THE ENDOCRINE CONTROL OF EMBRYONIC DIAPAUSE IN THE AUSTRALIAN SHARPNOSE SHARK *Rhizoprionodon taylori*

Thesis submitted by
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ANIMAL ETHICS

This research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004 and the QLD Animal Care and Protection Ethics Committee Approval Number #A1508.

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Abstract

Embryonic diapause, the temporary suspension of development at any stage of embryogenesis, is a reproductive strategy widespread among all vertebrates, including elasmobranchs. Prolonging the gestation period is believed to be an adaptation to cope with unfavourable environmental conditions and therefore, allowing parturition to occur in conditions that are more suitable for newborns. Although it has only been confirmed in two elasmobranchs (*Rhizoprionodon taylori* and *Dasyatis say*), evidence suggests that at least 14 species of rays and two sharks undergo diapause, suggesting this form of reproduction exists within a wide range of reproductive modes, including lecithotrophs and matrotrophs. Where it has been studied, embryogenesis is arrested at the blastodisc stage and preserved in the uterus from four to ten months.

Endocrine systems have remained relatively conserved throughout the vertebrate evolutionary history. Therefore, current models on vertebrates in which diapause have been extensively studied provide the basis to formulate theories for the control mechanisms existent in elasmobranchs. In mammals and reptiles, the ovary represents an important source of steroid hormones for the regulation of reproduction. The corpora lutea is an indispensable gland that mainly produces progesterone (P_4), the key hormone associated with gestation in vertebrates. Similar to other taxa, the ovarian follicles of Chondrichthyes may give rise to secondary structures homologous to the corpora lutea by mean of atresia or ovulation. These follicles may be very similar, thus distinguishing these structures may be difficult and problematic, in part because of the confusing literature. Considering the importance of these temporary structures as possible sources of steroid hormones and their control of diapause, ovarian follicles and circulating hormone levels were investigated throughout the reproductive cycle of *R. taylori*.

Three secondary ovarian follicles occur in the ovary of *R. taylori* throughout the reproductive cycle. At late diapause, a new ovarian cycle is restarted and atresia may occur at early non-vitellogenic oocytes, giving rise to atretic previtellogenic follicles. During the diapausing period however, a number postovulatory follicles (POF) and usually only one vitellogenic atretic follicle (AF) compose the ovary during the diapausing period. These structures persist in the ovary from 7 to 10 months, however

their steroidogenic capacity and possible role in reproduction remain unknown. The macroscopic identification of AF and POF is only possible during part of embryonic diapause due to the relatively different sizes of these structures. However, extrapolation of these characteristics to identify AF and POF i other species is discouraged due to the high morphological variability among species.

This study is the first report of steroid hormones in a diapausing elasmobranch and therefore contributes to a better understanding of the endocrine control of reproductive processes within this group. Levels of 17ß-estradiol (E_2), testosterone (T) and P_4 are reported in plasma samples of wild female *R. taylori* captured throughout the reproductive cycle and correlated with internal morphological changes. Levels of P_4 and T were elevated through most of the embryonic diapause period, suggesting a role of these hormones in the maintenance of this condition. Increasing T plasma concentrations from late diapause to early active development were associated with a possible role of androgens in the termination of embryonic diapause. As in other elasmobranchs, a concomitant increase of E_2 with ovarian follicle size suggested a direct role of this hormone in regulating vitellogenesis. Significant correlations between photoperiod or water temperature and maximum follicular diameter and hepatosomatic index could suggest that these abiotic factors play a role triggering and regulating the synchrony and timing of reproductive events.

Chapter 1 General Introduction

Plate 1: Mature female Australian sharpnose shark *Rhizoprionodon taylori*

The class Chondrichthyes is a diverse group of cartilaginous fishes that has evolved from the extinct Placoderms over 400 million years ago. Chondrichthyes are believed to have arisen from the extinct Placoderms in an early offshoot of the vertebrate evolutionary tree in the late Silurian-lower Devonian (Carroll, 1988; Miller et al., 2003). This class, comprised of approximately 1200 extant species, is subdivided into two sister taxa: Elasmobranchii (sharks, rays and skates) and Holocephali (chimaeras). During their lengthy evolutionary history, Chondrichthyes have evolved numerous complex reproductive modes and mechanisms comparable to those of advanced tetrapods (Wourms, 1977; Carrier et al., 2004).

