaying a banding pattern that is similar to that of the highly venomous black banded sea snake, which inhabit the same reef platforms as neonate sharks, the neonatal grey carpet sharks would be more likely to deter predation. As the neonates grow and mature, this banding slowly disappears. In two individuals an irregular pigmentation was observed, where the regular banding of the neonates was accompanied by black speckling along the length of the body (Figure 2.15). The exact cause of this irregular pigmentation pattern is unknown, and further studies are required to determine if there is a genetic component causing the irregular pigmentation, or if it confers an advantage, or if an environmental factor was involved.

Figure 2.15. Grey carpet shark neonate upon hatching, irregular pigmentation.

During **stage ten**, the widening of the trunk was accompanied by a decrease in yolk volume (see Chapter 2 for further details on yolk volume change and embryo length variation), reflecting a combination of the yolk being internalized as well as yolk being used to meet metabolic requirements as the embryo prepares to hatch (Onimaru *et al.*, 2018; Tullis and Peterson, 2000). The final stage of development prior to hatching was the complete internalization of the yolk, which ensures that the embryo has energy reserves to sustain it after hatching, until it is able to acquire its own food (Kraemer and Bennett, 1981; Troyer, 1983). If internalization of yolk does not occur before hatching, the yolk could become damaged or be severed completely from the body as a result of movement through varying terrain on the coral reef flats.

In conclusion, my study clearly shows that a non-invasive observation and criteria-based staging of developing oviparous tropical sharks is possible without detrimental effects on the embryo's survival. This non-invasive study was able to outline 11 developmental stages: i) appearance of the embryo from the blastodisc; ii) formation and emergence of external gill filaments; iii) formation of fin buds; iv) appearance of the eyes; v) development

of heterocercal tail; vi) transition to buccal pumping; vii) appearance of pigmentation banding on tail; viii) resorption of the external gill filaments; ix) completion of pigmentation reaching the pectoral fins; x) widening of the trunk region; and xi) internalization of the yolk in the grey carpet shark. All of these developmental stages were reliably visible despite the embryo's position and the dark opaque nature of the egg case, and have been previously used as key traits of development through albeit invasive methods (Onimaru *et al.,* 2018). Being able to identify developmental milestones in a non-invasive manner, is a necessity in the field, and particularly when working with endangered species, such as the grey carpet shark. This newly developed set of stages for a non-invasive study was also useful to observe the impact of cyclic thermal stress on the appearance of developmental milestones as well as the time to hatching.

CHAPTER 3

The Impact of Cyclic Thermal Stress on Embryo Development in the grey carpet shark (*Chiloscyllium punctatum*)

INTRODUCTION

Embryo development is a vulnerable phase in the life cycle of most animals (Di Santo, 2015; Eme *et al.,* 2015; Flint *et al.,* 2018; Latif *et al.,* 1999; Primmett *et al.,* 1988; Roy *et al.,* 1999), and many environmental factors have the potential to alter any of the developmental phases to the point of threatening their survival (Rosa *et al.,* 2014; Roy *et al.,* 1999; Wargelius *et al.,* 2005; Weiss and Devoto, 2016). Specifically, changes in temperature correlate with many biological processes in elasmobranchs (Di Santo, 2015; Hume, 2019; Magnuson *et al.,* 1979); and this is particularly true for elasmobranchs during the life stages where individuals are unable to move, such as during the long sessile developmental period (Hume, 2019; Luer and Gilbert, 1985). Furthermore, changes caused by temperature variation can have a greater impact on sharks because they take a long time to reproduce (Dulvy *et al.,* 2008; Dulvy *et al.,* 2014; Stevens *et al.,* 2000); they have extremely low population growth rates (Braccini *et al.,* 2006; Dulvy *et al.,* 2008; Dulvy *et al.,* 2014; Stevens *et al.,* 2000); and most importantly, they have a weak density-dependent compensation in juvenile survivorship (Cortes, 2000). Hence, if embryo development and neonates decline or are reduced, shark recruitment could be compromised, and the global shark population could reach threatening levels (Barnett *et al.,* 2019; Powter and Gladstone, 2008).

Recent studies have explored the following environmental stressors and how they affect shark embryo development: (i) temperature; (ii) ocean acidification; (iii) dissolved oxygen content; and (iv) predation (Anderson and Podrabsky, 2014; Dahlke *et al.,* 2017; Kempster *et al.,* 2013; Kobayashi *et al.,* 2017). However, very little is known about the effects of these environmental factors on the developing embryos of oviparous species such as the grey carpet shark, that inhabits dynamically changing shallow coastal environments. Since their eggs are

sessile, the embryos are more susceptible to changes in environmental conditions (Wheeler *et al.,* 2020). Contrary to viviparous shark embryos, which develop under constant internal environmental conditions (Hamlett and Hysell, 1998), grey carpet shark embryos, receive no maternal care once the eggs are laid on the coral reef flats, and can be subjected to stress provided by alterations in environmental factors (Kempster *et al.,* 2013; Rosa *et al.,* 2014), referred to as stressors. Environmental factors include but are not limited to: lower water level, diminished dissolved oxygen availability; temperature changes; as well as a high predation risk (Kempster *et al.,* 2013; Rosa *et al.,* 2014).

To accurately discern whether any specific developmental stage in sharks is more vulnerable to environmental stressors than others, we need to first examine each stressor separately at different developmental stages; secondly, we need to examine whether potential synergistic effects exist between the stressors. Because there are only a few studies on shark development that examine the effects of environmental stressors on the development of the embryo, we must use the information on exposure to thermal stress from other vertebrates such as teleosts, birds, or amphibians to create the experimental design.

Previous research on walleye embryos (*Sander vitreus*) under their optimal developmental temperature of 10 ºC, showed that organogenesis was the stage of development most likely to display deformities or mortality (Latif *et al.,* 1999). Up to 80% of the observed mortality of eggs occurred during gastrulation and early organogenesis (Latif *et al.,* 1999). Mortality at other points of development included 0.2% when the blastodisc was dividing into blastomeres (day one), while the remainder of the mortality spiked at 3.5% before gastrulation on day two (Latif *et al.,* 1999). While it is beneficial to know the stage at which the embryos are vulnerable without any environmental stressors, it is highly unlikely that habitat selected by females to oviposit their eggs is still at the optimal temperatures for the developing embryos

as a result of climate change. So, it is critical to explore the vulnerability of embryos when facing varying environmental factors and a combination of factors.

The impact of thermal stress has been explored in relation to organogenesis (Iida *et al.*, 2016; Lugowska and Witeska, 2018). When the common barbel (*Barbus barbus*) was exposed to varying temperature treatments, both below and above their thermal optima, there were three types of embryonic body malformations: (i) yolk sac oedema; (ii) spine curvature; and iii) shortening of the body (Lugowska and Witeska, 2018). These abnormalities along with lower survivorship, occurred at both thermal extremes of 12°C and 22°C, while those closer to their optimal temperatures (14 ºC and 18 ºC) showed the highest survivorship and least body abnormalities (Lugowska and Witeska, 2018). Recent studies continue to show that the offspring of many different organisms become shorter as the temperature continues to rise (Gardner *et al.*, 2011; Lema *et al.*, 2019; Wonglersak *et al.*, 2020; Wu *et al.*, 2019). In the amargosa pupfish (*Cyprinodon nevadensis amargosae*, a reduction of body length of about 7.4%, and a decline in body mass of about 36% was observed between the year 2008 and 2016 when water temperature increased from 24 $\pm$ 5.3 °C to 33.0 $\pm$ 3.6 °C (Lema *et al.*, 2019). All these studies on the effects of temperature change on different aspects of embryo development in teleosts, strongly suggest that temperature should also solicit some type of changes and alterations on the embryo development of sharks.

Rather than expose developing shark embryos to continual thermal stress as already done in other studies (Gervais *et al.*, 2018; Musa *et al.*, 2020; Rosa *et al.*, 2014; Rosa *et al.*, 2016), a novel method was developed to investigate thermal stress as it is experienced by fish in shallow coastal environments such as reefs and estuaries. The periodicity of intermittent cycles of exposure to elevated temperatures was calculated based upon an examination of the day-time low tide cycles which fell between 11:00 A.M. and 3:00 P.M on the Heron Island

reef platform, inside the fringing reef, over a 12-month period (Renshaw Personal Communication). The rationale for this approach is supported by reports of cyclic changes in temperature and dissolved oxygen occurring on the reef platforms during high and low tide (Reid *et al.,* 2019; Zhu *et al.,* 2014). The nadir of the low tide is approximately 2 hours, then the incoming tide equalises the water temperature with that of the surrounding ocean. In summary, the duration of the exposure time for cyclic thermal stress was 2 hours and the calculated periodicity between 11:00 am and 3 pm was 5 days of day-time low tides followed by 9 days without day-time low tides (Renshaw Personal Communication). An increase in temperature is normally associated with a reduction in water dissolved oxygen (Schmidtko *et al.,* 2017). Hence, under natural conditions embryos are not only exposed to the increase in temperature during low tide, but also to a decrease in dissolved oxygen (Harborne, 2013), which could detrimentally affect embryo development by this synergistic effect (Anderson and Podrabsky, 2014).

In this chapter, I explored the impact of high-temperature exposure on grey carpet shark embryos in a manner that mirrored the natural cyclic pattern of low tides in their natural habitat. More specifically, I aimed to determine which key developmental milestones were vulnerable to thermal stress during embryogenesis. The time of appearance of the following specific milestones was examined: formation of external gill filaments; formation of fin buds; formation of eye placodes; the heterocercal shape of caudal fin; buccal pumping; pigmentation banding on the tail; resorption of the external gill filaments; pigmentation of the pectoral fins; widening of the trunk region; the rate of yolk utilisation; internalization of yolk; the time until hatching; as well as the survival rate of embryos and neonates. These developmental milestones were determined by using characteristic traits described for other elasmobranch species (Ballard *et al.,* 1993; Harahush *et al*., 2007; Musa *et al*., 2018; Musa *et al*., 2020;

Onimaru *et al*., 2018). However, only the developmental milestones that could be seen without opening the egg cases were compiled into a developmental atlas for embryos that were kept at their normal rearing temperature (24 ºC). This developmental atlas is described in detail in Chapter 3, and it was used to assess the impact of cyclic thermal stress on developing embryos as presented in this chapter.

Since previous literature has indicated that grey carpet shark embryos exposed to a constant temperature of 30ºC hatch on average 10 days faster than embryos exposed to an optimal temperature of 26 ºC (Rosa *et al.*, 2014), I hypothesized that cyclic exposure to high temperature decreases the time to hatching. Based on this hypothesis, I made the following predictions:

1. If shark embryos are susceptible to high temperatures as observed in teleost embryogenesis, mortality should differ between the two temperatures (24 ºC and cyclic 28 ºC).
2. The use of yolk reserves should differ between the two temperature treatments (24 ºC and cyclic 28 ºC).
3. If embryos under cyclic thermal stress resorb yolk reserves at a faster rate under high temperatures, then a difference in time of hatching should be observed between the two temperature treatments (24 ºC and cyclic 28 ºC).
4. If exposure to higher temperatures alters the time of hatching, then the time at which each developmental milestone appears should differ between thermal treatments (24 ºC and cyclic 28 ºC).

The data collected will build on existing knowledge to provide specific information about the impact of thermal stress on grey carpet shark embryo development.

MATERIALS AND METHODS

Specimens: source of egg cases and maintenance.

Fertile eggs of the grey carpet shark (Figure 3.1) were used. This oviparous species was selected as a model because it has a relatively short development period taking approximately 118 days to hatch when incubated at 25 ºC (Onimaru *et al.*, 2018; Rosa *et al.*, 2014) compared to other oviparous species such as the lesser spotted dogfish (*Squalus acanthias*) in which hatching takes place after approximately 2 years. The relevance of using the grey carpet shark is that it is currently listed as near threatened by the international union for the conservation of nature (IUCN) and in Australia, it is well represented in reef ecosystems and sea grass beds (Kempster *et al.*, 2013) at present.

Figure 3.1. The Grey Carpet Shark (*Chiloscyllium punctatum*). Grey Carpet Shark key life stages, neonate (left) and adult (right).

Eggs were provided by Sea World, Gold Coast, Australia. From a total of 686 egg cases obtained for the experiments, only 461 were suitable for incubation. Some eggs were discarded because they: (i) had fungal infections, n=50; (ii) were blank (no yolk present), n= 58; (iii) showed signs of predation, n= 7; or (iv) had a disintegrated yolk, n= 110. Predation occurred as a result of the adult grey carpet sharks sharing an artificial bay with other organisms that are known to predate on them such as other sharks, and teleost fishes (Cox and Koob,

1993; Sisneros and Tricas, 2002). At the end, a total of 30 egg cases were haphazardly assigned to a (i) control group (24 ºC), or (ii) thermal stress group (28 ºC), or (iii) extreme high temperature group (32 ºC). Unfortunately, about 40 days prior to the predicted hatching period a severe storm caused a power outage and the entire project setup needed to be relocated. As a result, due to space restrictions, time constraints and other logistic situations, the extreme high temperature group (32 ºC) had to be stopped, and so, it was eliminated from the study. This resulted in 15 of the individuals being removed from the treatments (24 ºC: 1; 28 ºC: 3; 32 ºC:11). From the total of 30 egg cases assigned to temperature treatments few died at one point before the end of the experiment (24 ºC: 3; 28 ºC: 2). Nevertheless, we were able to salvage the rest of the experiment (Control group = 5; Thermal stress group = 5), and maintained the sharks until they completed 80 days post-hatching. When individuals undergoing thermal treatment died, they needed to be replaced; the new egg needed to have no embryo visible, ruling out individuals in the holding tank which were past this point in development. Because sharks only laid few eggs at a time, the eggs were assigned to treatments throughout the year haphazardly. Furthermore, the time required to run the embryos through heat treatment, combined with a limited number of tanks and heaters available, the treatments were run at various times throughout the year until all embryos hatched, and after the 80 days post-hatching.

Each egg case was carefully scraped using a scalpel blade so that the embryo and yolk were visible before placing the egg case into the quarantine tank (10000 L) under a controlled temperature of 23.8 $\pm$ 0.11 ºC with a natural photoperiod where they remained until they were determined to be fertile. Each egg case was then labeled, using a combination of coloured zip ties and an individual number on an identification tag. Egg cases were then suspended vertically, about 5 cm below the water surface using plastic pegs. Once egg cases were

determined to be fertile (1-2 weeks), they were transferred to a 300 L holding tank, and placed into individual containment units until hatching. Water temperature in the holding tank was maintained at 24.6 $\pm$ 0.07 ºC using a Hailea hc-250a® chiller during the summer months; while during the winter months, it was maintained at 23.6 $\pm$ 0.29 ºC. Throughout the study, dissolved oxygen was maintained at normoxic levels (5.7 $\pm$ 0.28 mg O_2 L^{-1}) in the holding tank that eggs were kept in when not in treatment. Temperature and dissolved oxygen levels for egg cases used here were monitored every day and on alternate days at 15-minute time intervals for 24 hours using a WinTPS WP82Y dissolved oxygen meter equipped with a YSI probe®.

In the holding tank, the egg cases were placed into individual containment units. The eggs were kept in these containment units after they had been identified to be fertile. The purpose of these containment units was to limit contact of egg cases with each other, to prevent the spread of the fungal infection which sometimes resulted in the death of the embryo. The embryos were always kept in the containment units when not being put through their respective cyclic thermal stress treatments, and up until the point of hatching.

Embryogenesis at two temperatures

The temperatures used in my study represent the natural thermal profile that embryos experience on reef flats and seagrass beds during high and low tide (Harahush *et al.,* 2007; Payne and Rufo, 2012; Rosa *et al.,* 2014). Egg cases were exposed to intermittent cycles of thermal stress as follows: two hours of exposure per day to the embryo's respective temperature (24 ºC, 28 ºC, or 32 ºC) and then placed back into a holding tank at 24 ºC for the remainder of the day. This intermittent cyclic exposure was repeated daily for a period of 5 days, followed by 9 days of rest in their common holding tank at 24 ºC (Figure 3.2). Because of the elimination of the 32 ºC group, only the data for the control and high temperature are analyzed in this

chapter. The final treatment groups were: the **control group**, for which a mean water temperature of **24 ± 0.3 ºC** was maintained for the two hours of treatment; and the high temperature group is called from now on the **thermal stress group,** for which a temperature of **28°± 0.3 ºC** was maintained for the two hours of treatment. Each treatment group was moved to an alternate tank (15 L) for their respective treatment to account for handling and ensure that the only variation across the two treatments was temperature. The temperature in the treatment tanks for the thermal stress group was increased at a rate of 0.26 ± 0.0026 ºC min^{-1}, until the target temperature was reached, and then maintained for the two hour exposure.

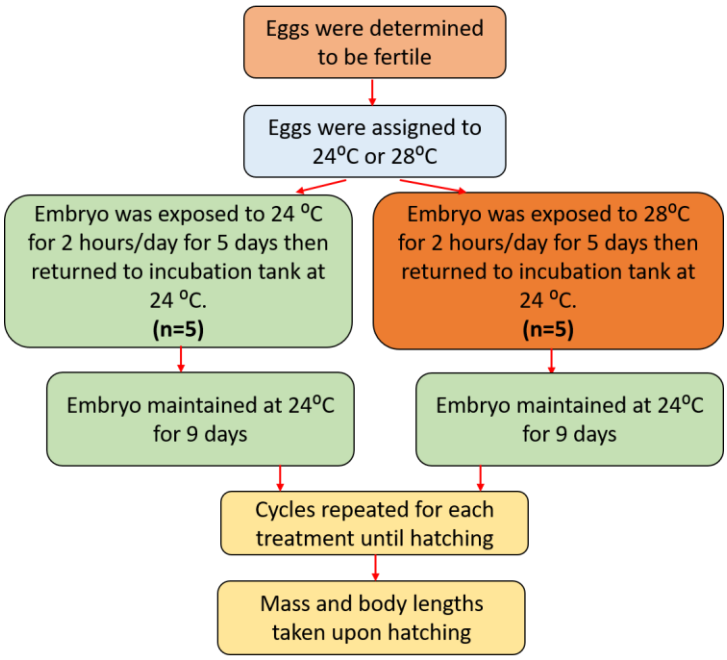

Figure 3.2. Schematic of the experimental design from top to bottom, illustrating the treatment cycle for grey carpet embryos (thermal stress or control). Egg cases were haphazardly assigned to the two indicated temperature treatments.

For the heating and maintenance of treatment tank temperature, Wiepro HBIDO heaters (100W) were used. After the two hours of exposure, the embryos were gradually acclimated back to the control temperature of 24 ºC, by gradually adding water from the control tank (24 ºC) to the thermal stress tank over the span of 20 minutes. It is important to note that all

embryos regardless of thermal stress treatment were held in the same holding tank when not in their thermal stress treatment. These cycles were repeated until the embryos hatched. The intermittent cycles of exposure to elevated temperatures were based upon the natural frequency of day-time low tide cycles which occurred between 11:00 a.m. and 3:00 p.m. over a 12-month period at Heron Island (Renshaw Personal Communication).

Experiments were conducted in an area that was partially covered at Sea World. A divider was put in place to limit direct sun exposure to treatment tanks (Figure 3.3). However, the divider was unable to stop all radiation from the sun. Thus, to reduce confounding factors due to tank placement, such as the proximity to electrical outlets or the level of UV exposure, the egg cases were placed in different treatment tanks so that all embryos were rotated through all tanks for the treatment sessions on a continual basis until the end of the experiment.

Figure 3.3 Divider to limit UV exposure during thermal stress treatments and to protect from elements (Eg. Rain, wind, etc).

Due to the inverse relationship between water dissolved oxygen and temperature (Schmidtko *et al.*, 2017), dissolved oxygen levels were closely monitored using a WinTPS WP82Y® dissolved oxygen meter equipped with a YSI probe® in the experimental tanks, and were maintained above 4.69 mg O_2 L^{-1} (which is not considered to be hypoxic) using a 5-watt submersible water pump.