The modification of the male pelvic fins into claspers, male copulatory organs, early in the evolution of this group allowed for internal fertilization and the flexibility of reproductive modes, ranging from oviparity (egg laying) to viviparity (live bearing) (Carrier et al., 2004; Musick and Ellis, 2005). Reproductive modes can be divided in two main types according to the style of foetal nutrition: lecithotrophy (development of the embryo supported exclusively by the yolk), for example oviparity and yolk-sac viviparity; or matrotrophy (embryo development is supported completely or in parts by maternal input of nutrients), including histotrophy, oophagy and placental viviparity (Manire and Rasmussen, 1997; Grogan and Lund, 2004; Musick and Ellis, 2005).

While reproductive modes are conservative adaptations selected early in the species' history, numerous reproductive strategies more easily adaptable to environmental changes have evolved in Chondrichthyes and in vertebrates as a whole (Angelini and Ghiara, 1984). These strategies relate to the adaptation of efforts directed to reproduction in relation to external pressures, which may arise from abiotic (e.g. photoperiod and temperature) and/or biotic factors (e.g. predators or interspecific competition) (Angelini and Ghiara, 1984). Numerous external pressures may be responsible for the temporal pattern of breeding of a species. For example, reproductive synchrony, a mechanism that maximizes reproductive effort, is an adaptive response of individuals selecting the most favourable time of the year to reproduce and increase mating success. This seasonal pattern of breeding typically results in a cluster of births occurring over a short period of the year (Angelini and Ghiara, 1984; Ims, 1990; Bradshaw and Holzapfel, 2007). The majority of viviparous Chondrichthyes are seasonal breeders commonly displaying this same pattern, where near synchronized mating, gestation and parturition is a common

feature (Lutton et al., 2005). As in vertebrates, it is believed that seasonal chondrichthyans have reproductive cycles closely tuned to certain ecological factors (e.g. temperature or relative abundance of prey for the neonate) in order to synchronize the parturition events with the best conditions for the survival of the young (Hamlett and Koob, 1999).

The timing of reproductive events is attributed to a sophisticated endocrine system capable of identifying changes in the cyclic behaviour of environmental events (Heldmaier et al., 1989; Norris, 2007). As in all other vertebrates, reproduction in Chondrichthyes is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The perception of natural changes in environmental factors (e.g. temperature, photoperiod) and the presence of suitable breeding sites (e.g. resource availability, presence of predators) are perceived by the nervous system and HPG axis that regulates secretion of steroid hormones and other bioregulators (Gelsleichter, 2004; Norris, 2007). In turn, these secretions, or their absence, induce or promote reproductive events such as ovulation, mating, embryonic growth and parturition. During unfavourable periods, however several mechanisms have evolved to block reproductive processes, avoiding energy waste. Different strategies evolved to optimize reproductive effort are widespread among seasonal breeders and include sperm storage, delayed fertilization and embryonic diapause (Angelini and Ghiara, 1984).

Early reproductive studies in Chondrichthyes have drawn attention to the similarities in reproductive endocrinology, which closely resembles that of higher vertebrates (Wourms, 1977; Dodd, 1983; Callard et al., 1989). It appears that a number of primary regulatory hormones were first established in the elasmobranchs and retained throughout evolution in vertebrates (Gelsleichter, 2004; Callard et al., 2005). Therefore, due to their phylogenetic position at the base of vertebrate endocrine system evolution, studies on the Chondrichthyes' endocrine control of reproduction can provide a better understanding of the hormonal control in higher vertebrates (Dodd, 1972b; Callard et al., 1989; Gelsleichter, 2004). However, much less is known about the endocrine regulation of the chondrichthyan biology than most other vertebrate groups (e.g. mammals, teleosts and reptiles; Callard et al., 2005; Norris, 2007).

Numerous recent studies have helped identify the general roles that ovarian steroids play in regulating reproduction. Current knowledge is largely based on inferences from studies undertaken in a small number of placental viviparous (e.g. Rasmussen and Gruber, 1993; Manire et al., 1995), aplacental viviparous (e.g. Tsang and Callard, 1987b; Snelson et al., 1997; Mull et al., 2010) and oviparous elasmobranch species (e.g. Koob et al., 1986; Sulikowski et al., 2004; Awruch et al., 2008b). However, less common reproductive strategies that separate events otherwise occurring very close together have been shown to provide a unique opportunity to assess hormonal changes separately. In the bonnethead shark *Sphyrna tiburo* hormone variations could be associated to mating and ovulation individually due to prolonged sperm storage (five months; Manire et al., 1995). Similarly, embryonic diapause (a temporary arrest or retardation of embryonic development) may provide a better insight into the control of gestation and embryonic development in this group because it separates the events of fertilization and embryonic development. However, endocrine studies have never been undertaken in a cartilaginous diapausing fish.

Embryonic diapause is widespread in vertebrates of unrelated taxa (Podrabsky and Hand, 1999; Desmarais et al., 2004; Ewert, 2004) including elasmobranchs (Lessa, 1982; Simpfendorfer, 1992) in which this trait has been poorly studied. The endocrine control of embryonic diapause has been extensively studied in tetrapods, especially mammals and reptiles (Ewert, 2004; Ptak et al., 2012). Where it has been studied, the control of diapause and gestation is closely associated with levels of progesterone. This steroid hormone, mainly produced by a temporary endocrine gland in the ovary (corpora lutea), is essential for the normal embryonic development and therefore, diapause occurs when P_4 synthesis is reduced or suppressed (Lopes et al., 2004; Murphy, 2012). The presence and steroidogenic capacity of structures homologous to the corpora lutea (atretic and postovulatory follicles) have been widely reported within matrotrophic and lecithotrophic elasmobranchs (e.g. Chieffi, 1962; Lance and Callard, 1969; Tewinkel, 1972). Due to the similarities observed in the reproductive endocrinology across all vertebrate taxa, current knowledge in better-studied vertebrates can provide clues to understand the processes of endocrine control of diapause in elasmobranch species.

Rhizoprionodon taylori and embryonic diapause

The Australian sharpnose shark *R. taylori* is endemic to the tropical inshore waters of northern Australia and southern Papua New Guinea (Compagno et al., 2005; Last and Stevens, 2009), and is one of the most abundant small elasmobranch in the near shore regions of Queensland (Simpfendorfer, 1993a; Harry et al., 2011). This small carcharhinid (maximum length of males 690 mm, and females 790 mm) reaches maturity at one year of age (males 560 and females 575 mm), during which animals grow 140% the size of birth (Simpfendorfer, 1993a). Females annually produce litters ranging from 1 – 10 after a gestation period of 11.5 months. However, unlike other Carcharhiniformes, embryonic development is halted soon after fertilization, at the blastodisc stage. The gestation in this species is divided into a long period of embryonic diapause (7 months) followed by a relatively short active gestation (4.5 months) (Simpfendorfer, 1992). The embryonic source of nutrients during this developmental phase varies from yolk and uterine secretions at the release from embryonic diapause, followed by the formation of placenta during the second half of active gestation (Simpfendorfer, 1993b).

R. taylori was the first elasmobranch in which diapause was confirmed (Simpfendorfer, 1992). The lack of macroscopic development of uterine ova for extended periods is common to a number of elasmobranchs and it suggests a period of embryonic diapause. However, the confirmation of this trait still remains largely uninvestigated. The exceptions are *R. taylori* and the bluntnose stingray *D. say*, where diapause has been confirmed through microscopy (Simpfendorfer, 1992; Morris, 1999). Considering the relative abundance of a diapausing species in coastal environments throughout the year, and the need for more endocrine studies in chondrichthyans, *R. taylori* provides an excellent opportunity to investigate the endocrine control of embryonic diapause. Furthermore, the separation of fertilization from active embryonic development allows the association of hormonal variation to each independent event and therefore possibly allowing a better comprehension of the control of the reproductive cycle as a whole.