General measurements and observations

During development, each individual embryo was photographed three times per week to record key developmental milestones, as described by Onimaru *et al.*, (2018). For this, egg cases were moved to an alternate tank (15 L tank) held at 24°C and then candled using a 400-lumen light that was placed below the egg and tank, so that photos could be taken and the key developmental stages noted. Viewing embryos through their natural egg case does pose some restrictions, due to the dark and opaque nature of the egg cases. It was not possible to view the earlier stages of development such as gastrula, blastula, neurula and somitogenesis using the candling technique. Hence, the primary stage noted was the first visible sign of an embryo which can be reliably observed when the embryo is developing in the egg case. The photographs were taken using a Canon Powershot® G12 Digital Camera with a ruler in the field of view to enable measurement of the key body traits. Since yolk utilization is a key indicator of embryo metabolic capacity while coping with thermal stress (Rodda and Seymour, 2008), the photos were used to calculate the average yolk surface area as well as the yolk volume. The egg yolk surface area and volume were measured using Image J, version 1.52a egg tool (NIH, 2018). Information on key body traits were later used to determine whether differences existed in the time at which developmental milestone appear based on temperature treatment.

Upon hatching, the neonates were photographed with a scale bar and weighed. Throughout literature and across taxa, there are numerous ways being explored to accurately evaluate the overall fitness of organisms (Falk *et al.,* 2017; Warner *et al.,* 2016; Weideli *et al.,* 2019). There are however, two primary methods of evaluating fitness in fish, which are the body mass length relationship (Relative Condition) and Fulton's Condition (Jin *et al.,* 2015;

Weideli *et al.,* 2019). Other means of fitness evaluation have been explored in sharks, such as hepatosomatic index, however, it requires the euthanization of the animal to weigh the liver (Barley *et al.,* 2017; Hussey *et al.,* 2009). Hepatosomatic index was not an ideal way to calculate fitness as individuals had their fitness evaluated upon hatching and 30 days post-hatching. Hence, both Fulton's Condition and Relative Condition were calculated as follows:

$$Relative\ Condition = \frac{Body\ Mass\ (g)}{Body\ Length\ (cm)}$$

$$Fulton's\ Condition = \left(\frac{Body\ Mass\ (g)}{Body\ Length\ (cm)^3}\right) \times 100$$

Neonates were weighed (± 0.01g) within 24 hours of hatching using a Mandel® TX2202L Top Loading Scale. Neonates were placed into a home tank (85 L), and fed a varied diet consisting of pieces of prawn, squid and young molluscs three times per week to satiation. Neonates were followed for 30 more days to monitor their survival rate. Measurements of body mass and length (measured from photographs) were taken once a week, prior to being fed, to monitor neonate growth. Photographs were also used for individual identification using the unique banding pattern of each shark.

The photographs taken following hatching, with a scale bar included, were used to measure key body trait measures using Image J version 1.52a (NIH, 2018). The body traits measured were: total length (TL ± 0.01 mm), snout length (SNL ± 0.01 mm), thoracic length (THL ± 0.01 mm), eye to eye (EE ± 0.01 mm), pectoral Fin Length (PFL ± 0.01 mm), pelvic fin length (PVL ± 0.01 mm), the first dorsal fin span (D1FS ± 0.01 mm), second dorsal fin span (D2FS ± 0.01 mm), area of pectoral fin (APF ± 0.01 mm) and distance between dorsal fins (DBD ± 0.01 mm).

Developmental staging

In order to observe the development of embryos inside of the egg cases, each egg case was scraped with a scalpel upon collection to remove outer fibrous material. Embryos were photographed using a Canon Powershot G12®. To develop a non-invasive field atlas, only the developmental stages easily observable and recognizable to the naked eye within the egg case *via* candling were determined (Table 3.1), and modified based on Onimaru *et al.*, (2018). The new field atlas based on the developmental milestones for embryos that were not exposed to thermal stress is described in full in the previous chapter, Chapter 2.

Table 3.1. Sequential developmental milestones of grey carpet shark Embryo Development under 24 ºC (Control; n=5) or 28 ºC (Thermal Stress; n=5).

Stage	Developmental Milestone
1	Embryo visible
2	Formation of External Gill Filaments
3	Formation of Fin Buds
4	Formation of Eye Placodes
5	Heterocercal Tail Shape
6	Buccal Pumping
7	Banding on Tail
8	Resorbed Gill Filaments
9	Pigmentation to Pectoral Fins
10	Widened Trunk
11	Internalized Yolk

In Onimaru and colleagues' 2018 study, invasive techniques had to be used to obtain developmental details, a window for viewing the embryo was cut into the egg cases, allowing greater visibility of early embryo changes, but potentially increasing the chances of embryo death. As I kept the embryos contained within their egg case, the number of stages that can be clearly observed with the naked eye are less than those reported in Onimaru *et al.*, (2018), but

similar to those reported by Musa and colleagues (2018), without the risk of compromising results due to an unforeseen contamination and potential death of the embryos. Development time was determined at different stages by the summing of development time (in days), starting from the determined date of oviposition to the developmental milestone in question or time of hatching. It is important to note that my study aimed to explore the developmental milestones of the grey carpet shark in a non-invasive, non-destructive manner, because, the survival of embryos through studies was of paramount importance to be able to identify mortality due to treatment. This approach is particularly useful when working with endangered species in the field, where it is not viable to open the egg cases without causing mortality of the embryos, and lastly in studies which need the egg case to stay intact to explore the impact of other variables such as temperature or hypoxia on embryo development. Hence, the developmental atlas outlined in Chapter 2, provides a strong foundation for this chapter to observe key developmental milestones in the grey carpet shark in a non-invasive manner, as well as provide a guideline for other species of shark. The research protocol was approved by the Animal Ethics Committee at Griffith University (AHS-03-15-AEC).

Statistical analysis

Statistical analyses were conducted using SPSS v.23. Normality of data was determined using a Kolmogorov-Smirnov test. Non-parametric data were log-transformed for statistical analysis. Survival between treatments was analyzed using a Chi-square test, including embryo mortality, as no mortality was observed in either treatment after hatching. Characteristics of embryo development (i.e. rate of yolk consumption, development time, and time to developmental milestones) were analyzed using an Independent two-tailed t-test with temperature as the independent variable. A comparison of yolk volume across developmental

stages was conducted using a Chi-square test. To determine the best predictor of time to hatching, a stepwise linear regression was conducted using temperature, body mass and body length at hatching as well as developmental milestones that occurred later in development (buccal pumping and milestones that followed). Before running the stepwise analyses, data was analyzed for possible collinearity, and hence, traits with high collinearity values were excluded from the model. Buccal pumping displayed high collinearity with resorption of gills, and hence only buccal pumping was kept in the model. The stepwise analysis produced two models with the internalization of the yolk as the primary explanatory variable followed by pigmentation to the pectoral fin. Then, a second stepwise linear regression was repeated with the same variables, but this time removing those which were significant in the first analyses, in order to determine if any other variables could serve as predictors of time to hatching. In addition, because the internalization of the yolk and the time at which pigmentation reached the pectoral fins were the main predictors of time to hatching, a third and fourth stepwise linear regression was performed to identify which of the traits studied were the best predictors for internalization of yolk and the pigmentation reaching the pectoral fins. Finally, the morphological traits (body mass, length, snout length, thoracic length, eye to eye, pectoral Fin Length, pelvic fin length, the first dorsal fin span, second dorsal fin span, area of pectoral fin and distance between dorsal fins) upon hatching and 30 days post-hatching were compared between treatments using an Independent two-tailed t-test. The overall growth of the morphological traits were also analyzed up to day 30 post-hatching using a repeated measures analysis. The Relative Condition and Fulton's Condition upon hatching and on day 30 post-hatching were compared between temperature treatments using Independent two-tailed t-tests with temperature as the independent variable. Differences were considered significantly different when $P \leq 0.05$.

RESULTS

Cyclic exposure to temperatures of 24°C (control) and 28°C (thermally stressed) did not have a significant impact on the survival of grey carpet shark embryos ($\chi = 2.00$; df = 1; P = 0.157). Three individuals in the control group and two individuals in the thermal stress treatment died, all of which died during embryogenesis. These individuals which died were replaced with new egg cases that were deemed fertile but did not yet have a visible embryo. The trend of appearance of the various developmental milestones can be observed in Figure 3.4. Overall, the results strongly suggest that temperature has a greater impact on the developmental milestones that appear later during development than those that occur earlier (Table 3.2).

Yolk consumption during embryogenesis

Cyclic exposure to thermal stress did not cause a significant difference in yolk utilisation throughout development ($T_{(8)} = -0.993$; P = 0.350; Figure 3.5). However, it was noted that the degree of inter-animal variation in yolk utilization was remarkably consistent at approximately 0.2 $\pm$ 0.003 cm^3 day^{-1} in the embryos exposed to cyclic thermal stress, while the control group yolk consumption was 0.18 $\pm$ 0.046 cm^3 day^{-1} with a range varying between 0.12 cm^3 day^{-1} and 0.24 cm^3 day^{-1}. Independently of the thermal stress, variation in yolk volume was observed between most developmental stages (Table 3.3).

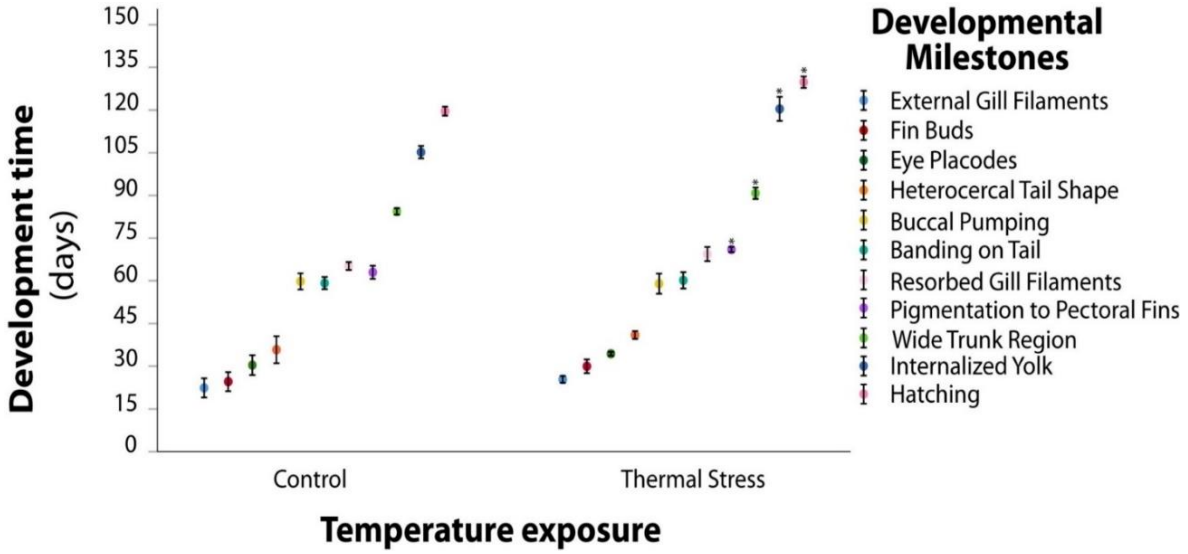

Figure 3.4. Developmental milestone appearance in grey carpet shark embryos exposed to two treatment cycles during development. **Control** represent individuals exposed to a constant 24°C (n=5), while **Thermal Stress** represent individuals exposed to periodic cycles of exposure to 28°C (n=5) for two hours day^{-1} for five days. Significant differences are indicated by the asterisk (*) revealing that appearance of pigmentation reaching the pectoral fins, the trunk region widening, the time of internalization of yolk and time of hatching was significantly different between the control and thermal stress groups.

Table 3.2. Difference in time (Day post-fertilization) of appearance of developmental milestones of grey carpet shark embryo development under 24 ºC (Control; n=5) or 28 ºC (Thermal Stress; n=5) using independent two-tailed t-tests for each stage with temperature as the independent variable.

Stage	Day Mean ± s.d.	T-value	P-value
2	22 ± 7.6 (25 ± 2.7)	-0.835	0.428
3	25 ± 7.5 (30 ± 5.3)	-1.315	0.229
4	30 ± 7.9 (34 ± 1.5)	-1.117	0.296
5	36 ± 10.6 (41 ± 3.1)	-1.050	0.325
6	60 ± 6.38 (59 ± 7.84)	0.177	0.864
7	59 ± 4.8 (60 ± 6.5)	0.078	0.788
8	65 ± 3.1 (69 ± 5.6)	-1.467	0.191
9	63 ± 5.1 (71 ± 2.1)	-3.213	**0.022**
10	84 ± 2.6 (91 ± 4.6)	-2.704	**0.034**
11	105 ± 4.9 (120 ± 9.4)	-3.212	**0.012**

Note: Values in parentheses represent averages for the thermal stress group. **Bold** indicates statistical significance between temperature treatments. Data are shown as $\bar{X}$ ± s.d.

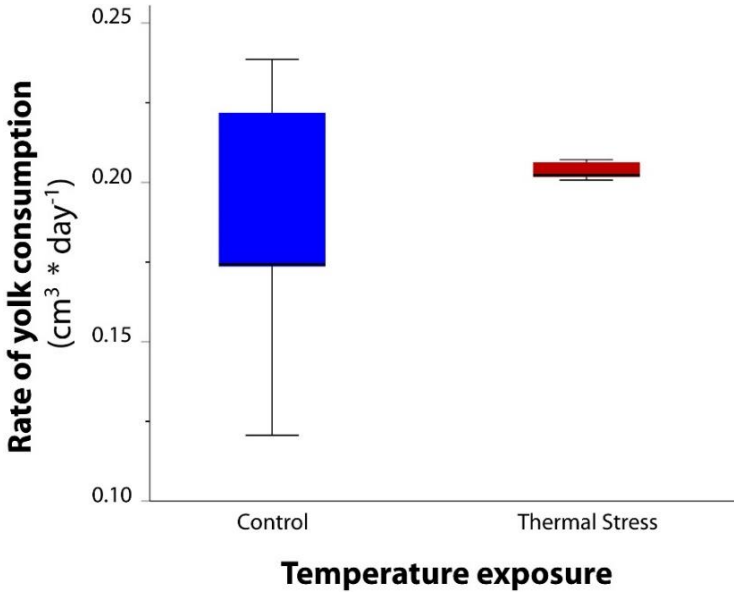

Figure 3.5. Grey carpet shark embryo rate of yolk consumption in two cyclic temperature treatments analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable. . **Control** represent individuals exposed to constant 24°C (blue; n=5) while **Thermal Stress** represent individuals exposed to cyclic thermal stress at 28°C (red; n=5) for two hours day^{-1} for five days. While there was no difference in the means there was a large reduction in inter-animal variation in the thermally stressed embryos.

Table 3.3. Chi-Square test comparison of yolk volume difference across developmental stages of grey carpet sharks, as outlined in Chapter 3. The post-hoc comparison between developmental stages are indicated under the stages subheading.

Stages		Chi-square	P	Degree of Freedom
3	4	8.16	0.004	1
	5	8.271	0.004	1
	7	7.367	0.007	1
	9	12.96	<0.001	1
	10	14.5	<0.001	1
4	5	2.573	0.109	1
	7	0.004	0.949	1
	9	9.158	0.002	1
	10	15.503	<0.001	1
5	7	2.363	0.124	1
	9	22.022	<0.001	1
	10	30.005	<0.001	1
7	9	9.528	0.002	1
	10	15.503	<0.001	1
9	10	23.393	<0.001	1

Note: Due to the lack of difference on yolk volume between the two thermal groups (24°C and 28°C), at each specific developmental stage, data was combined n=10.

Time to hatching

Temperature did significantly affect the time until hatching, with the embryos exposed to cyclic 28 °C taking 10 days longer on average than the individuals exposed to 24 °C constantly (Figure 3.6; $T_{(8)} = -3.97$; $P = 0.004$). The control embryos maintained at 24 °C took a mean of 120 $\pm$ 3.2 days until hatching, compared to 130 $\pm$ 4.0 days to hatch in the cyclic thermal stress group (28 °C). If complete yolk internalization is taken as a common baseline, then, the control group exposed to 24 °C, hatched with a mean of 11.9 $\pm$ 5.88 days after yolk internalization; while the group exposed to cyclic thermal stress (28 °C), hatched sooner at 9.4 $\pm$ 5.32 days after yolk internalization. However, the time between the internalization of the yolk and the time of hatching did not vary across the two temperature treatments ($T_{(8)} = 1.419$; $P = 0.194$).

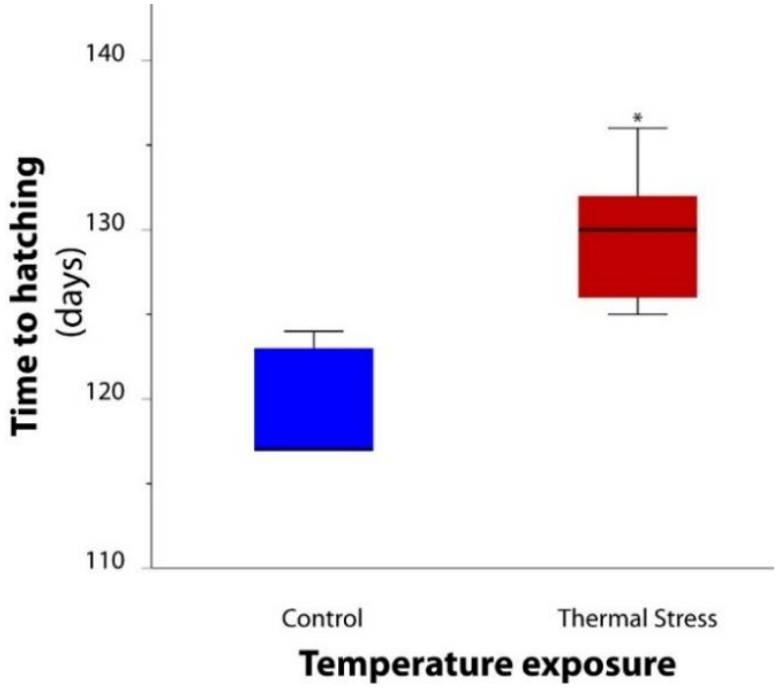

Figure 3.6. Number of days required to hatch by grey carpet shark embryos in two treatment cycles. **Control** represents embryos exposed to 24°C (blue; n=5) while **Thermal Stress** represents embryos exposed to cycles of elevated temperature at 28°C (red; n=5) analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable. Significant differences are indicated by the asterisk (*) revealing that the time to hatching significantly varied between the control and thermal stress group.

Thermal stress induced delay of three key milestones during development

The present study aimed to determine whether temperature had a significant effect on the development of key milestones as well as on whether hatching was delayed. The data revealed that cyclic thermal stress significantly delayed three developmental milestones compared to embryos exposed to the control temperature: time to develop pigmentation of the pectoral fins; time to trunk widening and time to full internalization of yolk (Table 3.4). The time to develop pigmentation of the pectoral fins was delayed in embryos exposed to cyclic thermal stress (Figure 3.7).

Table 3.4. Time (developmental day) at which key developmental milestones appeared when grey carpet shark embryo were exposed at 24 ºC (Control; n=5) or 28 ºC (Thermal Stress; n=5) and their statistical difference using independent two-tailed t-tests for each stage with temperature as the independent variable. **Bold** indicates statistical significance.

Developmental Milestone	Control	Thermal Stress	T	P
Formation of External Gill Filaments	22 ± 7.6	25 ± 2.7	-0.835	0.428
Formation of Fin Buds	25 ± 7.5	30 ± 5.3	-1.315	0.229
Formation of Eye Placodes	30 ± 7.9	34 ± 1.5	-1.117	0.296
Heterocercal Tail Shape	36 ± 10.6	41 ± 3.1	-1.050	0.325
Buccal Pumping	59 ± 7.8	60 ± 6.4	0.177	0.864
Banding on Tail	59 ± 4.8	60 ± 6.5	0.078	0.788
Resorbed Gill Filaments	65 ± 3.1	69 ± 5.6	-1.467	0.191
Pigmentation to Pectoral Fins	63 ± 5.1	71 ± 2.1	**-3.213**	**0.022**
Widened Trunk	84 ± 2.6	91 ± 4.6	**-2.704**	**0.034**
Internalized Yolk	105 ± 4.9	120 ± 9.4	**-3.212**	**0.012**
Days to Hatch	120 ± 3.6	130 ± 4.5	**-3.970**	**0.004**

Note: Data are shown as $\bar{X}$ ± s.d.