1.1. Project structure and objectives

This study aimed to better understand the mechanisms underlying endocrine control of embryonic diapause in the Australian sharpnose shark *R. taylori* using four approaches: (1) review diapause in elasmobranchs and construct hypothesis on how it is controlled based on knowledge from other vertebrate taxa; (2) examine fluctuations in reproductive

hormone levels throughout the reproductive cycle (including diapause); (3) identify and describe the morphology of ovarian follicles that may contribute to the supply of endocrine products; and (4) correlate morphological gonadal development with plasma levels of gonadal steroids.

1.2. Thesis outline

Chapter 2 is a literature review of embryonic diapause in elasmobranchs that develops theories that are systematically tested in Chapter 3 and Chapter 4.

Chapter 3 briefly summarizes the literature on elasmobranch atretic and postovulatory follicles before addressing the identification these temporary structures in the ovary of *R. taylori*.

Chapter 4 examines, for the first time, the circulating levels of reproductive hormones in the diapausing *R. taylori*. Variations in annual profile of these hormones are correlated with changing morphology and environmental factors to determine the possible mechanisms controlling the embryonic diapause in this specie.

Chapter 5 synthesises the findings of the previous chapters, and indicates where progress has been made, what has been learned, and where future research needs to occur.

Chapter 2 Embryonic diapause in the elasmobranchs

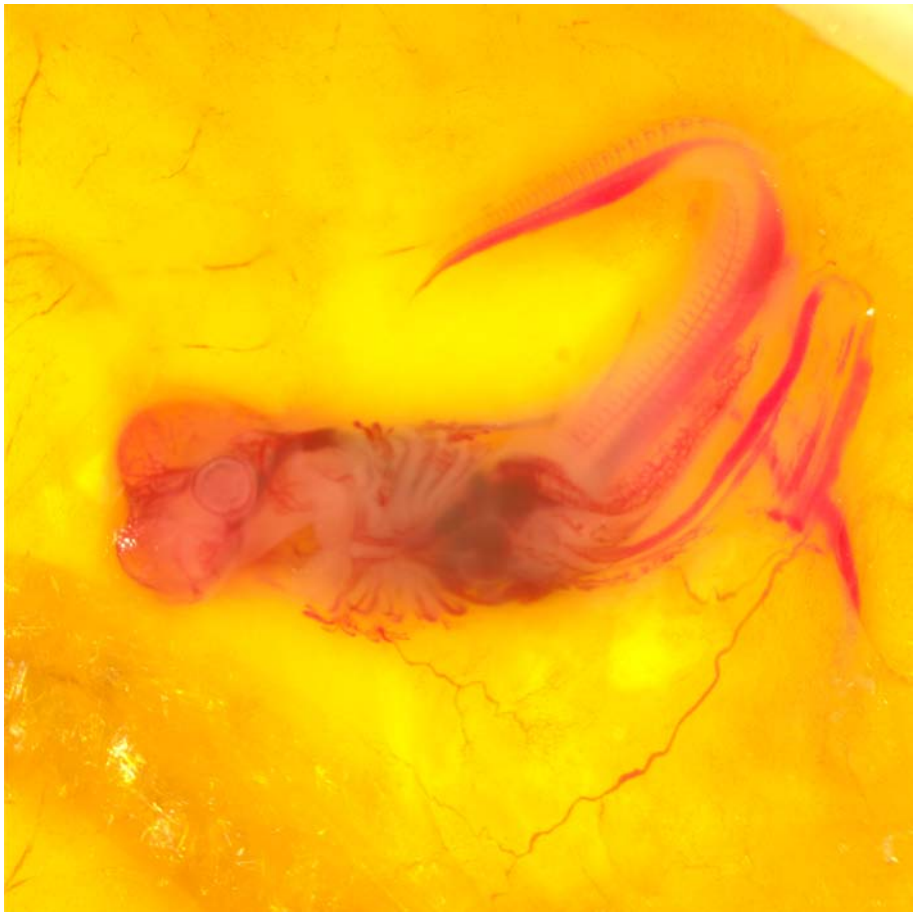

Plate 2: Actively developing embryo (Stage 1) of *Rhizoprionodon taylori* after 7 months of embryonic diapause