The time to trunk widening was significantly delayed in response to cyclic thermal stress (Figure 3.8). The time of trunk widening was determined by a noted increase of trunk width of 40% in the control group between stage nine and stage ten (0.89 ± 0.08 cm, which increased to 1.46 ± 0.13 cm), while the thermal stress group displayed a 35% increase in the width of the trunk (0.90 ± 0.07 cm to 1.39 ± 0.18cm). However, the width of the trunk did not significantly differ between the control group and the thermal stress group prior to the dramatic widening of the trunk ($T_{(8)}$ = -0.259; P = 0.802), or after the trunk had significantly widened ($T_{(8)}$ = 0.737; P = 0.484). Furthermore, the yolk was internalized earlier in the control group compared to the thermal stress group (Figure 3.9).

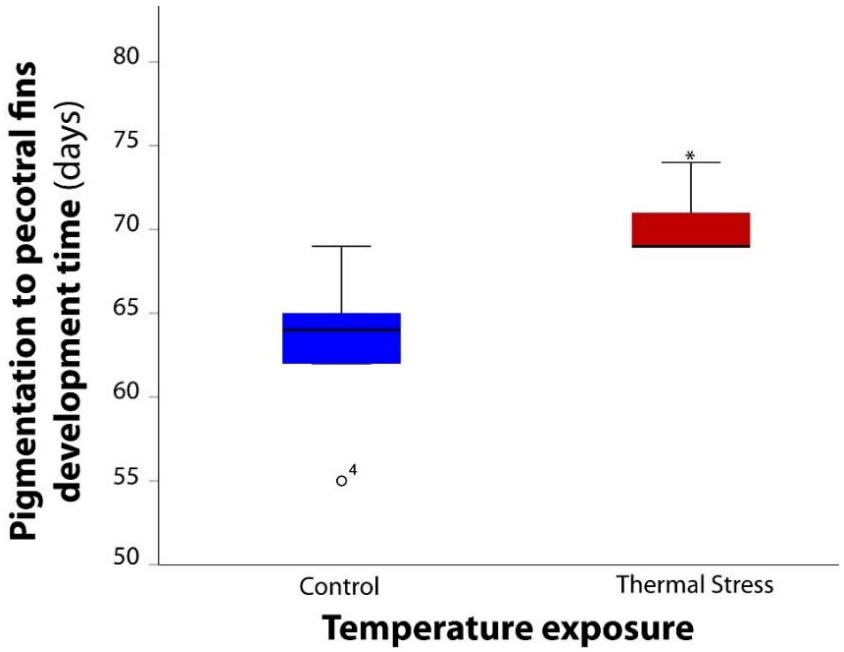

Figure 3.7. Time to develop pigmentation on the pectoral fins in grey carpet shark embryos in two treatment cycles analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable. **Control** represents embryos exposed to 24°C (blue; n=5) while **Thermal Stress** represents embryos exposed to cycles of elevated temperature at 28°C (red; n=5). Significant differences are indicated by the asterisk (*) revealing that pigmentation of the pectoral fins took longer in embryos exposed to cyclic thermal stress.

The best predictor of the number of days until hatching was the time to complete pectoral fin pigmentation and yolk internalization (Table 3.5). It is important to reiterate that these two factors were significantly impacted by temperature (Figure 3.7 and Figure 3.9).

Table 3.5. Stepwise regression analysis of time until hatching in grey carpet shark embryos.
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Variable	β	P	R^2
Internalization of Yolk	0.598	0.007	
Pigmentation of Pectoral Fins	0.449	0.027	
			0.855

Note: Control 24°C (n=5); Thermal Stress 28°C (n=5).

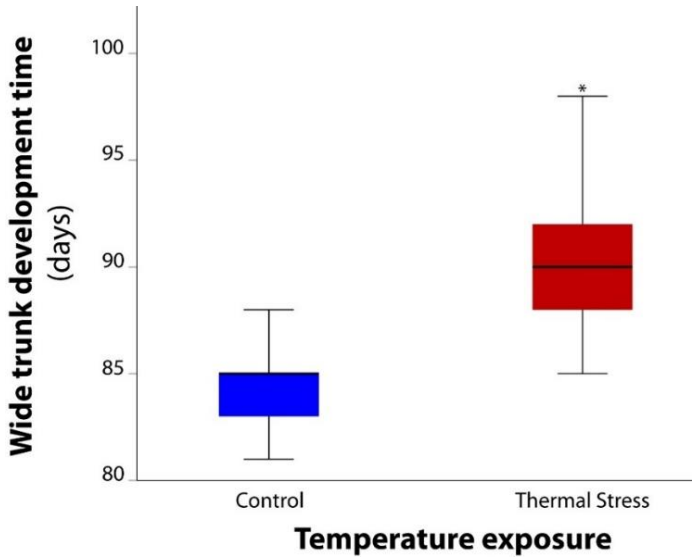

Figure 3.8. Time to development of a wider trunk in grey carpet shark embryos exposed to two different treatment cycles analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable. **Control** represents embryos exposed to 24°C (blue; n=5) while cyclic **Thermal Stress** represents embryos exposed to 28°C (red; n=5). The significant difference is marked with an asterisk (*) indicating that widening of the trunk occurred later in embryos exposed to cyclic thermal stress.

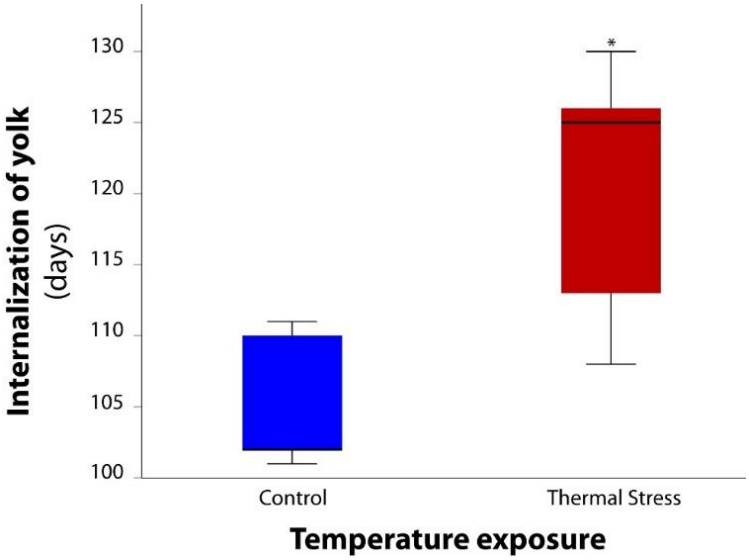

Figure 3.9. Days until the internalization of the yolk in grey carpet shark embryos in two different treatment cycles analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable. **Control** represents embryos exposed to 24°C (blue; n=5) while **Thermal Stress** represents embryos exposed to cycles of 28°C (red; n=5). The significant difference is marked with an asterisk (*) indicating that the internalisation of the yolk took significantly longer in embryos exposed to cyclic thermal stress.

A second stepwise regression was used to determine how much of the variance of the number of days until hatching was accounted for by cyclic thermal stress alone. Temperature accounted for 62% of the variance in hatching time when the two primary predictors (Time of pigmentation reaching the pectoral fins and internalization of yolk) were excluded from the analysis ($\beta = 0.814$; $P= 0.004$; $R^2= 0.6210$).

Because both the internalization of the yolk and the time to pigmentation of the pectoral fins were strong predictors for the time until hatching, the next step was to determine if any of the remaining variables would serve as predictors for these two milestones. This analysis included the developmental milestones prior to the specific stages in question, as well as temperature. The time at which pigmentation reached the pectoral fins was best predicted by temperature ($\beta= 0.703$; $P= 0.005$) and by the appearance of pigmentation in the form of banding on the tail ($\beta= 0.483$; $P= 0.026$), model $R^2= 0.736$; while, the time of internalization of yolk was best predicted by temperature only ($\beta= 0.750$; $P= 0.012$; $R^2= 0.509$).

Embryo Length & Yolk Volume Across Developmental Stages

From the post-oviposition period until hatching, changes in the length of the embryos and their yolk volume across time was gradual in both temperature treatments (Figure 3.10; Appendix 3.1). This regression between weeks post-deposition and embryo length and yolk volume revealed that in the group exposed to cyclic thermal stress**,** the time of deposition explained 93% of the variation in yolk volume, and 88% of the variation in embryo length; while in the control group**,** the time of deposition explained 91% of the variation in yolk volume, but only 54% of the variation in embryo length. The slopes of the yolk volume through time did not differ between the temperature treatments ($\chi = 2.000$, $df = 1$, $P = 0.157$). Similar results were observed for the embryo length between the two temperature treatments

(χ = 2.000, df = 1, P = 0.157). It is important to acknowledge the lack of data for the later development of the embryos in the control group following week 12. This lack of data is a result of the embryos curling up around the yolk sac frequently which made it extremely difficult to get photos that allowed for accurate measurement of the yolk volume and embryo length. All individuals regardless of the thermal treatment, did internalize their yolk completely prior to hatching.

In week 10 post-deposition, for embryos in the control group and week 11 for embryos cyclically exposed to 28 °C, there appeared to be an interaction between embryo length and yolk volume. The order in which some of the development stages were reached differed between the two temperature treatments. Two individuals in the control group and one individual in the thermal stress group displayed stage nine (banding to the pectoral fins) before stage eight (resorption of external gill filaments). While one individual cyclically exposed to thermal stress (28 °C), displayed stage four (eyes) before stage three (fin buds). Due to the slight deviation in the order some milestones appeared in some individuals, the developmental stages are presented in the chronological order followed by most individuals and occurs in the same sequence as indicated by Onimaru and colleagues (2018).

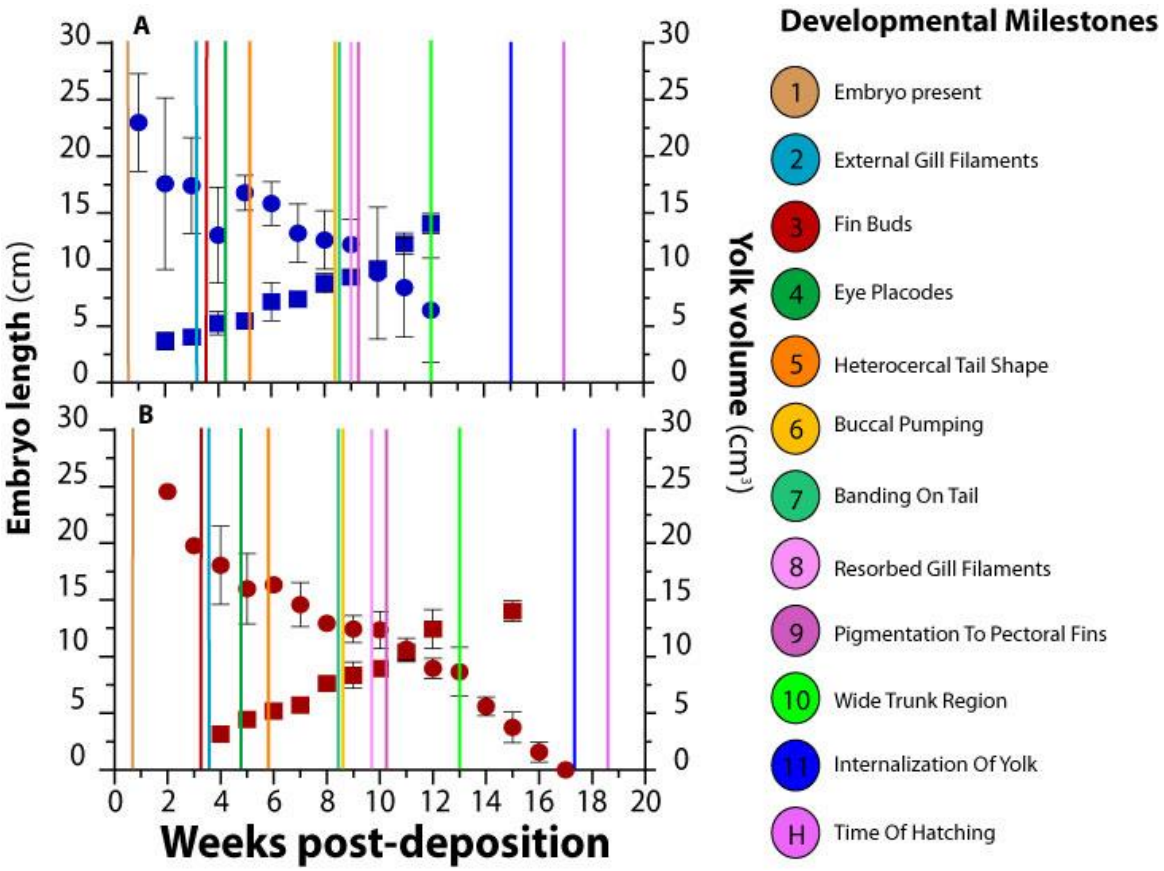

Figure 3.10. Grey carpet shark embryo length and yolk volume through weeks post-deposition. Developmental stages are identified by the different line colours. **A** represent embryos in the control group exposed to 24°C (n=5) while **B** represents embryos cyclically exposed to 28°C (n=5). Circle data points represent yolk volume and square data points represent embryo length.

Neonatal length at hatching

There was a striking difference observed between groups at hatching. Upon hatching, neonates at the higher temperature were smaller in total length compared to neonates at the control temperature (24 °C: 18.1 $\pm$ 0.50 cm, 28 °C: 17.6 $\pm$ 0.85 cm; $T_{(8)}$ = 2.38; P = 0.048; Figure 3.11).

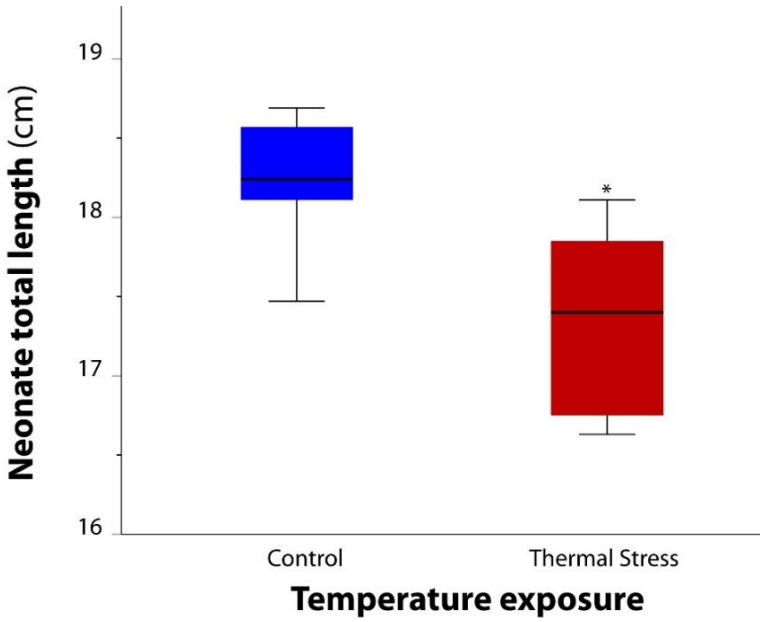

Figure 3.11. Total length of neonates upon hatching, following thermal stress exposures throughout development analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable. **Control** represents embryos exposed to 24°C (blue; n=5) while **Thermal Stress** represents embryos exposed to cycles of 28°C (red; n=5). The significant difference is marked with an asterisk (*) indicating that the thermally stressed neonates were significantly shorter than the controls.

However, when the length was examined with a repeated measures analysis and over the first thirty days post-hatching, the length of neonates did not vary significantly between the temperature treatments ($F_{(1,7)}$ = 0.204; P = 0.665; Figure 3.12 A). On the other hand, there was no significant difference in the body mass of the neonates upon hatching (24 °C: 22.5 $\pm$ 4.53 g, 28 °C: 20.4 $\pm$ 1.04 g; $T_{(8)}$ = 0.88; P = 0.407) or on day 30 post-hatching ($T_{(8)}$ = 0.633; P = 0.552; Figure 3.12 B) between the two temperature treatments.

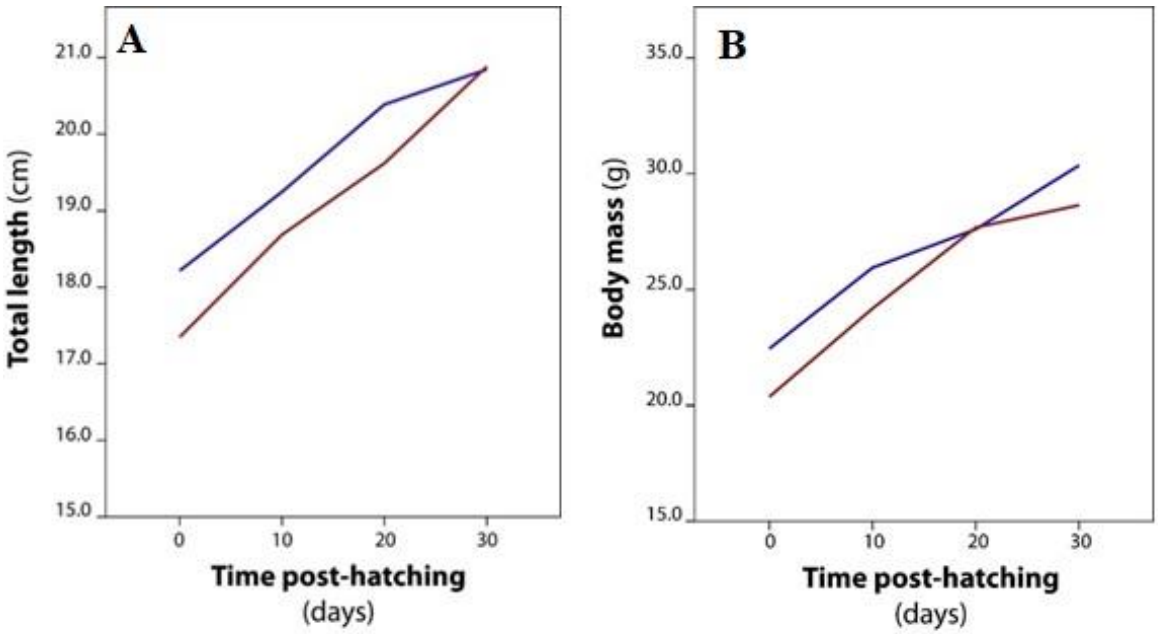

Figure 3.12. Total length (A) and body mass (B) of neonates upon hatching up to day 30 post-hatching, following thermal stress exposures throughout development analyzed using independent two-tailed t-tests for each stage with temperature as the independent variable at the different time intervals. **Control** represents embryos exposed to 24 °C (blue; n=5) while **Thermal Stress** represents embryos exposed to cycles of 28 °C (red; n=5).

The overall fitness (Fulton's Condition) of the sharks did not vary significantly between the two temperature treatments upon hatching ($T_{(8)}$ = - 0.318; P = 0.762), or by day 30 post-hatching ($T_{(7)}$ = -0.013; P = 0.990). Similar results were observed when analyzing Relative Condition between the temperature treatments on day zero ($T_{(8)}$ = 0.473; P = 0.649; Figure 3.13 A), and on day 30 post-hatching ($T_{(7)}$ = -0.091; P = 0.930; Figure 3.13 B). This data indicated that at hatching there was no relationship between length and body mass of the embryos, but it should be noted that the resorbed yolk mass would be included in the body mass at hatching. However, by 30 days post-hatching the expected length to body mass ratios noted were not significantly different between treatment groups.

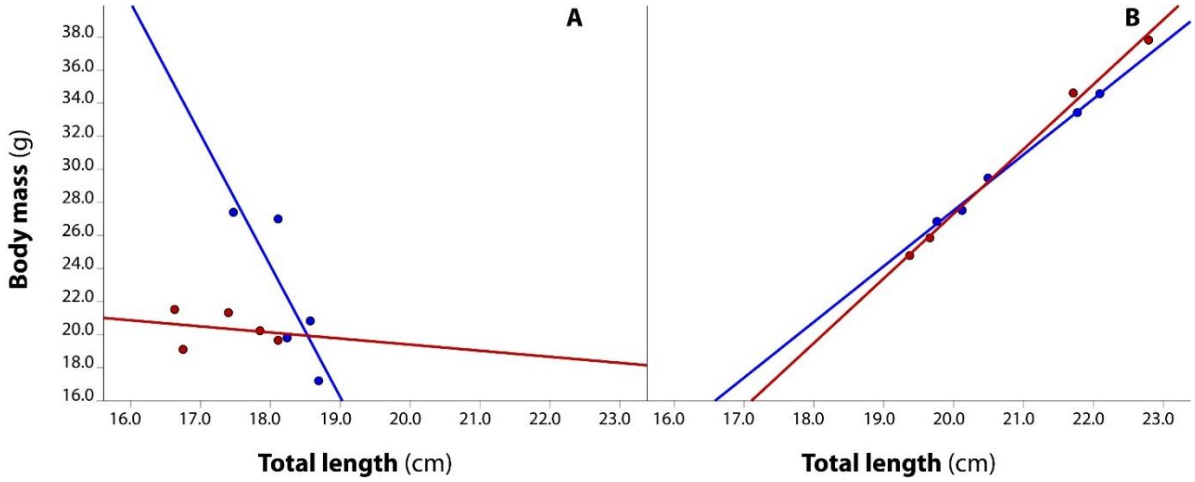

Figure 3.13. Body mass – length relationship upon hatching (**A**) and at day 30 post-hatching (**B**), following thermal stress exposures throughout embryo development. Blue lines represent the **Control** (n=5) embryos exposed to 24°C (**A**: $R^2 = 0.71$, $y = 1.67^2 - 7.94 * x$; **B**: $R^2 = 0.994$; $y = -39.91 + 3.37 * x$); while red lines represent the **Thermal Stress** (n=5) embryos exposed to cycles of 28°C (**A**: $R^2 = 0.05$; $y = 26.75 - 0.37 * x$; **B**: $R^2 = 0.995$; $y = -50.86 + 3.91 * x$).

In summary, cyclic thermal stress appears to more heavily impact the time at which later developmental milestones appear than the earlier milestones. The only developmental milestones that were significantly impacted by the thermal stress were the three final developmental milestones (pigmentation reaching the pectoral fins, widening of the trunk region and internalization of the yolk). Upon hatching, the neonates that were exposed to the cyclic thermal stress, were significantly shorter upon hatching but appear to make up that shorter body length relatively quickly, with their total length being comparable to the control group by day 30 post-hatching. Similarly, Fulton's Condition and Relative Condition, did not vary between the two temperature groups at hatching or 30 days post-hatching.

DISCUSSION

The mortality of an organism is impacted by countless factors, especially when concerning embryos that develop *in ovo* and are unable to escape stressors (Rosa *et al.*, 2014). Even under conditions that are ideal, not all embryos will survive until hatching, as was shown by Harahush and colleagues (2007), who found that grey carpet shark embryos developed under a temperature range of 21-25 °C, displayed a mortality of 11.6% (80 of 228 egg cases). Furthermore, under constant thermal stress (30 °C), grey carpet shark embryos displayed increased mortality when compared to their control counterparts, 26 °C (Rosa *et al.*, 2014). Individuals exposed to 30 °C had a survival rate that ranged from 80-89% compared to a 100% survival when embryos were developed at a present-day temperature of 26 °C (Rosa *et al.*, 2014). As the first study of its kind to explore exposure to cyclic thermal stress on embryonic development, it was interesting to find that cyclic thermal stress did not affect the overall mortality of embryos. However, cyclic thermal stress did significantly impact the rate of embryo development, the timing of developmental milestones, and hatching time. However, the temperature of 28°C can only be considered a mild source of thermal stress, because previous studies have shown that in the tidal pools of the reef flats at Heron Island, temperatures ranged from 20.2 °C up to 34.5 °C (Davies and Kinsey, 1973).

Individuals incubated cyclically to elevated temperature (28 °C) took on average 10 days longer to hatch (Average hatching days: 130), than individuals incubated in the lower temperature (24 °C). These results are particularly interesting as they are opposite of results obtained by Rosa *et al.*, (2014) under a constant high temperature of 30 °C for the same species. Embryos incubated at the constant temperature of 30 °C hatched on average 19 days faster than individuals maintained at a control temperature of 26 °C, average hatching days: 79 $\pm$ 11

(Rosa *et al.,* 2014). In the lesser spotted dogfish, embryos hatched after 15 weeks under normoxia and a constant temperature of 20 °C, while embryos maintained under normoxic conditions and 15 °C took approximately 23 weeks to hatch (Musa *et al.*, 2020). The discrepancy in the time to hatching (51 days) between the present study and Rosa *et al.,* (2014), could be explained by the type of thermal exposure the embryos underwent during development (cyclic exposure vs. constant exposure). While the discrepancy in trend between the current study and Musa *et al.,* (2020) on the lesser spotted dogfish, could also be attributed to the difference in the type of thermal exposure as well as natural environmental niches that the species inhabit (temperate *v.s* tropical).

Embryos exposed to cyclic thermal stress would automatically elevate their metabolic rate during the heat cycle because the metabolic rate is increased at higher temperatures (Zhang *et al.*, 2020). Normally, as temperature increases, the metabolic rate increases creating a rise in respiratory demand in terrestrial and aquatic organisms (Clarke and Fraser, 2004). It is generally accepted that a 10 ºC increase in temperature normally doubles or triples the oxygen demand (Tullis and Baillie, 2005). If the increase in the metabolic rate is not satisfied by an increase in oxygen uptake, a reduction in the organismal aerobic scope for different activities such as swimming, feeding, digestion among others could be further reduced and can have a detrimental effect on growth and reproduction capacity (Moulton *et al.,* 2020; Pistevos *et al.,* 2015; Rosa *et al.,* 2014). It is known that stress-activated proteins (protein kinases) are turned on by environmental stress and stress-activated proteins act to lower the metabolic rate (Storey, 1998). Therefore, the 10-day delay in hatching of the individuals under cyclic thermal stress (28 °C), could also potentially be related to the stress exerted by the increased temperature on the metabolic demands (Jiang *et al.,* 2019; Sandersfeld *et al.,* 2017; Song *et al.,* 2019). Future studies should measure the metabolic rate of embryos during and post cyclic thermal stress.

It is well established that there is a strong relationship between dissolved oxygen and temperature, in both fresh and saltwater (Manaspah *et al.,* 2006; McDonnell *et al.,* 2019). Specifically, when water temperature increases, dissolved oxygen decreases, and this as a result of decreased oxygen solubility (Jeffries *et al.,* 2018; Jiang *et al.,* 2019; Nilsson *et al.,* 2009). Hence, it is plausible that while embryos were subjected to the increased temperature cycles, they were also exposed to a concomitant reduction of dissolved oxygen, even if it was not to a hypoxic level. For aquatic organisms that utilize buccal pumping, ventilation is energetically costly when compared to passive diffusion, such as is used by external gill filaments (Tomita *et al.,* 2014). Due to the fact that buccal pumping requires the use of the musculature of the buccal cavity, while the use of passive diffusion in external gill filaments requires no use of musculature (Pelster and Bemis, 1992; Rodda and Seymour, 2008; Tomita *et al.,* 2014). Obtaining oxygen is particularly expensive for marine organisms, due to the lower levels of oxygen (20% less), as well as the higher density and viscosity of seawater when compared to freshwater (Li *et al.,* 2018; Schmidtko *et al.,* 2017). Since dissolved oxygen levels are already lower in seawater, a further decrease in oxygen levels during periods of increased temperature, could have potentially exacerbated the level of stress placed on embryos (Del Rio *et al.,* 2018; Delage *et al.,* 2014). The change in oxygen levels in my study was from approximately 5.74 mg O_2 L^{-1} at 24 ºC to 4.69 mg O_2 L^{-1} at 28 ºC. While this drop in dissolved oxygen did not constitute a hypoxic exposure, it is necessary to consider whether it could have induced the observed delay in hatching time.

High levels of dissolved oxygen are critical during embryo development, as oxygen plays a key role in maintaining organismal metabolism, leading to increased mortality when oxygen requirements are not met (Ciuhandu *et al.,* 2007; Wood *et al.,* 2019a). The lesser spotted dogfish under hypoxic conditions at 15 °C displayed a survival rate of 63.2% compared

to the 100% survival rate under normoxic conditions and 15 °C (Musa *et al.*, 2020). On the other hand, embryos at 20 °C under hypoxia had a survival rate of 61.9% compared to the embryos under normoxia and 20 °C, with a survival rate of 85.7% (Musa *et al.*, 2020). Generally in the absence or shortage of oxygen, a restriction of ion movement, protein synthesis and transcription, and other anabolic pathways can dramatically affect the overall organismal metabolic rate (Bickler and Buck, 2007; Polymeropoulos *et al.*, 2016; Richards *et al.*, 2007). When an organism experiences hypoxia or anoxia, there are two means of survival to meet its energy demands: (i) increase Adenosine Triphosphate (ATP; a key energy molecule) supply by using anaerobic pathways or (ii) reduce ATP demand (Bickler and Buck, 2007; Boutilier and St-Pierre, 2000; Currie and Boutilier, 2001). The drawback associated with the use of anaerobic pathways to generate ATP is that it creates a large amount of anaerobic waste, which reduces the survival time of the organism during the hypoxic or anoxic exposure (Boutilier and St-Pierre, 2000). In contrast, a reduction of the energetic demand (metabolic depression) leads to a reduction in the overall production and need of ATP (Bickler and Buck, 2007; Currie and Boutilier, 2001; Richards *et al.*, 2007). This reduction in energetic demands also reduces the demand for oxygen, as oxygen is central to aerobic respiration to avoid the production of dangerous waste (Bickler and Buck, 2007). However, this reduction in energy demand can also result in the prioritization of energy allocation to basic key tasks in the survival of the organism (Cheung *et al.*, 2008; Wei *et al.*, 2009). For instance, in Atlantic salmon embryos, hypoxia has been linked to delays in growth and embryo development (Wood *et al.*, 2019a). Particularly in fish, it has been suggested that limitations of oxygen availability are generated by the presence of the egg chorion (egg capsule), which acts as a limiting barrier for embryo oxygen uptake in Atlantic salmon and rainbow trout (Rombough, 1988; Wood *et al.*, 2019a). This suggestion is further bolstered by the fact that the limitation of growth

observed in the embryos was not apparent in the alevin life stage (early life stage which remains in the protection of the gravel until the yolk-sac is completely utilised) of the Atlantic salmon and rainbow trout (Wood *et al.,* 2019a). If one extrapolates the oxygen limitation of the egg capsule in Atlantic salmon and rainbow trout to the shark egg case, a similar hypothesis could be proposed. As the egg case of oviparous chondrichthyans is composed of an intricate laminated collagenous shell (Wourms, 1977), it offers minimal oxygen intake due to its insoluble nature; however, prior to the dissolving of the seal on the egg case, there are respiratory pores to allow some degree of oxygen exchange with the surrounding medium (Wourms, 1977). The seal of the egg case opens at approximately stage 26-31 in the lesser spotted dogfish as outlined by Ballard *et al*., (1993). In the grey carpet shark, the seal of the egg was observed to open at approximately day 35 post-deposition and seawater circulates through the egg case (Ballard *et al.,* 1993; Harahush *et al.,* 2007). So, while the dissolved oxygen levels in my study were recorded to be above 4.69 mg O_2 L^{-1} in the tanks, which is considered to be normoxic for seawater, the rate of actual oxygen able to diffuse into the egg case could have been restricted to the respiratory pores initially, until the opening of the egg case occurred later during development (Harahush *et al.,* 2007; Wourms, 1977). Hence, with the potentially increased metabolic rate, as a result of the thermal stress treatments, the egg case could be responsible for a limited oxygen availability. A reduction of oxygen levels available to the embryo within the egg case, despite apparent high oxygen levels in the water surrounding the egg, could be detrimental for the development of the embryo. It is worth noting that in a closely related species, the white-spotted bamboo shark (*Chilioscyllium plagiosum*), the level of oxygen consumption of the embryos was reported to be 1.4 μL O_2 g^{-1} min^{-1} (Tullis and Peterson, 2000). So, it is possible to suggest that even when the dissolved

oxygen falls to 4.69 mg O_2 L^{-1} during cyclic thermal stress, the low oxygen consumption rate of the embryos may not have been impaired. It would be interesting and important that future studies explore oxygen levels within chondrichthyan egg cases to determine whether the egg case is in fact limiting oxygen diffusion.

Through my study, during the later stages of development, an increased pattern of tail beating was observed. The tail beating has been also reported in the little skate (*Leucoraja erinacea*) when subjected to gradual hypoxia and undergoing metabolic depression (Di Santo *et al.,* 2016). These increased tail beats are suggested as an effective mechanism used by individuals to draw in oxygen-rich water and alleviate the stress caused by any oxygen reduction (Di Santo *et al.,* 2016). As individuals became larger in length, and their body occupied a greater volume of the egg case, their movements became restricted, and perhaps a greater limitation of oxygen flux into the egg case occurred. Different studies have demonstrated that being small under hypoxic stress is an advantage (Pan *et al.,* 2016; Pauly, 1981). However, other studies suggest that individuals of different masses could still be susceptible to hypoxia (Nilsson and Ostlund-Nilsson, 2004, 2008). There is a consensus indicating that small individuals have a large surface area to volume ratio, and this is an advantage to easily satisfy organismal oxygen diffusion and demand while under oxygen limiting conditions (Flint *et al.,* 2015; Pauly, 1981; Reardon and Chapman, 2012). Hence, shorter embryos exposed to cyclic thermal stress could have an advantage while undergoing some limitations in oxygen availability. Nevertheless, further studies would need to be performed to confirm that being smaller is in fact an advantage for sharks under hypoxia, by exploring the point at which the embryos can no longer maintain their ventilation rate (P_{crit}). Furthermore, future studies should also explore the influence of temperature variation on oxygen consumption rates through development, in order to obtain evidence regarding

metabolic stress and metabolic depression. Perhaps video recordings and respirometry could be made while during the exposure to the cyclic temperature treatment to quantify behavioural changes inside the egg case, the tail movement, as well as the aerobic metabolic scope.

There was significant variation in the time of appearance of some key developmental milestones. The first difference was observed in the time of appearance of the pigmentation reaching the pectoral fins of the embryos. This appearance of pigmentation occurred 8 days later in the group exposed to cyclic thermal stress, compared to the control group. The significant difference in the time of appearance of pigmentation reaching the pectoral fin could be a result of the cyclic exposure to 28 °C, which can alter the time at which the hormonal pathways are triggered that promote pigmentation, as was proposed for epaulette sharks reared at 32 ºC (Gervais *et al.,* 2016). Other studies have found links between hormones and the development of pigmentation (Busca and Ballotti, 2000; Visconti *et al.,* 1999). For instance, cAMP (cyclic adenosine 3'-5'-monophosphate) has been identified as a key messenger for the regulation of skin pigmentation (Busca and Ballotti, 2000). Specifically, it is suggested that the cAMP pathway plays a pivotal role in melanocyte differentiation and regulation of melanogenesis (Busca and Ballotti, 2000). Furthermore, cAMP has been identified as a key pathway in elasmobranchs pigmentation, specifically in the smooth back river stingray, *Potamotrygon reticulatus* (Visconti *et al.,* 1999). The specific hormones involved were α- melanocyte-stimulating hormone (α-MSH) and prolactin which both caused a darkening of the skin (Visconti *et al.,* 1999). α-MSH was also identified as a key hormone in the lesser spotted dogfish (Sumpter *et al.,* 1984). Hence, future studies could explore the influence of temperature on cAMP pathways in oviparous elasmobranch species.

The complete pigmentation of the embryo could be strongly associated with the survival of the newly hatched embryos. The appearance of the black and white banding pattern

on hatchlings and juveniles may be an adaptive Batesian mimicry strategy that protects hatchlings if they resemble the black-banded sea snake (*Laticauda semifasciata*; Figure 3.14).

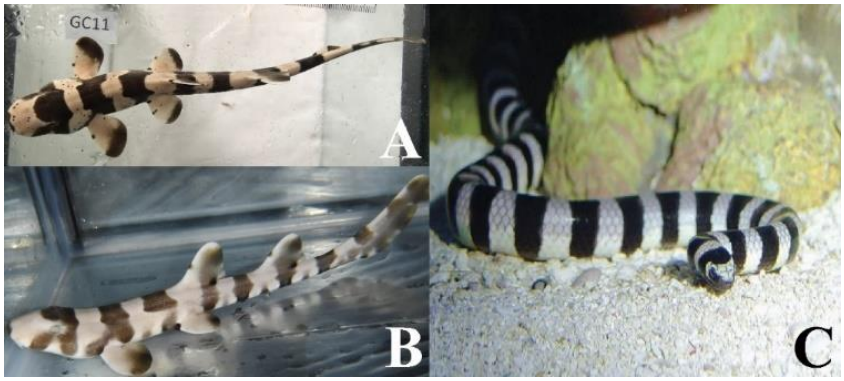

Figure 3.14. Pigmentation Pattern in grey carpet shark neonates (A & B), compared to the banding and colouration pattern of the black-banded sea snake (C). Image A, B Jessie Walton; image C: http://www.aquariumofpacific.org

This banded colour patterning in the grey carpet shark is normally displayed prior to hatching and could have evolved as a survival strategy because it mimics a venomous sea snake (Tu 1973). By using this specific type of mimicry, the grey carpet shark neonates could avoid predation (Dudgeon and White, 2012; Kempster *et al.*, 2013). Perhaps, because of the apparent antipredator benefit that the pigmentation pattern can offer to neonates, it is not surprising to find this trait as a strong predictor of the time of hatching. The use of mimicry colouration has been reported in other species such as the scarlet kingsnake (*Lampropeltis elapsoides*) which displays a red, yellow and black pattern that is apparent in the coral snake *Micrurus fulvius*; (Kikuchi and Pfennig, 2012). Similar colour mimicry has also been reported in various species of butterflies, such as the *Dismorphia*, which mimics the *Ithomiini* species known for their impalpable taste (Kikuchi and Pfennig, 2010; Kleisner and Saribay, 2019). However, further studies would be required to determine the effectiveness of this banding as a form of Batesian mimicry for grey carpet shark neonates and embryos.

For the embryo to develop this pigmentation and other developmental milestones, it relies on the yolk sac as a key energy source (Regnier *et al.*, 2012; Vidal *et al.*, 2002). A widely accepted method of estimating energy use in fish embryos is derived from monitoring the rate at which individuals consume their yolk (Regnier *et al.*, 2012; Vidal *et al.*, 2002). The yolk is composed of lipoproteins, neutral lipids, and monounsaturated fatty acids (Heming and Buddington, 1988; Wiegand, 1996). In teleosts, phospholipids, wax ester, and triacylglycerol are used as a catabolic substrate for breaking down the lipoproteins to provide energy for the embryo (Henderson and Almatar, 1989; Wiegand, 1996). Once the catabolism has occurred, the yolk is absorbed by the embryo by endocytosis *via* the yolk syncytial layer (Heming and Buddington, 1988; Krieger and Fleig, 1999; Wiegand, 1996). Typically, as an embryo is exposed to higher temperatures, their metabolism increases, which is observed in their increased rate of oxygen consumption and generally in their increased rate of yolk consumption (Mueller *et al.*, 2011; Rosa *et al.*, 2014). Nevertheless, in my study, the developing embryos did not display an increased rate of yolk consumption at higher temperatures compared to the control group (24 °C). The lack of variation in the yolk volume could be due to changes in the composition of the yolk and not the volume. While the rate of yolk consumption is a strong indicator of metabolic activity in most teleost fish, minimal to no changes of yolk composition throughout embryo development were reported in *Sardinops caerulea* and rainbow trout (Lasker, 1962; Smith, 1957). However, other species of teleosts have displayed a change in their yolk composition throughout development, such as in *Scophthalmus maximus*, *Ammodytes personatus*, and *Stizostedion vitreum* (Moodie *et al.*, 1989; Ronnestad *et al.*, 1992; Yamashita and Aoyama, 1985). Temporal change in yolk composition has not yet been analysed in oviparous sharks and should be addressed in future studies. The significant delay in the internalization of the yolk sac, in response to cyclic thermal stress compared to control

conditions, as a key developmental milestone was the strongest predictor of time to hatching. This internalization of yolk occurred approximately 15 days later in the embryos exposed to cyclic thermal stress compared to the control individuals.