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2.1. Introduction

Elasmobranchs are a successful group of fishes that have persisted in the world's oceans over hundreds of millions of years (Grogan and Lund, 2004), proving resilient to waves of extinction that saw many other groups vanished (Carroll, 1988). One reason suggested for the resilience of this group over this long time period is the diversity of their reproductive modes (Carrier et al., 2004). Within the approximately 1100 extant species in the group there are two forms of egg-laying (oviparity) and six forms of live-bearing (viviparity) (Hamlett and Koob, 1999; Carrier et al., 2004; Musick and Ellis, 2005), which are estimated to have evolved on six independent occasions from oviparity (Musick and Ellis, 2005). This diversity of reproductive modes and the large number of occasions on which some have arisen through evolutionary time suggests that the elasmobranch reproductive system is highly adaptable and that novel reproductive specialisations may still await discovery within this taxa.

One reproductive specialisation that has been observed in a small number of viviparous elasmobranchs is embryonic diapause (Simpfendorfer, 1992; Wyffels, 2009), the temporary ceasing or retardation of development during any stage of embryogenesis, also known as discontinuous development or embryonic quiescence (Mead, 1993; Renfree and Shaw, 2000). Within the elasmobranchs it was first hypothesised to occur in the Brazilian shovelnose shark *Rhinobatos horkelli* (Lessa, 1982), and first conclusively demonstrated to occur in the Australian sharpnose shark *Rhizoprionodon taylori* (Simpfendorfer, 1992). However, despite the knowledge of this phenomenon over several decades, it remains poorly identified and studied. The purpose of this review was to synthesise information available on the occurrence of embryonic diapause in elasmobranchs, examine the implications of diapause for life histories, construct a hypothesis of how it is controlled, and identify future areas where research is needed to improve the base of knowledge.

2.2. Embryonic diapause in vertebrates

Diapause has been reported in all vertebrate classes in a number of species within unrelated taxa, *e.g.* mammalia (Desmarais et al., 2004), reptilia (Jones and Guillette, 1982; Ewert, 2004), Osteichthyes (Podrabsky and Hand, 1999) and Chondrichthyes

(Simpfendorfer, 1992; Wyffels, 2009). This wide distribution amongst disparate taxa implies that embryonic diapause originated independently within each group during evolution (Mead, 1993; Lopes et al., 2004) and suggests it is an advantageous strategy across a wide range of species and life histories, and that the broader vertebrate reproductive system must be amenable to allow this adaptation to readily evolve.

Embryonic diapause has been best studied in mammals, and much of the information about diapause comes from this taxa. Within mammals, delayed implantation occurs when embryonic development is arrested prior to implantation of the embryo into the uterine wall (Mead, 1993) and has been classified in two separate types. Firstly, facultative diapause which is dependent on seasonal changes in the environment (*e.g.* food availability) and external stimuli (*e.g*. stress of lactation, deprivation of food and/or water) that may cause the onset of diapause, and thus results in a diapause period of variable length. In mammals, this trait is known to occur in rodents, insectivores and marsupials (Mead, 1993; Lopes et al., 2004). The second form is obligate diapause, which occurs regularly at a specific stage of development and for a set period in every generation of a given species in spite of environmental conditions. This type of diapause is observed in species from a number of mammalian groups including mustelids, bears, pinnipeds, marsupials and some bats (Bleier, 1971; Mead, 1993; Lopes et al., 2004).

Reptiles are the only egg layers within the higher vertebrates (mammals, reptiles and birds) to display embryonic diapause. Reptilian diapause occurs in several viviparous lizards (Chamaleonidae and Gekkonidae) and freshwater turtles in which development is arrested at gastrula stage (Ewert, 2004). Diapause may occur prior to oviposition (Shanbhag et al., 2003), subsequent to oviposition (Kennett et al., 1993; Andrews, 2005) or even remain in diapause from pre-oviposition until after eggs have been laid (Booth, 2002). In addition, some viviparous lizards prolong gestation after the completion of embryonic development, possibly awaiting better environmental conditions to guarantee the survival of the young (Cree and Guillette, 1995; Girling et al., 2002; Atkins et al., 2007). Within this group, external stimuli, especially temperature and rainfall, play an essential role in the onset, maintenance and termination of diapause (Andrews, 2005).

Embryonic diapause also occurs in some bony fishes with annual life cycles inhabiting temporary aquatic habitats, such as numerous species of killifish (Cyprinodontiformes)

(Wourms, 1972; Podrabsky et al., 2010). Seasonal drying of these habitats kills all adult and juvenile forms and, until the next rainy season, the population exists only in the egg stage, which undergoes diapause while embedded in the sediment (Wourms, 1972; Hand and Podrabsky, 2000). The hatching of the eggs occurs several months later, on the return of the rainy season, when embryos are released from diapause and the life cycle proceeds (Wourms, 1972; Mead, 1993; Hand and Podrabsky, 2000). The type of arrest may be facultative or obligate, depending on the fish genera and stage of development (Wourms, 1972). The mechanisms controlling diapause are understudied in this group but may involve several environmental cues in addition to the presence of water, including photoperiod (Podrabsky and Hand, 1999) and temperature (Matias and Markofsky, 1978; Podrabsky and Hand, 1999).

2.3. Embryonic diapause in elasmobranchs

Rhinobatos horkelli was the first elasmobranch species described with embryonic diapause as a possible reproductive strategy (Lessa, 1982). Studying the embryonic development of this species, the author divided the gestation cycle in two distinct stages: a period of lethargy which extends over nine months after fertilization of the eggs, where no macroscopic signs were observed for development of the uterine eggs (the embryonic diapause period); and a second period of three months of normal embryonic development during the summer. The author hypothesised that, although it was the first report of embryonic diapause in an elasmobranch, this phenomenon could not be exclusive to *R. horkelli* amongst this group. This conclusion was corroborated by subsequent studies by Simpfendorfer (1992) and Morris (1999) in which diapause has been positively identified in *R. taylori* and *Dasyatis say* respectively.

Embryonic diapause in the elasmobranchs has been identified by the occurrence of fertilized eggs without visible embryos during extended periods (months) within populations with synchronous seasonal reproductive cycles (Wyffels, 2009). Techniques such as histology and scanning electron microscopy have been employed to demonstrate the existence of diapausing elasmobranch embryos and the stage of their arrest (Simpfendorfer, 1992; Morris, 1999; Wyffels, 2009). For species where these techniques have not been employed the presence of fertilized eggs in the uteri for extended time periods (Figure 2.1) has been used to indicate the likely existence of diapause. This

phenomena, however, has not yet been found in asynchronous species, and would presumably be more difficult to determine due to the inconsistent embryonic developmental stages among specimens at each given time of the year, which could potentially obscure long periods of arrested development. The assessment of diapause in these species would be possible through serial assessment of individuals throughout pregnancy, perhaps using a non-destructive method such as ultrasonography (Carrier et al., 2003; Daly et al., 2007). Nevertheless, the likelihood of an asynchronous species having diapause is presumably low given that diapause would take away any seasonal advantages that asynchronous reproduction may provide to the species.

Obligate embryonic diapause has only been confirmed in two species of elasmobranch, but there is evidence for its occurrence in as many as 16 species: 14 batoids from two orders and five families, and two selachians from two orders and two families (Table 2.1). There is no evidence for facultative embryonic diapause in elasmobranchs. Simpfendorfer (1992) and Morris (1999) used histology to confirm diapause in *R taylori* and *D. say*, respectively. In both species, development was arrested at the blastodisc stage (Figure 2.2) for a period of approximately nine (*D. say*) and seven (*R. taylori*) months before a short period of active development. Wyfells (2009) also confirmed diapause in *D. say* using scanning electron microscopy.

Diapause is spread throughout elasmobranch taxa and can exist in a wide variety of reproductive modes. All elasmobranchs with embryonic diapause (confirmed or with strong evidence of its occurrence) are lecithotrophic (e.g. *R. horkelli*, *Trygonorrhina dumerilii*) or matrotrophic aplacental species (*e.g. D. say*, *Trygonoptera imitata*). The only exception is *R. taylori*, which is matrotrophic placental. The reproductive cycle within all diapausing species lasts approximately 12 months, except for *Pristiophorus nudipinnis*, which has a two-year cycle (Walker and Hudson, 1999).