In many egg-laying vertebrates, hatchlings emerge from the eggs without depleting their yolk reserve (Pezaro *et al.*, 2013; Radder *et al.*, 2002). It has been hypothesized that the remnant-yolk is used as reserve energy for post-hatching activities. Nevertheless, the residual yolk has also being considered non-nutritive in other ectotherms, and could indicate that the key molecules such as the oil globules and lipoproteins were largely consumed throughout development, and the residual yolk contains minimal energy-rich molecules (Radder *et al.*, 2007). So, further research is required in order to determine if grey carpet shark hatchlings are able to find food as soon as they hatch, or if they need to disperse first, and thus rely on their residual yolk. Future studies on the movement of grey carpet shark neonates, the time until their first meal, and their habitat use upon hatching could provide some insight into the role that residual yolk plays in energy availability upon hatching. In spite of the fact that all of the neonates had resorbed the yolk sac at the time of hatching, neonates would typically accept food within the first 1-2 days after hatching (Personal Observation).

The yolk volume significantly decreased across most stages, with the exception of stage four, five, and seven; which were not significantly different from one another. The decline in yolk volume during the early and late developmental stages could be a result of increased use of the lipid reserves for initial tissue development leading up to stage three, and then, during the stages eight through eleven. It seems that between stages four, five, and seven, which occur after organogenesis, there is a lower energy demand (lower rate of yolk consumption) compared to the other stages of development. In previous literature, organogenesis was noted to be complete at stage 31 in the lesser spotted dogfish (Stage three in my study), which was

identified by the gill filaments reaching their maximum length and the dorsal fins ceasing sagittal growth (Ballard *et al.,* 1993). A rapid decline in yolk volume was also observed in the greater spotted catshark, *Scyliorhinus stellaris* (Musa *et al.,* 2018). Similar to the findings of the present study, most of the decrease in yolk volume for the greater spotted catshark was observed during the latest stages of development (stage six). In my study, the grey carpet shark embryos exposed to 24 °C had utilized 75.51 $\pm$ 1.98% of their yolk by stage ten, while embryos cyclically exposed to thermal stress at 28°C had utilized a similar 75.42 $\pm$ 0.15% of their yolk, indicating no significant difference between treatments. A similar decline in yolk volume was also observed later in development in the white-spotted bamboo shark at approximately day 80 post-oviposition (Tullis and Peterson, 2000). By the end of stage six, the greater spotted catshark had used 98.79 $\pm$ 1.05% of the initial yolk (Musa *et al.,* 2018). The measurement of the yolk volume during later stages in my study was limited by the position of the embryo, which due to their increased length, started coiling inside the egg case and around the yolk sac. Hence, the accuracy of measurements of the yolk volume during the late developmental stages could not be as accurate of measurements at the early stages. However, further studies would need to be completed to confirm the timing of completion of organogenesis and the relationship between organogenesis completion and changes in the rate of yolk consumption.

Finally, from all the morphological traits of the neonates that were compared between the two temperature treatments, neonate total length (cm) was the only trait that varied significantly at the time of hatching (See Appendix 4.1. for more information on all traits). There was also no difference in Fulton's Condition or the Relative Condition of the neonates upon hatching between temperature treatments. A similar observation was made on lake whitefish, exposed for only one hour to constant high temperature (+3°C to 2, 5, and 8°C) compared to controls (Lee *et al.,* 2016). Whitefish at the pre-hatch stage were lighter in mass

and smaller in length; and with a reduction in total length occurring progressively from 2°C to 5-8°C (Lee *et al.,* 2016). In the lesser spotted dogfish exposed to thermal stress, upon hatching, neonates were significantly shorter in total length and displayed a lower body mass, which resulted in an elevated Fulton's Condition (Musa *et al.,* 2020). Furthermore, in the Arctic cod and walleye pollock similar trends on size were attributed to the increased temperature exposure (Laurel *et al.,* 2018). Prior literature on walleye pollock reported a 0.4 mm difference in length at temperatures which differed by 3.9 °C (Blood *et al.,* 1994). However, when the difference in incubation temperature was 12.8 °C, walleye pollock total length at hatching was greater by 0.6 mm (Laurel *et al.,* 2018). On the other hand, in the Arctic cod, a dramatic change in total length, up to 1.55 mm was observed across 5.4°C, ranging from -0.4°C to 5°C (Laurel *et al.,* 2018).

In my study, I found that a 4 °C variation between the two experimental temperature treatments can solicit a change in growth. Individuals of the thermal stress group (28 °C) were on average 8.68 mm shorter than the individuals at the lower temperature group (24 °C). A similar change was observed in the clearnose skate (*Raja eglanteria*) which when incubated at 14.5 and 16.5 °C individuals were 3.5-7% smaller than the control individuals reared at 12.5 °C (Hume, 2019). Clearnose skates incubated at 16.5 °C were on average 137.21 $\pm$ 2.25 mm long, while individuals incubated at 14.5 °C were 143.76 $\pm$ 3.77 mm on average and the control group was significantly longer at hatching, with a total length of 148.82 $\pm$ 1.03 mm (Hume, 2019). The observed change in total length between the two thermal treatments could be a result of multiple factors. For instance, the grey carpet shark embryos exposed to 28 °C could have triggered a change in energy allocation for growth as a result of the temperature increase acting as a stressor as previously reported on other animal species (Storey, 1988). Comparatively, the fish *Cyprinodon macularius* displayed a significant

decrease in total length in response to thermal stress, a temperature difference, with individuals at 24 °C and 28°C averaging 5.3 mm in length while individuals at higher temperatures of 34 °C were on average 3.9 mm and at 35 °C were 3.65 mm (Storey, 1988). When considering energy allocation, there are a large proportion of biological processes which require energy such as growth, routine movement, reproduction, avoidance of predators and coping with stressors (Griffen, 2018; Kempster *et al.,* 2013; Polymeropoulos *et al.,* 2016; Ropke *et al.,* 2019). For the purpose of my study, reproduction and avoiding predation were not considered as potential factors, because the embryos were maintained in a controlled environment away from predators during the formation of embryos, and individuals were not at a life stage where reproduction is a point of high energy investment. This leaves growth, movement, and coping with stressors as the primary sources for energy allocation. Because individuals were maintained under controlled environmental conditions while in their holding tanks: dissolved oxygen (5.74 $\pm$ 0.28 mg O_2 L^{-1}) and water temperature (23.97 $\pm$ 0.2°C), individuals in the control group would have undergone minimal stress. The more possible potential stress that the control group would have gone through is limited to handling (movement of the embryos between the holding tank and the temperature treatment tank), but it was important for the experimental design to expose all egg cases to the same type of handling. On the other hand, only one group of embryos were exposed to the higher temperature treatment, and hence, this increased temperature exposure may have been sufficient to increase their metabolic rate. In a previous study, the grey carpet shark embryos raised under two different temperatures, 26 °C and 30 °C, results showed that embryos under the higher temperature had a significantly higher metabolic rate of ~160 $\mu mol^{-1}h^{-1}$ compared to the metabolic rate of ~80 $\mu mol^{-1}h^{-1}$ on the control individuals (Rosa *et al.,* 2014). Hence, in my study, embryos incubated under cyclic thermal

stress may have also experienced an increase in metabolic rate that solicited a stress response (including hormonal signals), which resulted in a decrease in length.

In conclusion, this is the first study to use cyclic thermal stress at a periodicity that more accurately reflects the type of thermal challenges that embryos face under natural conditions. Results show that exposure to cyclic elevated temperature can significantly delay key milestones in grey carpet shark embryo development and result in neonates of diminished length without affecting survival. The data supports two proposals: (i) that cyclic thermal stress could have bioenergetic consequences in terms of elevated metabolic rate during exposure to elevated temperature, as reported in turtle eggs (Reid *et al.*, 2009); and (ii) that the repeated temperature transitions could have triggered the increased expression of stress-activated proteins which are known to lower metabolic rate, as first described by Storey (1998). Both of these proposals need to be tested in specifically designed experiments.

Previous studies using constant incubation temperatures reported results differed from those here reported. Further experiments using a cyclic thermal exposure need to be carried out in order to investigate the contrasting results obtained after embryos are exposed to a constant elevation in temperature (Johnson *et al.*, 2016; Rosa *et al.*, 2014). Cyclic thermal stress was found to increase embryo development time and alter the time of appearance of different developmental milestones, such as pigmentation reaching the pectoral fins, widening of the trunk, and internalization of yolk. While there was not a significant difference in mortality between temperature treatments, future studies should explore a wider range of high temperatures (32 or 36°C), which reflects the presently projected temperatures for coral reef flats under climate change (Hobday and Lough, 2011). Since an embryo's ability to develop and survive can be compromised by the synergistic effects of several factors, potential exposure to hypoxia during elevated temperature was avoided by maintaining dissolved

oxygen levels above 4.69 mg O_2L^{-1}. Future studies could investigate the effect of allowing the dissolved oxygen level to fall with increased temperature, to investigate the potential synergistic effect of both elevated temperature and hypoxia. Future studies could also explore the regulatory pathways and mechanisms used during the development of specific milestones in order to determine the time periods at which grey carpet shark embryos are most vulnerable to environmental stressors. Identification of environmental stressors in a manner that reflects their natural habitat would yield valuable information to enable us to understand the threats that the global warming component of climate change poses to grey carpet sharks, and other reef sharks, embryo development.

APPENDIX 3.1.

Appendix 3.1. Embryo length and yolk volume across developmental stages one to nine in grey carpet shark embryos exposed to 24 ºC (n=5) continuously or to 28 ºC (n=5) in a cyclic manner of two hours a day for five days, followed by nine days recovery at 24 ºC.

	Temperature			
	24 ºC		**28** ºC	
Stage	**Embryo Length (cm)**	**Yolk Volume (cm^3)**	**Embryo Length (cm)**	**Yolk Volume (cm^3)**
2	3.7 ± 0.8			
3	4.4 ± 0.9	18.3 ± 2.8	3.9 ± 1.0	19.55 ± 1.2
4	5.4 ± 1.0	15.7 ± 6.0	4.3 ± 1.1	15.1 ± 1.8
5	7.4 ± 1.85	15.8 ± 2.7	5.5 ± 0.6	16.0 ± 2.4
6	8.6 ± 1.0	11.3 ± 2.1	9.2	13.4
7	9.0 ± 0.7	11.7 ± 0.8		13.9 ± 1.4
8	11.9	10.0	9.2 ± 1.0	12.0 ± 0.8
9	11.5 ± 1.6	10.5 ± 2.5	11.3 ± 2.1	10.4 ± 1.6
10	14.7	10.8	13.0 ± 1.9	5.9 ± 1.8

Note: Data are shown as $\bar{X}$ ± s.d. Data with no s.d. had only one individual due to curling of embryos around the yolk sac and thus limiting possible measurements.

CHAPTER 4

Does Thermal Stress Post-Hatching, Affect The Hypoxia Tolerance of grey carpet sharks (*Chiloscyllium puncatum*)?

INTRODUCTION

In marine ecosystems, global warming has been linked to increased water temperature, decreased dissolved oxygen levels, and increased ocean acidification (Belkin, 2009; Camp *et al.*, 2018; Cheung *et al.*, 2009; Rosa *et al.*, 2017; Rosa *et al.*, 2014; Rosa *et al.*, 2016; Rummer and Munday, 2017). Recent research has demonstrated that environmental effects can be synergistic, and have the potential to alter the ability of an organism to respond or cope with another stressor (Del Rio *et al.*, 2018; McDonnell *et al.*, 2019; Rodgers *et al.*, 2019). For instance, in the dwarf Victoria mouthbrooder, *Pseudocrenilabrus multicolor victoriae,* adults exposed to both high temperature (29 ºC) and hypoxia (14% air saturation), exhibited a lower tolerance to temperature, by displaying a larger thermal agitation window (temperature range between their loss of equilibrium and point of displaying agitation), and lower body condition, compared to individuals maintained at normoxic conditions (95% air saturation) and at a cooler temperature of 21 ºC (McDonnell *et al.*, 2019). Comparatively, in adult coral reef damselfish (*Acanthochromis polyacanthus*), exposed to an increased temperature of 33 ºC, fish displayed a reduced absolute aerobic scope, as well as the maximum scope of their oxygen uptake, while their routine oxygen uptake increased when compared to the control of 30 ºC (Rodgers *et al.*, 2019). On the other hand, chinook salmon fry (*Onchorhynchus tshawytscha*) exposed to hypoxia (5.5 mg O_2 L^{-1}) displayed an improved ability to tolerate acute thermal stress as they had a higher critical maximum temperature of 29.2 ºC (CT_{Max}; temperature at which organism responds with unorganized locomotion), compared to the CT_{Max} of 28.8ºC observed in fry maintained in normoxic conditions (Del Rio *et al.*, 2018).

Despite some individuals being more tolerant to thermal stress and hypoxia, there is evidence that suggests that a shift to small-sized individuals within a population could

potentially reduce its overall survival. In different fish species such as chinook salmon and those in the Cyprinidae family such as *Barbus bocagei*, *Achondrostoma occidentale*, *Iberochondrostoma lusitanicum*, and *Iberochondrostoma almacai*, it has been demonstrated that smaller fish have slower swimming speeds, which result in a reduced capacity for catching prey and avoiding predators, with an overall reduction of survivorship (Del Rio *et al.*, 2018; Mameri *et al.*, 2020; Mateus *et al.*, 2008; Pepin, 1991). Nevertheless, there is also scientific evidence that strongly demonstrates that having a smaller body size can be advantageous for organisms when coping with stressors such as thermal stress and hypoxic stress (Landsman *et al.*, 2011; Robb and Abrahams, 2003; Stierhoff *et al.*, 2009). For example, the fathead minnow (*Pimephales promelas*), are able to tolerate exposure to hypoxia for a significantly longer period of time than their primary predator, the yellow perch, *Perca flavescens* (Robb and Abrahams, 2003). Specifically, fathead minnows were able to withstand on average 350 minutes under extreme hypoxia (1.57 mg O_2 L^{-1}), while the small yellow perch tolerated approximately 210 minutes, and larger yellow perch were able to tolerate this condition for only 25 minutes (Robb and Abrahams, 2003). Overall, this research shows that within these two species, there is also a clear trend of smaller individuals being more tolerant to hypoxia (Robb and Abrahams, 2003). Similarly, in the red drum (*Sciaenops ocellatus*), the critical oxygen level (P_{crit}) was higher in larger individuals than smaller ones (Pan *et al.*, 2016). This lower P_{crit} for smaller individuals could be ecologically significant in allowing smaller individuals to find refuge from predators in hypoxic waters (Chapman *et al.*, 2002; Pan *et al.*, 2016).

Because embryos and juveniles exposed to quickly changing environments before and after hatching are extremely vulnerable (Rosa *et al.*, 2014; Rosa *et al.*, 2016), it is important to further our knowledge about the potential stress caused by natural environmental factors on

the ability of embryos and neonates to survive. Although prior studies on the adult grey carpet shark have explored the impact of thermal stress on adults (Chapman *et al.*, 2011; Rosa *et al.*, 2017), there is still a large gap in knowledge in understanding how grey carpet shark neonates would cope with climate change. Hence, in this chapter, grey carpet shark neonates were used to examine the effects of post-hatching thermal stress on their ability to tolerate hypoxia, a characteristic of the shallow coastal environments that these sharks encounter on a cyclic basis. The aim was to explore whether prior exposure to thermal stress for 5 days within 24 hours of hatching would alter the ability of grey carpet shark neonates to cope with a severe hypoxia challenge. Specifically, my study compared exposure of untreated neonates to neonates exposed to 5 days of cyclic thermal stress (32°C) to determine whether:

(i) variation exists in the survival of neonates between control individuals exposed to 24 °C and cyclic thermal stress individuals;

(ii) variation exists in morphological traits (fin lengths, total length, body mass, etc.) between control individuals exposed to 24 °C or cyclic thermal stress;

(iii) variation in growth rate exists between control individuals exposed to 24 °C compared to cyclic thermal stress;

(iv) variation in the response of neonates to a hypoxic challenge (time to loss of righting reflex) between control individuals exposed to 24 °C compared to cyclic thermal stress;

(v) variation in ventilation rate stability during a hypoxia challenge occurs between control exposed to 24 °C and thermal stress individuals, as seen in their adult counterparts when subjected to a hypoxia challenge, .

MATERIALS AND METHODS

Thermal Stress in Neonates

I used a total of 10 neonates to explore the effect of their thermal acclimation history and subsequently the effect of prior thermal stress on their response to a hypoxia challenge. Fertile egg cases were provided by Sea World, Gold Coast, Australia. All egg cases were maintained in seawater at 24 ºC from arrival up to hatching and were not exposed to thermal stress during development (Figure 4.1). However, within 24 hours of hatching, neonates were haphazardly placed into two holding tanks (85 L) and assigned to one of two temperatures: 24 ºC (control group) or 5-daily episodes of cyclic thermal stress for 2 hours at 32 ºC (thermal stress group).

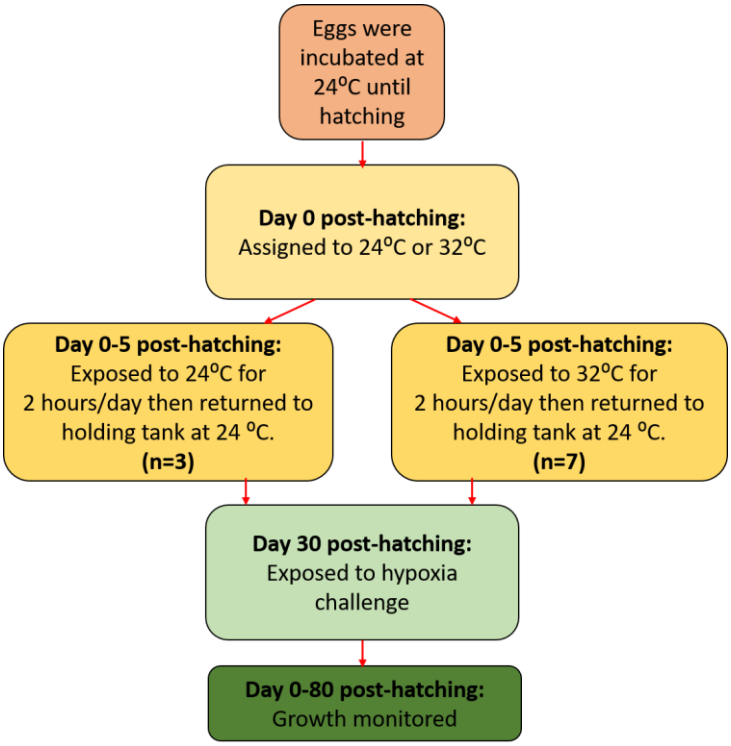

Figure 4.1. Grey carpet shark neonate treatment cycle, conducting from the initial step at the top to the final step. Eggs were haphazardly assigned to the two temperature treatments.

Post-hatching measurements of neonates pre and post thermal stress or control treatment

Upon hatching, the neonates were weighed (± 0.01g) using a Mandel® TX2202L Top Loading Balance and photographed using a Canon Powershot® G12. The photographs were taken with a ruler at the base of the tank to facilitate the measurement of the body traits of interest. These photographs were also used for individual shark identification using the unique body band pattern of each shark. Body trait measures were made using Image J version 1.52a (NIH, 2018). The body traits measured were the following: total length (TL ± 0.01 mm), snout length (SNL ± 0.01 mm), thoracic length (THL ± 0.01 mm), eye to eye (EE ± 0.01 mm), pectoral fin length (PFL ± 0.01 mm), pelvic fin length (PVL ± 0.01 mm), the first dorsal fin span (D1FS ± 0.01 mm), second dorsal fin span (D2FS ± 0.01 mm), area of pectoral fin (APF ± 0.01 mm) and distance between dorsal fins (DBD ± 0.01 mm).