Since embryonic diapause is not a rule among all members of the same taxa, the reproductive trait has likely evolved independently in each species. There are at least two possible exceptions to this. Firstly, embryonic diapause occurs in at least six species of the genus *Rhinobatos* (Table 2.1), suggesting that it may have been an ancestral condition within the genus. However, it does not occur in all species in this genus, which may be due to loss of diapause in some branches. However, the genus is undergoing taxonomic

revision, so the phylogeny of the species currently in this genus is uncertain. Specifically, embryonic diapause has not been reported in species from the genus *Glaucostegus* that now contains some of the species that were formally part of *Rhinobatos* (Last and Stevens, 2009). If embryonic diapause has evolved separately within each of the rhinobatids then this genus would appear to have a pre-disposition to easily evolving this trait. The second possible exception is within the two species of *Dasyatis.* Both *D. brevis* (Melendez, 1997) and *D. say* (Morris, 1999) have been reported to employ embryonic diapause. These are sister species that are believed to have separated after the formation of the Isthmus of Panama 3 million years ago (Rosenberger, 2001), suggesting that embryonic diapause was an ancestral trait that has existed more than 3 million years.

Ovulation and fertilization in elasmobranch species often take place immediately after mating (Callard et al., 2005). It is well documented in viviparous species that**,** as the oocytes pass through the oviducal gland and into the uterus, the eggs are fertilized and then enclosed in a membranous egg case (Wourms, 1977; Carrier et al., 2004). Where it has been examined in diapausing elasmobranchs, embryogenesis is arrested at the blastodisc stage and preserved in the uterus for periods from four to ten months (Table 2.1). The ovulated oocytes are encased in a gelatinous olive-green (Abdel-Aziz et al., 1993), delicate brown membranous (White et al., 2002; Marshall et al., 2007; Kume et al., 2009; Trinnie et al., 2009) or keratinized case (Marshall et al., 2007) assumed to be impervious to sperm (White et al., 2002) due to its thick, multilayered nature and the absence of pores and channels (Morris, 1999), indicating that delayed fertilization is not a possible explanation for the observed state of the uterine eggs. Generally speaking, embryonic diapause in elasmobranchs lasts for longer periods than the active development of the embryos. Within the species examined, embryonic diapause lasts up to five times longer than active development in at least 10 species, however the opposite has been observed in three species where diapause is up to two times shorter than active development (Table 2.1). Embryonic diapause in elasmobranchs has independently evolved as an obligate state in a number of species with a range of reproductive strategies, suggesting that its control may occur via a number of different mechanisms.

2.4. Possible control mechanisms in elasmobranchs

The arrest of embryonic development within distinct groups of animals occurs in three phases: (1) entry into diapause and the arrest of cell division (2) maintenance and (3) reactivation of development after the diapausing period (Renfree and Shaw, 2000). Embryonic diapause starts when environmental conditions are optimal and would provide normal metabolism and development; in general, it precedes the commencement of an environmental change such as winter or the dry season (Hand and Podrabsky, 2000). Environmental cues provide stimuli to arrest and restart the embryonic development in diapausing species, however, these factors are species-specific and vary widely (Morris, 1999).

While the control mechanisms of embryonic diapause within elasmobranchs remain uninvestigated, other vertebrate taxa in which diapause has been further studied provide clues to understand the process in this group. Chang (1968) provided evidence that the uterine milieu, and not the embryo genetic programming, is the key to maintain mammalian diapause. In his experiments, reciprocal egg transfer between ferrets (non-diapausing) and minks (diapausing) resulted in the delayed implantation of the ferrets but not the minks. Similarly, dormant blastocysts of rodents become active when transferred into an active uterus (Dickmann and De Feo, 1967).

Although the control of embryonic diapause in mammals is under maternal control (Lopes et al., 2004), the endocrine mechanisms vary widely among species. The main hormones associated with the termination of diapause in several species are prolactin and progesterone. Pituitary secretions of prolactin terminate diapause and induce implantation in the mink (Fasano et al., 1989; Henningsen et al., 2008), however a single injection of cabergoline, a synthetic dopamine agonist that suppresses prolactin secretion, is sufficient to induce the termination of diapause and implantation in tammars (Hearn et al., 1998). Progesterone, on the other hand, is required for successful implantation in ferrets (Manire et al., 2004), mink (McMillan, 2007), and the Australian sea lion (Podrabsky et al., 2010).