During the 80 days post-hatching, neonates were photographed, measured for length, and weighed once a week (Appendix 4.1.) and their growth rate and survival were monitored. Through the 80 days post-hatching, the neonates were fed a varied diet consisting of squid, baby mussels, and pieces of prawn until satiation was reached. Satiation was determined when neonates stopped consuming food for a period of 30 minutes despite food still being available in the tank and food being directly offered to their mouths. Fulton's Condition and Relative Condition were calculated at 30 days post-hatching as follows:

$$Relative\ Condition = \frac{Body\ Mass\ (g)}{Body\ Length\ (cm)}$$

$$Fulton's\ Condition = \left(\frac{Body\ Mass\ (g)}{Body\ Length\ (cm)^3}\right) \times 100$$

Thermal stress exposure

Immediately after the photo record and first body mass after hatching was taken, the neonates were moved from the holding tank into their designated temperature treatment tank either maintained at 24 ºC (controls) for 2 hours or slowly heated to 32 ºC for two hours. These treatments were administered once a day for 5 days. The water temperature in the treatment tank always started at the temperature of their shared holding tank (24 ºC). For the cyclic thermal stress group, the water temperature in the tank was increased at a rate of 0.26 ± 0.0026 °C min^{-1}, while water temperature was maintained at 24 ºC in the tank for the control individuals. After two hours at the treatment temperature, each neonate was gradually acclimated back to 24 ºC by adding water from the control tank (24 ºC) into the thermal stress tank over a 20 minute time period. Then the photographs and body mass measurements that were recorded initially were continued for up to day 80 post-hatching.

Hypoxic challenge

After the first 30 days of monitoring neonatal growth and survival, each animal was given a hypoxic challenge (0.35 mg O_2 L^{-1}) to assess hypoxia tolerance. For this, a 15 L acclimation tank was filled with seawater from the ultraviolet sterilized seawater system, approximately 5 centimeters from the lip of the tank. A TPS WP82 ® Dissolved Oxygen Meter equipped with a YSI probe® was used to monitor temperature and oxygen levels through the hypoxic challenge. In order to measure the DO levels in a systematic manner, the YSI probe was secured to the experimental tank approximately 5 cm below the water surface at a 45º angle, just above a 5-watt submersible pump, to ensure that the reading was accurate to the circulating oxygen levels. Oxygen was at normoxic levels ($\bar{X}$ 7.37 $\pm$ 1.19 mg O_2 L^{-1}) at the start of the experiment. A gas diffusion ball was placed in the tank in order to slowly diffuse

nitrogen into the tank to displace the dissolved oxygen levels to administer the hypoxic challenge (0.35 mg O_2 L^{-1}).

Then, the hatchling was transferred from the holding tank to the treatment tank and a layer of bubble wrap was placed at the interface with the water to limit gas equilibration with the external environment. The neonate was left to habituate in the treatment tank for five minutes before commencing oxygen displacement. Prior to commencement, the ventilation rate, behaviour, and duration of neonate activity were monitored and recorded to take note of the resting behaviour of each fish. Then, the dissolved oxygen levels were decreased over the span of 17 min to reach the target of 0.35 mg O_2 L^{-1}. The target hypoxic challenge was reached by introducing nitrogen gas through the diffusion ball, at an average rate of 5 L min^{-1}. The timing of the hypoxic challenge started as soon as the oxygen level reached this target value.

The subsequent behaviour of the neonate was closely monitored throughout the hypoxic challenge, classifying swimming behaviour as exploratory (EXP) or escape behaviour (ESB). EXP was defined as a natural slow movement, typically leisurely swimming or tetrapodal walking on the bottom of the tank; while ESB was defined as rapid directional movement, typically across the tank or forcibly seeking for escape at the surface. Immediately after the target DO level was reached, the ventilation rate was recorded every five minutes for 30 sec. until loss of righting reflex, at which time the experiment was terminated, or until the experiment was terminated at three hours after commencement. The ventilation rate was recorded by counting the number of times the buccal cavity expanded over thirty seconds during which the neonate was resting on the bottom of the tank to ensure accuracy. The time to loss of righting reflex (**TLRR**) was determined by first testing for responsiveness and if sharks were unresponsive then the righting reflex was tested. Premature testing for the righting

reflex would artificially increase energy expenditure as a confounding variable so care had to be taken to determine when the brain shut down had commenced.

After an initial exploratory phase, sharks tended to reduce movement and remain almost immobile during a hypoxic challenge, in order to determine whether a neonate was still responsive, a plastic probe was used to deliver a soft poke on one of their pectoral fins. This fin responsiveness test was only begun after all spontaneous movement had ceased and ventilation rate had reached a plateau before decreasing or increasing. The fin responsiveness test was always performed after the ventilation rate count. When neonates no longer responded to the fin responsiveness stimulus, they were slowly rotated onto their back using two fingers at the level of the caudal peduncle, to determine whether the neonate had lost its righting reflex, providing evidence of cerebellar shut down (Chapman *et al.,* 2011). If the shark attempted to right itself, the rotation was stopped and the hypoxic challenge continued until it no longer had its righting reflex this time was designated the TLRR, if it did not lose its righting reflex the experiment was terminated at 3 hours. Immediately after the termination of the hypoxic challenge, neonates were placed into a recovery container (1L), of well-aerated normoxic seawater until their rate of ventilation returned to its resting rate. The neonates were then returned to their holding tanks. The research protocol was approved by the Animal Ethics Committee at Griffith University (AHS-03-15-AEC).

Statistical Analysis

Statistical analyses were conducted using SPSS version 23. Normality of data was determined using a Kolmogorov-Smirnov test. Non-parametric data was log-transformed for statistical analysis. Survival between treatments was compared using a Chi-square test. To analyze neonate growth following hatching, repeated measures analysis was used with

temperature treatment as the covariate. The body morphology of the neonates upon hatching was examined using an Independent two-tailed t-test to ensure there was no initial difference of groups prior to treatment. Fulton's Condition and the Relative Condition of the neonates were analyzed at hatching, at 30 days post-hatching, and at 80 days post-hatching using an Independent two-tailed t-test. Repeated measures analysis was also used for determining the variation in breathing rate during the hypoxic exposure with temperature treatment as a covariate. To determine whether the time to loss of righting reflex was significantly different between temperature treatments, an Independent two-tailed t-test was used, with time to loss of righting reflex as the dependent variable, and prior temperature treatment as the independent variable.

Because the fins of the shark can increase the overall shark surface area, and due to the potential correlation among the four pairs of fins in the sharks, we initially explored the correlation among them. The variation of the correlation among the fins changed through time post-hatching, and it was difficult to account for the impact of this variation. Hence, in order to reduce the number of variables and data redundancy, a principal component analysis (PCA) was performed including the fin length measurements (pelvic fin, pectoral fin, first dorsal fin and second dorsal fin). The PC scores were analyzed using an Independent two-tailed t-test to determine if there is any difference across temperature treatments. The PC1 and PC2 scores from the fin length measurements along with the physiological observations (intensity and frequency of movement (number of events over time), defecation, and regurgitation), ventilation rates, total length, body mass, the distance between dorsal fins, thoracic length, area of the pectoral fin, separate ratios of the length of each of the four fins in relation to total length, the distance between eyes and snout length were used in a stepwise linear regression in order to determine the best predictors of TLRR. Finally, the relationship between neonate body mass

and time to loss of righting reflex was explored *via* a regression with prior temperature treatments being considered as the independent variable. Differences were considered significantly different when $P \leq 0.05$.

RESULTS

Neonatal Growth

In my study, a Chi-squared test revealed that there was no significant difference in mortality between the neonates exposed to cyclic thermal stress (32 °C for 2 hours day^{-1} for 5 days post-hatching and the controls (24 °C) up to day 80 post-hatching (χ = 2.000; df = 1; P = 0.157). From the initial 13 individuals that were placed into the treatments, two individuals in the thermal stress group died, one due to unknown causes 83 days post-hatching and the other dying after two days of thermal stress. While one individual was removed from the control group as it was displaying neurological issues upon hatching (swimming in loops, weak righting reflex). Due to the unforeseen circumstance of the grey carpet sharks at Sea World Gold Coast underwent a period of 67 days where no egg cases were laid due to a disturbance, there was a delay in access to egg cases, and thus a delay in the development of embryos. This in combination with a severe storm, made it non-feasible to add more individuals to the control group and to explore other temperatures.

The body length and mass of the neonates in each group were not significantly different upon hatching (**Total length**: $T_{(8)}$ = 0.789; P = 0.466; **Body mass**: $T_{(8)}$ = 1.825: P = 0.106). Neonates exposed to 32 °C during the first five days after hatching had minimal changes in body morphology when compared to their controls. When examining the change in neonate total body length over 80 days post-hatching with a repeated measures analysis, there was no significant variation observed in total body length between the thermal stress group and their

controls ($F_{(1,8)}$ = 0.006; P = 0.941; Figure 4.2). Similarly in the repeated measures analysis, no significant differences were found in body mass over the 80 days post-hatching ($F_{(1,8)}$ = 0.004; P = 0951; Figure 4.2).

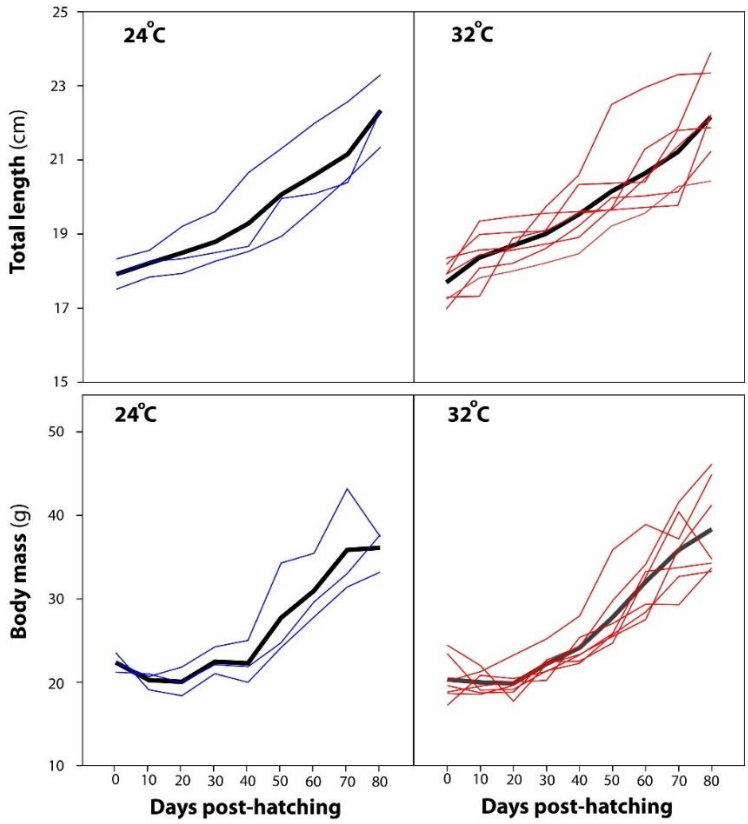

Figure 4.2. Grey carpet shark growth in total length and body mass over 80 days post-hatching analyzed using a repeated measures analysis with temperature as an independent variable. Neonates were exposed for five days to 24 °C in blue (n=3) constantly or 32 °C in red (n=7) for 2 hours day^{-1}. The mean value for total length in cm and body mass in g at each thermal treatment is indicated by the bold black line in each respective section of the figure; individual lines represent each individual in thermal treatment.

Based upon the lack of significant difference in the total length and the body mass between temperature treatments, it was not surprising to find that both the Relative Condition and Fulton's Condition did not significantly vary between treatments at hatching (Respectively: $T_{(8)}$ = 1.047; P = 0.326; $T_{(8)}$ =0.551 ; P = 0.596). This trend continued on day

30 post-hatching (Fulton's: $T_{(8)}$ = 0.651; P = 0.533 ; Relative: $T_{(8)}$ = 1.020; P = 0.338) and day 80 post-hatching (Fulton's: $T_{(8)}$ = 0.926; P = 0.382 ; Relative: $T_{(8)}$ = 1.034; P = 0.323).

Despite the fact that individuals were haphazardly placed into the two temperature treatments, there was a significant difference in the pelvic fin length at hatching ($T_{(8)}$ = -2.923; P = 0.019) prior to treatment. The growth in pelvic fin length maintained the significant difference up to day 80 post-hatching ($F_{(1,8)}$ = 7.693;P = 0.024; Figure 4.3).

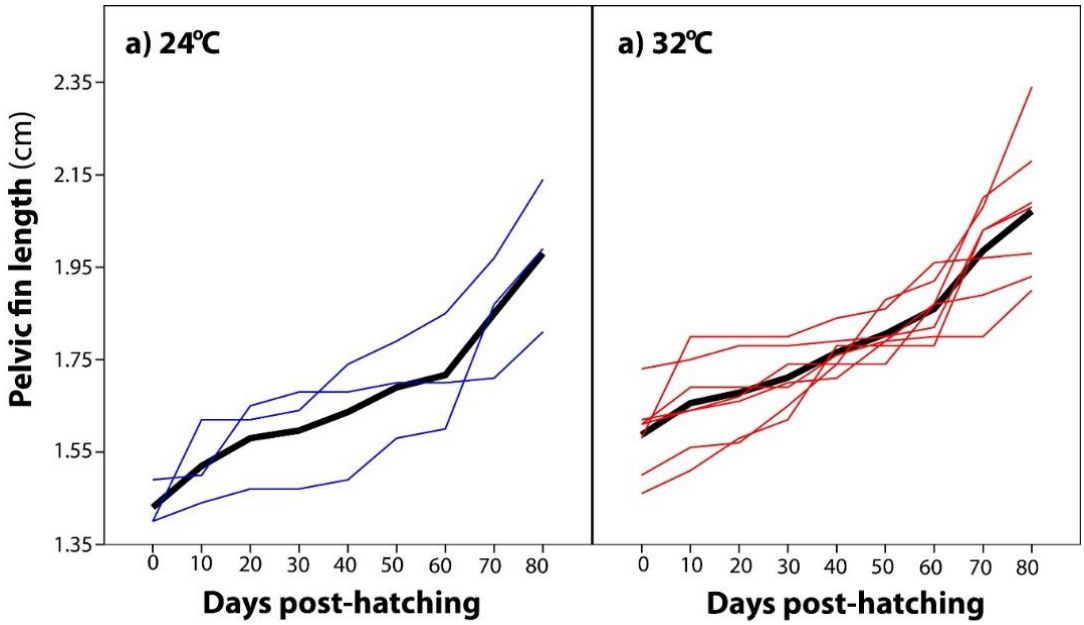

Figure 4.3. Grey carpet shark pelvic fin length over 80 days post-hatching. Neonates were exposed for five days to 24 °C (n=3) constantly (a) or 32 °C (n=7) for 2 hours day^{-1} (b) analyzed using a repeated measures analysis with temperature as an independent variable. The mean value for pelvic fin length at each thermal treatment is indicated by the bold black line in each respective section of the figure; individual lines represent each individual in each treatment.

Specifically, the pelvic fin was longer (up to 14.6%) in neonates exposed to 32 °C, compared to control individuals at day 80 post-hatching. However, the other fins did not display any significant variation between the time of hatching and prior to the hypoxic exposure (Table 4.1). All other morphological traits: Snout length, Eye to Eye, Thoracic

Length, Distance between Dorsal Fins, and Area of Pectoral Fin, were not significantly different between the two temperature treatments (Appendix 4.1.).

Table 4.1. Fin average length of grey carpet shark at hatching and 1-week post-hatching in neonates exposed to either 24 °C (n=3) or a thermal stress treatment (32 °C; n=7) cyclically for five days post-hatching. Fins includes: pectoral fin, pelvic fin, first dorsal fin and second dorsal fin. Significant differences between treatment groups upon hatching and a week after hatching are indicated by * and **bolded**.

	Control		**Thermal Stress**		**Significant Difference**	
Fin length (cm)	Day of Hatching	1 week Post Hatching	Day of Hatching	1 week Post Hatching	Day of Hatching	1 week Post Hatching
Pectoral Fin	1.85 ± 0.05	1.99 ± 0.12	1.94 ± 0.09	2.07 ± 0.07	P=0.150	P=0.325
Pelvic Fin	1.43 ± 0.05	1.60 ± 0.11	1.59 ± 0.09	1.71 ± 0.07	**P=0.019***	P=0.078
First Dorsal Fin	1.53 ± 0.09	1.76 ± 0.18	1.50 ± 0.15	1.74 ± 0.06	P=0.726	P0.779
Second Dorsal Fin	1.46 ± 0.18	1.65 ± 0.06	1.55 ± 0.10	1.70 ± 0.09	P=0.324	P=0.947

Note: Data are shown as $\bar{X}$ ± s.d.

Time to loss of righting reflex

The independent two tailed t-test shows that 5-daily episodes of cyclic thermal stress for 2 hours at 32 ºC, initiated within 24 hours of hatching, did not change the ability of grey carpet neonates to cope with a hypoxic challenge (0.35 mg O_2 L^{-1}) 25 days later. There was no significant difference in the TLRR of the thermally stressed group compared to their controls exposed to a constant 24 ºC ($T_{(8)}$ = - 0.430; P = 0.679; Figure 4.4).

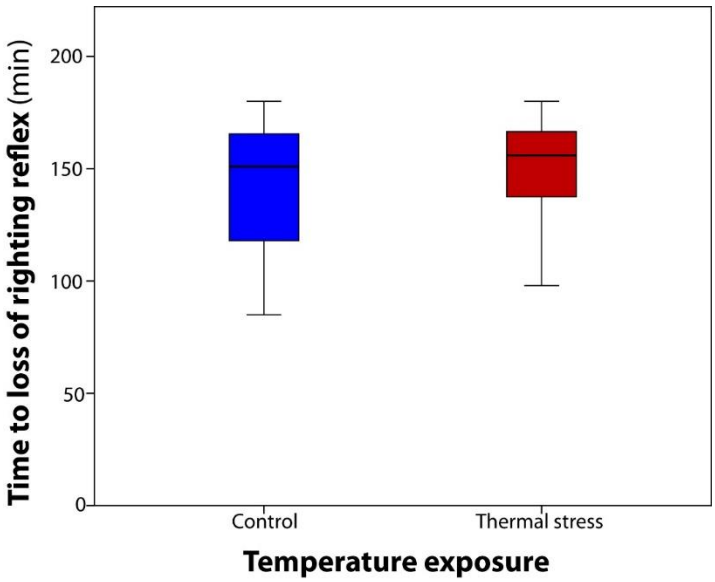

Figure 4.4. The response to hypoxic challenge, the time to loss of righting reflex (TLRR) of grey carpet shark hatchlings analyzed using an independent two-tailed t-test with temperature as the independent variable: Neonates were exposed for five days to 24 °C (blue; n=3) or cyclic thermal stress at 32 °C (red; n=7), then at post-hatching day 30, they were subjected to the hypoxic challenge (0.35 mg O_2 L^{-1}).

Surprisingly, the repeated measures analysis showed that all hatchlings greatly increased their ventilation rate during the hypoxic challenge independently of the post-hatching thermal exposure ($F_{(5,40)}$ = 63.847; P < 0.001). Specifically, within the first 20 minutes of hypoxic exposure, in the control group a steep increase in ventilation rate was observed from a resting rate of 30.0 $\pm$ 3 ventilations min^{-1} to 71.75 $\pm$ 5.3 ventilations min^{-1} (209% change). This increase in ventilation rate was lower in the thermal stress group from a resting rate of 33.4 $\pm$ 9.1 ventilations min^{-1} to 68.2 $\pm$ 6.2 ventilations min^{-1} (161% change). The dramatic increase in ventilation rate was followed by a plateau in ventilation rate between time 20 – 80 min exposure (control: 71 $\pm$ 0.75 ventilation min^{-1}; thermal stress: 67.5 $\pm$ 1.28 ventilation min^{-1}). Finally, a gradual decrease in ventilation rate was observed in both groups around 80 min exposure (control: 65.25 $\pm$ 18.74 ventilation min^{-1}; thermal stress: 54.19 $\pm$ 17.35 ventilation min^{-1}; Figure 4.5).

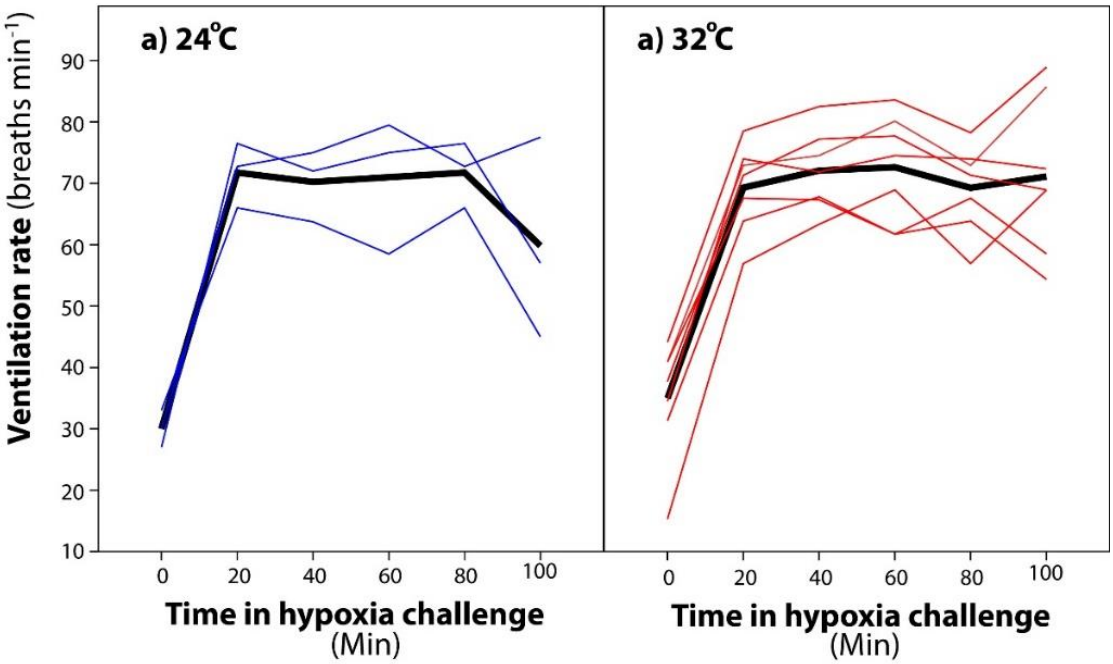

Figure 4.5. Changes in ventilation rate in grey carpet shark hatchlings exposed to a hypoxia challenge of 0.35 mg O_2 L^{-1} analyzed using a repeated measures analysis with temperature as the independent variable. Neonates were exposed for five days post-hatching to either (a) 24 °C (n=3), the controls or (b) 32 °C (n=7), induced cyclic thermal stress. The mean value for ventilation rate during the hypoxic challenge for each thermal treatment is indicated by the bold black line in each respective section of the figure; individual lines represent each individual in thermal treatment

While reducing the number of variables that could explain the TLRR, the PCA shows that PC1 and PC2 scores combined explained up to 82 % of the variation in fin size (Table 4.2). Specifically, the PC1 values explained 53 % of the individual variation in fin length, while PC2 values, explained an additional 29 % of the variation in fin length. There was no significant variation in the fin length PC1 values of the neonates between temperature treatments ($T_{(8)}$ = 1.615; P = 0.145). The lack of significant variation between temperature treatments was also observed in the fin length PC2 values ($T_{(8)}$ = 0.920; P = 0.384).

Table 4.2. Principal components analysis (PCA) for fin length morphology of grey carpet shark hatchlings exposed to 24 °C (n=3) or a 5-day burst of cyclic thermal stress at 32 °C (n=7). .

	Component	
Variable	**PC1**	**PC2**
PFL	0.926	0.048
PVL	0.871	0.247
DF1S	0.707	-0.474
DF2S	0.081	0.933
Eigen Value	2.166	1.145

Note: Factor loadings are shown for the retained component, Fin notation as follows: Pectoral Fin Length **(PFL)**, Pelvic Fin Length **(PVL)**, First Dorsal Fin Span **(DF1S)** and Second Dorsal Fin Span **(DF2S)**.

Finally, the stepwise analysis looking at traits that best explain TLRR showed that the ratio between the length of first dorsal fin and neonate total body length were the best predictors ($F_{(1,8)} = 14.84$; $P = 0.005$; $R^2 = 0.606$; Figure 4.6). The relationship between TLRR and body mass in my study was significantly altered by the temperature at which neonates were exposed cyclically (Figure 4.7). As it can be observed, individuals from the control group (24 °C) displayed a positive correlation between TLRR and body mass, while this correlation was negative in individuals exposed to 32 °C. However, this could be a result of the low n-value (n=3) in the control group compared to the thermal stress group (n=7).

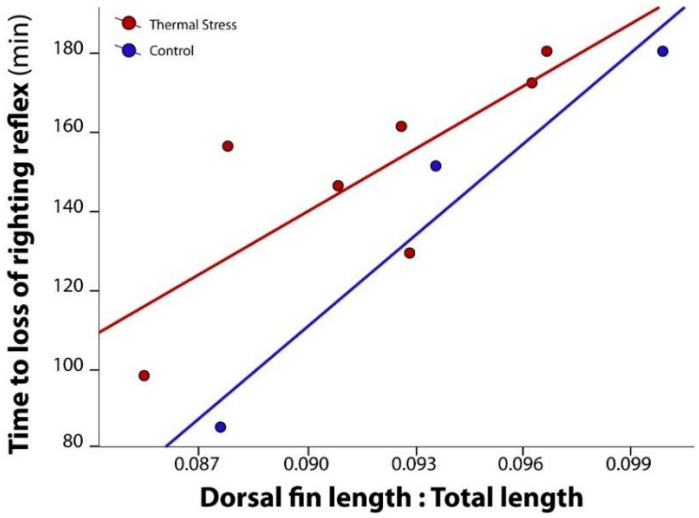

Figure 4.6. Inter-individual variation of the ratio of the first dorsal fin length to total length and time to loss of righting reflex (TLRR) in grey carpet shark hatchlings. The experimental group were held at 24 °C and exposed to cyclic thermal stress at 32°C (red; n=7), the control group were held and exposed to 24 °C (blue; n=3) for 2 hours, on five successive days post-hatching. Regression lines for TLRR and the dorsal fin length: total length ratio are as follows: control group (blue R^2=0.945; y=-5.78^2+7.64^3*x); thermal stress group (red R^2=0.607; y=- 3.32^2+5.24^3*x).

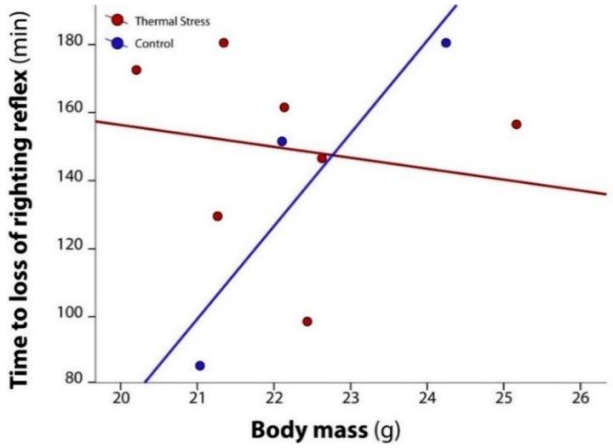

Figure 4.7. Inter-individual variation in the time to loss of righting reflex relative to body mass of grey carpet shark hatchlings. The experimental group were held at 24 °C and exposed to cyclic thermal stress at 32°C (red; n=7), the control group were held and exposed to 24 °C (blue; n=3) for 2 hours, on five successive days post-hatching. Regression lines for TLRR and body mass are as follows: control group (blue; R^2=0.840; y=-4.74^2+27.3*x) and thermal stress group (red; R^2=0.032; y=2.2^2-3.22*x).

DISCUSSION

The effect of cyclic thermal stress on neonates

The cyclic thermal stress exposure during the five days post-hatching (2 hours day^{-1}) did not significantly influence the growth in total body length and body mass of the neonates, which could indicate that the duration of exposure to cyclic thermal stress was not long enough, or severe enough, to elicit a change in the general morphology. While the exposure temperature of 32°C was above their natural thermal range of 18 - 29°C (Davies and Kinsey, 1973), it is possible that the temperature elevation was not long enough, comparable to the temperature elevations explored by others (Chapman *et al.,* 2011; Rosa *et al.,* 2014). Yet the intermittent nature of thermal stress more closely parallels the tidal conditions provided by the natural environment.

The effect of prior exposure to thermal stress on hypoxia tolerance

The remainder of my study focused upon the ability of grey carpet shark neonates to tolerate a hypoxic challenge, using TLRR as the key indicator of the brain shut down. The loss of righting reflex has been demonstrated to be a good indicator of energy conserving cerebellar shutdown (Renshaw *et al.,* 2002). Normally, when hypoxia tolerant fish are unable to meet oxygen-dependent metabolic demands, they undergo metabolic depression (Anderson and Podrabsky, 2014; Burggren *et al.,* 2019; Chippari-Gomes *et al.,* 2005; Delage *et al.,* 2014; Di Santo *et al.,* 2016; Renshaw *et al.,* 2002). Metabolic depression is a physiological response that allows organisms to lower ATP consumption during stressful events (Anderson and Podrabsky, 2014; Chapman *et al.,* 2011; Routley *et al.,* 2002). One of the hallmarks of

metabolic depression is a lowered ventilation rate, as demonstrated for the first time in a hypoxia tolerant shark species, the epaulette shark (Routley *et al.,* 2002).

The effect of temperature on TLRR has been previously explored in adult grey carpet sharks under three different temperatures (23, 25, and 27°C), with individuals at higher temperatures losing their righting reflex sooner in response to the 1.5-hour anoxic exposure (Chapman *et al.,* 2011); in contrast, the TLRR of neonates examined in my study were not affected by cyclic exposure to a similar temperature. In the Chapman study, individuals subjected to 23 °C were able to maintain their righting reflex on average for 172 minutes, while individuals exposed to 25 °C lasted approximately 50 minutes before losing their righting reflex, and individuals exposed to 27 °C, maintained their righting reflex for approximately 25 minutes (Chapman *et al.,* 2011). However, information was still missing regarding the potential effect of prior thermal history on ventilatory responses to low oxygen availability or on the TLRR of neonatal grey carpet shark. The difference in my study is that the neonates experienced periods of recovery after thermal stress which may have assisted with thermal acclimation.

The present study is the first study to my knowledge that exposed grey carpet shark neonates to a hypoxic challenge; hence it is difficult to compare results of similar life stages due to a lack of previous studies. Thus, comparisons between life stages were used here. I found no difference in TLRR after post-hatching thermal stress in grey carpet shark neonates, their ability to tolerate hypoxia was a new frontier for research of this life-history stage. It was observed that neonates displayed a bi-phasic change in their ventilation rate during the hypoxic challenge. This biphasic pattern of ventilation has also been previously observed in adult epaulette sharks in response to progressive hypoxia at 25 °C (Routley *et al.,* 2002). The epaulette sharks were assigned to a control group or a hypoxic group, with the control group

exposed to an oxygen concentration of 80 - 100% for 120 minutes twice day^{-1} for four days, and the hypoxic group was exposed to 0.36 mg O_2 L^{-1} for the same period of time (Routley *et al.*, 2002). In the progressive hypoxic treatment, the sharks took on average 7.7 hours to reach the target oxygen level of 0.3 mg O_2 L^{-1} (Routley *et al.*, 2002). The first phase of the biphasic ventilation rate in epaulette sharks as well as the grey carpet sharks neonates in my study was a dramatic increase (Routley *et al.*, 2002). The epaulette sharks exposed to the control treatment increased their resting ventilation from 36.4 $\pm$ 4.7 to 74.0 $\pm$ 2.5 ventilations min^{-1} (Routley *et al.*, 2002). This increase in ventilation rate by 203% (Routley *et al.*, 2002), is comparable to what was observed in the grey carpet sharks control group (209%) in my study. A less pronounced increase in ventilation rate was observed for the thermal stress groups. The increase in ventilation rate occurred within the first 20 minutes of exposure to hypoxia, with some individuals taking slightly longer to increase their ventilation rate to their peak ventilation rate. This increased rate of ventilation was maintained for most of the individuals up to 80 minutes on average. However, it needs to be recognized that two out of the ten total individuals, one from each temperature treatment were capable of maintaining the increased ventilation rate for the entirety of the 180-minute exposure, and this without losing their righting reflex.

The second phase of the biphasic ventilation was identified by the gradual decline in the ventilation rate. In adult epaulette sharks, a gradual decrease in ventilation rate was also observed from 74 $\pm$ 2.5 to 53.3 $\pm$ 6.3 ventilations min^{-1} until water oxygen concentration approached 0.3 mg O_2 L^{-1} (Routley *et al.*, 2002). Compared to results in my study, the decline in ventilation appears non-existent, with only an 8% reduction in ventilation rate in the control group alone. The thermal stress group displayed a greater reduction in their ventilation rate (19%). It is important to bear in mind that even though I have the same number of individuals

in the thermal stress group (n=7), compared to the pre-conditioned adult epaulette sharks study, the variance in ventilation rate in the current study was greater. This suggests a difference in the capabilities to sustain hypoxic stress between the two life stages (neonates and adults) as well as potential variation among species.

The increase in ventilation rate throughout the hypoxic challenge is a typical response in many fish (Chippari-Gomes *et al.,* 2005; Ern and Esbaugh, 2018; Scott *et al.,* 2017). For instance, in the red drum exposed to various levels of hypoxia (0 ppt, 10 ppt, 35 ppt, and 60 ppt), an increase of ventilation frequency, ventilation amplitude, and stroke volume was observed (Ern and Esbaugh, 2018). Specifically, the ventilation frequency increased by approximately 150% in the control group, compared to individuals exposed to 0 ppt O_2 (Ern and Esbaugh, 2018). Individuals exposed to 10 ppt O_2 increased their ventilation frequency by approximately 160% when compared to the ventilation frequency of the control individuals (Ern and Esbaugh 2018). In the mummichog killifish (*Fundulus heteroclitus*) exposed to hypoxia (10% air saturation) similar results were observed, with an increase in gill ventilation of approximately 120 breaths per min^{-1} in hypoxic seawater (~ 35% increase) from the baseline of 82 breaths per min^{-1} over the span of 1.5 hours before it started to decline (Wood *et al.*, 2019b). Increased gill ventilation is exceedingly costly and can be used as a good indicator of metabolic rate (Ern and Esbaugh, 2018; Fernandes and Rantin, 1994). So, most organisms would be able to maintain a point of relative stability where a plateau is observed but then a decline will follow (Burggren *et al.*, 2019; Wood *et al.*, 2019b). A similar response was also identified in the Mayan cichlid (*Cichlasoma urophthalmus*) exposed to severe hypoxia under three temperatures (23, 28 and 33°C), where an initial increase in the rate of gill ventilation occurred, followed by a plateau, and then a decline (Burggren *et al.,* 2019). From these examples, it is clear that for some organisms there is a point where maintaining the increased

gill ventilation becomes too expensive during a hypoxic challenge (Burggren *et al.,* 2019; Lai *et al.,* 2006). This decline in ventilation frequency was also observed in the marbled sole (*Pseudopleuronectes yokohamae*), which initially increased its ventilation rate as the oxygen levels were declined from 6.59 mg O_2 L^{-1} to 2.75 mg O_2 L^{-1}: however, when the oxygen levels declined past 2.75 mgO_2L^{-1}, the fish started to reduce their ventilation rate (Nishizawa *et al.,* 2017). These declines in the rate of ventilation indicate an inability to maintain the energy requirements due to the increased rate of buccal pumping and the commencement of metabolic depression (Burggren *et al.,* 2019; Nishizawa *et al.,* 2017).

When the neonates started diminishing their ventilation rate, they also decreased the depth of their gill movements. Initially, within the first few minutes of hypoxia, they were observed using pronounced movement of the musculature of the buccal cavity; while after reaching the plateau, they were observed to use a small discrete portion of the musculature of the anterior gill openings (Personal Observations). This difference in ventilation rate and depth of buccal pumping could be a result of a difference in oxygen reserves and has also been observed in grey carpet shark adults (Renshaw Personal Communication). The spleen of fish is a very important source of hematopoiesis (Chapman and Renshaw, 2009; Lai *et al.,* 2006; Randall, 1982). Different studies have shown that in fish subjected to hypoxia, there is an increase in red blood cells and hemoglobin concentration (Randall, 1982). The increase in oxygen-rich red blood cells has been associated with an increase in spleen erythropoietin (Lai *et al.*, 2018). Within the spleen, the trabeculae and the smooth muscle of the capsule allow the strong contraction of the spleen, which causes the ejection of the oxygen-rich blood to the rest of the body in times of hypoxic stress (Udroiu and Sgura, 2017). This same physiological response observed in fish seems to be present in the adults' grey carpet sharks when subjected to anoxia (Chapman and Renshaw, 2009). In adult grey carpet sharks, authors observed that

oxygen-rich red blood cell levels increased during the anoxia exposure, and remained elevated for at least two hours during re-oxygenation, which strongly supports the idea that a contraction of the spleen is occurring and releasing oxygen-rich red blood cells to the rest of their body (Chapman and Renshaw, 2009). In the neonates, an increased ventilation rate was observed which could indicate that the spleen has not yet acquired the capacity to store large amounts of red blood cells.

The size of the spleen determines the amount of blood that can be stored, so it can be expected that adults with a larger spleen volume would be capable of storing more oxygen-rich blood (Fange and Nilsson, 1985; Udroiu and Sgura, 2017). The storing capacity is also influenced by the cords of the spleen, which show a vast ability to extend, and as such store large quantities of blood (Udroiu and Sgura, 2017). In the rainbow trout, the spleen grows at a much slower rate than the overall body growth, with a negative allometry (Weatherley and Gill 1983). While in the common dentex (*Dentex dentex*) and the turbot (*Psetta maxima*), a positive allometric growth of their spleen in relation to their body size was observed post-hatching, followed by a negative allometric growth during the second point in growth inflexion (Sala *et al.,* 2005). Because fish follow an allometric growth pattern (Weatherley and Gill, 1983), it is quite possible that the growth of different body parts and organs, including the spleen, follows a differential growth rate as observed in rainbow trout, common dentex and turbot (Sala *et al.,* 2005; Weatherley and Gill, 1983). Due to the small size of the neonates (30 days post-hatching), neonates may not have yet developed their full capacity to sequester enough red blood cells from the circulation and store them for future use (Mannino *et al.,* 2019; Moore *et al.,* 2014; Noren *et al.,* 2005; Ponganis *et al.,* 2010). Furthermore, myoglobin reserves have been identified as an extra line of defense when facing a hypoxic challenge and

is stored in the striated muscles of nearly all vertebrates, and its key function is to facilitate oxygen storage and diffusion (Mannino *et al.,* 2019; Moore *et al.,* 2014; Noren *et al.,* 2005). Overall, an individual that is smaller, with less striated muscle, would have lower myoglobin stores to facilitate oxygen storage prior to the hypoxic exposure and thus, leaving the individual with less oxygen reserves to cope with the stressor (Moore *et al.,* 2014; Noren *et al.,* 2005; Ponganis *et al.,* 2010). So, the larger myoglobin content in adult grey carpet sharks is an asset when it comes to surviving hypoxic events compared to neonates; nevertheless, future studies would be required to confirm this hypothesis.

Unexpectedly, the best predictor of TLRR was the ratio between the first dorsal fin and the total length of the neonate. As the best predictor of TLRR, the ratio between the first and second dorsal fin and the total length of the neonates accounted for 60.6% of the variation between individual TLRR. While morphological traits can seldom be used to explain a physiological response, this relationship could indicate a role in energy consumption (Renshaw Personal Communication). In the white-spotted bamboo shark, a close relative of the grey carpet shark, it was found that the first dorsal fin generates thrust when neonates used their caudal fin to propel themselves forward (Maia and Wilga, 2013a). In the grey carpet shark, much like the white-spotted bamboo shark, there is a disconnect between the dorsal fins and the back of the neonate towards the posterior portion of the dorsal fin (Figure 4.8).

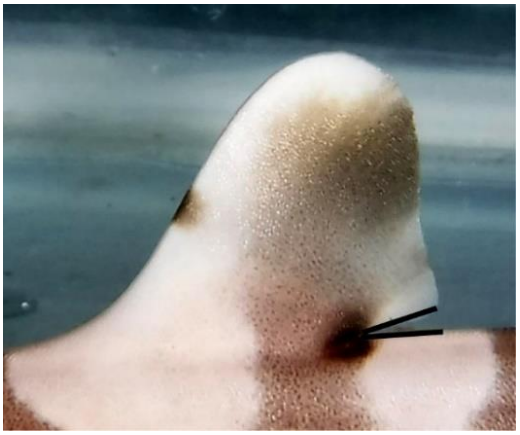

Figure 4.8. Dorsal fin disconnect from the body in the posterior portion of the fin in grey carpet shark hatchlings. Outlined by a black line.

This posterior portion of the fin, which is disconnected from the body, moves laterally in synchrony with the motion of the caudal fin, as was noted in the white-spotted bamboo shark (Maia and Wilga, 2013a; Maia *et al.,* 2017). From these findings, it was suggested that a greater deviation of the posterior section of the fin from the central body line of the neonate, the larger the amount of propulsion that can be generated (Maia and Wilga, 2013a). Hence, the forward propulsion could diminish the number of tail beats that the neonates must use to initiate escape behaviour, or simply to move across the substrate. Further studies would be needed to observe if there is in fact energy being saved by that hydrodynamic function of the dorsal fin, and if it does in fact reduce tail beat frequency.

Another key relationship that was explored was the relationship between body mass and TLRR. The concept of metabolic scaling, suggests that as the mass of an individual increases, the overall metabolism decreases (Banavar *et al.,* 2002; Burgess *et al.,* 2017; Jerde *et al.,* 2019; Thommen *et al.,* 2019). The exact pattern of how metabolism changes over different sizes of organisms, whether it is in an intraspecific or interspecific context is still highly debated, with several models explaining the variation observed (Burgess *et al.,* 2017; Glazier, 2015; Kooijman, 2010; Nakaya *et al.,* 2003; West *et al.,* 1997, 1999). In my study, I

explored the metabolic scaling of grey carpet shark neonates in hypoxia following cyclic thermal stress (32 °C) compared to control individuals (24 °C). The metabolic scaling of the neonates was evaluated by monitoring their TLRR while under hypoxic conditions (0.36 mg O_2L^{-1}). TLRR can be used to evaluate metabolic scaling, as TLRR indicates a point in time when an organism shuts down its brain to preserve ATP when their metabolic requirements can no longer be met (Renshaw *et al.,* 2002). It has been previously proposed in fish that their metabolic scaling coefficient is ~0.89 across various species (Jerde *et al.,* 2019). Prior to the study on various species of fish, a universal scaling coefficient was heavily debated (Jerde *et al.,* 2019), with a 0.75 scaling relationship being primarily suggested. However, in recent years, alternative hypotheses have put the scaling relationship between 0.5 and 1 (Bokma, 2004; Jerde *et al.,* 2019). A stronger metabolic scaling coefficient suggests that the trend of metabolism reducing per unit of mass is expected to be present in the grey carpet shark; particularly when the TLRR of each individual shark is plotted over their respective body mass. However, this was not the case, as there was not a clear relationship between the TLRR and the body mass (R^2= 0.03) of neonates exposed to cyclic thermal stress at 32 °C. However, in the control group, there was a strong relationship between these two traits, with an observed R^2= 0.84. This disparity across the two temperature treatments could be an artifact of the reduced number of control individuals available (n=3), compared to the high-temperature group (n=7). Nevertheless, the low value for the thermal stress group could suggest that individuals are responding in different ways, which increases the individual deviation from the regression line. Based on metabolic scaling models, it is possible that the thermal stress that the neonates experienced in the first five days post-hatching impacted the internal transport of resources, the animal's capacity to take up and use oxygen or its nutrients, or influenced the fluxes of metabolites and waste products. Further studies need to be done to explore the

metabolic coefficient of grey carpet shark neonates, the movement and use of metabolites, and other internal resources. Additionally, further studies are required to identify which model most accurately reflects the intraspecific variation in metabolic scaling in grey carpet sharks. Conducting further studies on more extreme temperature treatments such as 36°C, as well as the use of an increased number of individuals at 24°C, could be expected to clearly elucidate whether thermal stress interferes with the metabolic scaling relationship between body mass and metabolic depression and TLRR.

Overall, I found that a five-day bout of cyclic thermal stress did not significantly affect the ability of grey carpet shark neonates to maintain its righting reflex during a hypoxic challenge. However, the neonates appeared to show a coping mechanism similar to the adult epaulette sharks (Routley *et al.*, 2002). The grey carpet shark neonates displayed a dramatic increase in ventilation initially, maintained it for an extended period of time before they displayed a gradual reduction in their ventilation rate. The unique mechanism that was displayed in the grey carpet sharks compared to the study on adult epaulette sharks, was the noted reduction of the use of buccal cavity musculature as the neonates decreased their ventilation rate.

In summary, five days of cyclic thermal stress post-hatching resulted in no variation in the morphological characteristics of the neonates up to 80 days post-hatching. The five days of cyclic thermal stress post-hatching did not significantly impact the ability of the neonates to tolerate hypoxia. While individuals exposed to the cyclic thermal stress showed no clear relationship between their TLRR and body mass, the control individuals did show a relationship. A noteworthy observation during the hypoxic exposure was the biphasic ventilation displayed by the grey carpet shark neonates, with an initial phase showing a dramatic increase of ventilation rate which was maintained for 20 minutes on average until the

neonates were no longer able to maintain the increased rate of buccal pumping. At this point, the neonates underwent ventilatory depression, displaying a gradual decrease in the rate of buccal pumping and a more restricted and reduced use of the total musculature of the buccal cavity. Even though my study provided a snap-shot of the potential effects of thermal stress on grey carpet shark neonates, it also shed light on the capacity of grey carpet shark neonates to adapt to the imminent environmental threats imposed due to global warming and spreading hypoxic zones. I recognize that the low sample size in my study and particularly in the control group could produce different results if the number of individuals was increased. Recent projections of future ocean temperatures indicate that there will be an increase in temperature of approximately 5 °C by 2100 (Cheng *et al.*, 2019; IPCC, 2019; IPCC, 2014). As such, future studies should continue exploring cyclic thermal exposure at higher temperatures (36 °C) to explore how grey carpet shark neonates will tolerate future temperatures.

APPENDIX 4.1.

General morphometric parameters measured of grey carpet shark neonates exposed to two different temperatures of 24 °C and 32 °C. Measurements were made approximately every 10- from day of hatching up to 80 days post-hatching.

	Days Post-Hatching	24°C	32°C	P value
Body Mass (g)	0	22.37 ± 1.17	20.3 ± 2.64	0.221
	10	20.26 ± 1.03	19.96 ± 1.36	0.729
	20	20.05 ± 1.72	19.83 ± 1.73	0.849
	30	22.46 ± 1.63	22.16 ± 1.56	0.786
	40	22.28 ± 2.54	24.11 ± 1.98	0.227
	50	27.72 ± 5.69	27.75 ± 3.93	0.952
	60	30.97 ± 3.99	32.00 ± 3.97	0.716
	70	35.87 ± 6.39	35.83 ± 4.35	0.980
	80	36.11 ± 2.52	38.31 ± 5.61	0.575
Total Length (cm)	0	18.06 ± 0.64	17.70 ± 0.51	0.501
	10	18.25 ± 0.31	18.29 ± 0.71	0.785
	20	18.38 ± 1.39	18.39 ± 0.50	0.648
	30	19.25 ± 0.51	19.13 ± 0.78	0.649
	40	19.23 ± 1.23	19.64 ± 0.98	0.724
	50	20.21 ± 1.19	20.19 ± 1.26	0.977
	60	20.59 ± 1.23	20.46 ± 0.95	0.982
	70	21.42 ± 2.08	21.30 ± 1.46	0.969
	80	22.5 ± 1.02	22.72 ± 0.94	0.802
Thoracic Length (cm)	0	3.10 ± 0.15	3.05 ± 0.19	0.557
	10	3.15 ± 0.04	3.19 ± 0.16	0.749
	20	3.02 ± 0.41	3.09 ± 0.23	0.819
	30	3.27 ± 0.09	3.31 ± 0.04	0.288
	40	3.32 ± 0.15	3.36 ± 0.13	0.707
	50	3.59 ± 0.23	3.51 ± 0.20	0.532
	60	3.64 ± 0.34	3.60 ± 0.12	0.903
	70	3.71 ± 0.10	3.70 ± 0.38	0.752
	80	3.78 ± 0.26	4.14 ± 0.28	0.516
Eye to Eye (cm)	0	1.35 ± 0.01	1.29 ± 0.05	0.100
	10	1.36 ± 0.01	1.38 ± 0.04	0.775
	20	1.37 ± 0.08	1.41 ± 0.06	0.433
	30	1.45 ± 0.03	1.42 ± 0.04	0.658
	40	1.42 ± 0.03	1.46 ± 0.03	0.296
	50	1.51 ± 0.03	1.48 ± 0.07	0.891
	60	1.50 ± 0.03	1.51 ± 0.05	0.561
	70	1.58 ± 0.05	1.55 ± 0.08	0.618
	80	1.61 ± 0.05	1.66 ± 0.12	0.902

Snout Length (cm)	0	1.60 ± 0.05	1.47 ± 0.17	0.591
	10	1.63 ± 0.04	1.63 ± 0.12	0.237
	20	1.58 ± 0.14	1.68 ± 0.11	**0.026***
	30	1.74 ± 0.05	1.69 ± 0.10	0.070
	40	1.67 ± 0.08	1.73 ± 0.06	**0.018***
	50	1.75 ± 0.07	1.74 ± 0.13	0.073
	60	1.73 ± 0.05	1.77 ± 0.08	0.056
	70	1.88 ± 0.11	1.82 ± 0.16	0.148
	80	1.81 ± 0.03	1.98 ± 0.24	0.189
Pectoral Fin Length (cm)	0	1.86 ± 0.03	1.94 ± 0.09	0.150
	10	1.87 ± 0.10	1.98 ± 0.13	0.325
	20	2.00 ± 0.13	1.93 ± 0.10	0.211
	30	1.99 ± 0.12	1.99 ± 0.07	0.175
	40	2.00 ± 0.15	2.09 ± .012	0.052
	50	2.16 ± 0.12	2.15 ± 0.17	0.259
	60	2.28 ± 0.18	2.32 ± 0.09	0.561
	70	2.28 ± 0.20	2.42 ± 0.18	0.415
	80	2.40 ± 0.17	2.71 ± 0.45	0.389
Pelvic Fin length (cm)	0	1.45 ± 0.05	1.59 ± 0.09	**0.019***
	10	1.52 ± 0.09	1.61 ± 0.12	0.078
	20	1.63 ± 0.02	1.59 ± 0.04	0.150
	30	1.64 ± 0.05	1.63 ± 0.10	0.070
	40	1.61 ± 0.13	1.68 ± 0.11	**0.039***
	50	1.62 ± 0.06	1.69 ± 0.08	**0.037***
	60	1.72 ± 0.11	1.77 ± 0.09	**0.037***
	70	1.76 ± 0.19	1.92 ± 0.20	0.123
	80	1.98 ± 0.17	2.06 ± 0.17	0.414
1st Dorsal Fin Span (cm)	0	1.53 ± 0.09	1.50 ± 0.15	0.726
	10	1.67 ± 0.21	1.68 ± 0.14	0.779
	20	1.78 ± 0.20	1.78 ± 0.15	0.463
	30	1.73 ± 0.10	1.73 ± 0.09	0.813
	40	1.65 ± 0.06	1.73 ± 0.09	0.663
	50	1.79 ± 0.21	1.78 ± 0.15	0.525
	60	1.84 ± 0.11	1.83 ± 0.15	0.719
	70	1.86 ± 0.21	1.88 ± 0.17	0.790
	80	2.02 ± 0.06	1.95 ± 0.23	0.786
2nd Dorsal Fin Span (cm)	0	1.46 ± 0.18	1.55 ± 0.10	0.324
	10	1.61 ± 0.06	1.64 ± 0.08	0.947
	20	1.63 ± 0.05	1.64 ± 0.16	0.668
	30	1.67 ± 0.08	1.67 ± 0.14	0.485
	40	1.64 ± 0.05	1.67 ± 0.06	0.117
	50	1.66 ± 0.10	1.77 ± 0.17	0.290
	60	1.70 ± 0.09	1.78 ± 0.15	0.485

	70	1.79 ± 0.07	1.78 ± 0.09	0.290
	80	1.80 ± 0.14	1.94 ± 0.21	0.137
Distance Between Dorsal Fins (cm)	0	2.03 ± 1.78	2.06 ± 0.23	0.878
	10	2.07 ± 0.12	2.19 ± 0.22	0.435
	20	2.20 ± 0.19	2.13 ± 0.16	0.944
	30	2.23 ± 0.13	2.28 ± 0.15	0.881
	40	2.28 ± 0.20	2.36 ± 0.21	0.728
	50	2.37 ± 0.31	2.40 ± 0.25	0.662
	60	2.46 ± 0.22	2.49 ± 0.23	0.584
	70	2.57 ± 0.37	2.68 ± 0.35	0.693
	80	2.77 ± 0.16	2.95 ± 0.35	0.791
Area of Pectoral Fin (cm)	0	2.22 ± 0.20	2.09 ± 0.14	0.298
	10	2.24 ± 0.24	2.34 ± 0.22	0.814
	20	2.26 ± 0.32	2.27 ± 0.13	0.808
	30	2.51 ± 0.01	2.33 ± 0.10	0.729
	40	2.35 ± 0.30	2.53 ± 0.19	0.853
	50	2.55 ± 0.22	2.55 ± 0.26	0.468
	60	2.62 ± 0.29	2.72 ± 0.21	0.299
	70	2.85 ± 0.39	2.91 ± 0.45	0.672
	80	3.06 ± 0.33	3.34 ± 0.50	0.263

Note: Data are shown as $\bar{X}$ ± s.d.

CHAPTER 5

General Conclusion

Investigating the response of developing embryos to cyclic thermal stress, which mirrors the stress experienced in their shallow marine environments, is necessary to be able to predict the survival of this species and its implications for other oviparous elasmobranchs. In a wider sense, it has been proposed that understanding the effects of climate change is critical to the continuation of organisms across the globe (Anderson and Podrabsky, 2014; Belkin, 2009; Kobayashi *et al.,* 2017; Turbill and Prior, 2016). The experimental design used during my thesis allowed me to examine the potential changes that tropical grey carpet shark embryos and neonates will encounter if the global water temperatures along with the concomitant hypoxia events continue to rise as predicted.

My research also used an innovative cyclic thermal stress protocol that mimics what occurs in the natural habitat of grey carpet sharks, which has not been previously explored. By using cyclic thermal stress, the results of my research provided insight into how the response of embryos could vary under cyclic thermal changes compared to constant thermal stress, which was previously explored (Rosa *et al.,* 2014). Using a constant thermal stress, there was a direct relationship between increased temperature and increased organismal mortality, as well as an accelerated rate of development in grey carpet sharks (Rosa *et al.,* 2014). However, this was not the case under cyclic thermal stress in my study. Instead, I found no difference in the mortality of embryos, and the rate of development was significantly slower in embryos exposed to cyclic thermal stress. The time at which some developmental milestones appeared was also significantly altered by the cyclic thermal stress, with the banding reaching the pectoral fins, the widening of the trunk region, and the internalization of yolk occurring significantly later in individuals exposed to the higher temperatures. Individuals exposed to the cyclic thermal stress (28°C) were significantly shorter in length, by on average 8.7 mm upon hatching. This

finding is in agreement with the discovery that elevated temperatures are resulting in smaller animals (Baudron *et al.,* 2014; Lema *et al.,* 2019; Messmer *et al.,* 2017).

Even though the findings of my study provide a preliminary understanding of how grey carpet shark embryos will tolerate cyclic thermal stress on the coral reefs of Heron Island, future studies should explore elevated temperatures predicted, such as 36°C, which would be more likely to elicit a greater level of embryonic disruption than those observed in my study. Because an increase in temperature is accompanied by a decrease in water dissolved oxygen (Jeffries *et al.,* 2018; Jiang *et al.,* 2019; Nilsson *et al.,* 2009), and because oxygen is very important during embryogenesis (Ciuhandu *et al.,* 2007; Wood *et al.,* 2019a), hypoxia should also be explored in a cyclic manner with and without an increase in temperature in order to assess its impacts on embryo development.

I developed (Chapter 2) a photographic atlas for the key milestones which can be used while the embryo is developing inside the egg case, without the need to open egg cases (interfering with development), or the need for sophisticated equipment and validated it's use in Chapter 3 a photographic atlas for the key milestones which can be used while the embryo is developing inside the egg case, without the need to open egg cases (interfering with development), or the need for sophisticated equipment. The creation of this atlas is highly valuable, as the previous developmental atlas for grey carpet sharks removed the embryos from their egg cases (Onimaru *et al.,* 2018). The strategy of removing embryos from their egg cases for observations is not always feasible, for example for threatened species and it interferes with the assessment of embryo mortality in response to an environmental stressor. In the developmental atlas, I was able to clearly identify 11 developmental stages, plus a stage zero, to identify fertile egg cases. These developmental stages were determined based upon the 39 developmental stages for this same species outlined previously by Onimaru *et al.* (2018), but

also using studies on the embryo development of other sharks (Ballard *et al*., 1993; Musa *et al.,* 2018; Musa *et al.*, 2020).

For the latter portion of my study (Chapter 4), the effect of both thermal stress and hypoxia on grey carpet shark neonates was explored, because while they are able to swim to escape stressors, they are still vulnerable to environmental stress. In this part of my study, the five days of thermal stress for 2 hrs day^{-1}, did not have any effect on the survival or overall body growth of the neonates between the two temperature treatments. However, those that were exposed to 5 days of thermal stress immediately after hatching did not significantly differ in their time to loss of righting reflex in hypoxia when compared to controls. Interestingly, while previous literature indicated that adult grey carpet sharks do not undergo an adaptive ventilatory depression during anoxic exposure (Chapman *et al.*, 2011), results show that neonates were able to undergo ventilatory depression during the hypoxic stress test. Interestingly, the adults maintained a high rate of ventilation during exposure (Chapman *et al.*, 2011), while the neonates in my study did not maintain the rate of ventilation and started to decline their activity level. When exploring the cause for the variation in the loss of the ability of the neonates to stay upright (time to loss of righting reflex; TLRR), the strongest predictor was the ratio between the first dorsal fin span and the total body length of the neonates. While morphological traits are generally not used to explain physiological responses (Renshaw Personal Communication), this relationship could be explained as an energetic benefit. A previous study on the white-spotted bamboo shark showed that the dorsal fins played a key role in generating thrust, which could be expected to reduce the amount of energy needed during escape attempts and exploratory behaviours observed during the hypoxic exposure (Maia and Wilga, 2013a; Maia and Wilga, 2013b; Maia and Wilga, 2016; Maia *et al.*, 2017). However, to confirm this relationship and theory, further studies would be needed. Such

studies would benefit from exploring the hydrodynamic function of the dorsal fins and the total length of grey carpet shark neonates and adults. It would be also beneficial to explore the metabolic variation during the steady swimming of individuals with varying dorsal fin length and total length.

Furthermore, it would be highly beneficial for future studies to explore how other environmental factors associated with global warming could impact embryo and neonate grey carpet sharks, and other reef sharks' development and growth. Some environmental factors of interest are salinity, anoxia, and pollution. Because there is great variation in inter- and intra-specific responses to different environmental factors, depending on the regions that organisms inhabit, and the extent to which environmental factors change, we should explore other species of sharks, species of skates and rays, and species of chimera. Lastly, it would also be beneficial to explore and identify coping mechanisms of the various species and various life stages to these different stressors, to gain an understanding of how the organisms are responding internally.

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