Secretions of prolactin and progesterone are closely related. Prolactin secretions act to restrain the growth and secretions of the corpora lutea (Fasano et al., 1989; Sorbera and Callard, 1995), a temporary endocrine gland source of steroid hormones, mainly

progesterone (Mead, 1993; Koob and Callard, 1999; Martin and Ferreira, 2009). Although the presence of prolactin is known in elasmobranchs (Henningsen, 1999; Claes and Mallefet, 2009; Claes and Mallefet, 2010), its roles remain unknown within this group.

The elasmobranch ovary (Gelsleichter, 2004) and corpora lutea (Tsang and Callard, 1987a) have been described as a source of progesterone. Surges of this steroid in viviparous female elasmobranchs have been associated with the inhibition of follicular development (Koob and Callard, 1999; Gelsleichter, 2004; Mull et al., 2010) and the sexual maturation process (Rasmussen and Gruber, 1993). In pregnant animals it appears to be important for implantation (Manire et al., 1995; Sorbera and Callard, 1995), inhibition of myometrial contractions – maintaining a quiescent uterus in early pregnancy – (Sorbera and Callard, 1995) and parturition events (Snelson et al., 1997). Although levels of progesterone and other steroids have not been studied throughout the reproductive cycle of any diapausing elasmobranch, the presence of corpora lutea during the early stages of pregnancy suggests that progesterone affects the control of this reproductive trait (Dodd, 1972b; Tsang and Callard, 1987a).

Assuming that hormonal control maintains elasmobranch embryos in diapause, Simpfendorfer (1993b) reported that *R. taylori* possessed large and active corpora lutea in ovaries during the diapause period. However, the presence of corpora lutea have not been reported for other diapausing elasmobranchs such as *D. say* (Snelson et al., 1989; Morris, 1999), suggesting the potential for the existence of multiple control mechanisms among different elasmobranchs or simply that they were present but not observed.

With the knowledge of observed reproductive structures in *R. taylori* and the presumption that corpora lutea and progesterone are directly involved in the control of embryonic diapause, we propose a hypothesis for the control mechanisms in this species (Figure 2.3). The presence of large and active corpora lutea during the early stages of pregnancy suggests that progesterone is required to arrest embryonic development. The size of this temporary gland is substantially reduced during the first month of diapause and gradually degenerates until the end of this stage, which is possibly associated with a decline in progesterone. However, a second peak in progesterone levels is expected towards the end of diapause in order to induce the active development of the young and

induce implantation. At this stage, it is likely that the uterus becomes the main source of this steroid, since the corpora lutea is degenerating and its size very reduced or absent.

External stimuli such as photoperiod and temperature play an important role in the onset and termination of diapause in mammals, reptiles and bony fishes (Wourms, 1972; Hand and Podrabsky, 2000; Renfree and Shaw, 2000; Lopes et al., 2004); changes in key environmental components may trigger endocrine reactions in the mother that will control the embryonic development (Lopes et al., 2004; Wyffels, 2009). Amongst the diapausing sharks and rays, embryogenesis is usually arrested during late summer or autumn and terminated in the following spring or summer (e.g. Simpfendorfer, 1992; Melendez, 1997; Yamaguchi, 2006; Kume et al., 2009), thus displaying a fast active development when temperatures are higher. As in other taxa, it is likely that environmental changes, such as day length (Renfree and Shaw, 2000), play a role in the timing of diapause events acting as a trigger to the mothers endocrine system that will control embryonic diapause in elasmobranchs.

2.5. Benefits of diapause for elasmobranchs

Embryonic diapause allows the time between fertilization and parturition to be prolonged, possibly providing newborns with higher quality environmental conditions at the start of life (Mead, 1993; Renfree and Shaw, 2000). It was suggested by Simpfendorfer (1992) that diapause allows *R. taylori* to be born when sea temperature is at the highest, so higher growth rates can be achieved, reducing the risk of predation (Branstetter, 1990). Conversely, parturition in *Trygonoptera personata* (White et al., 2002) and *T. dumerilii* (Marshall et al., 2007) occurs when sea temperature is declining towards its minimum, when the neonates' main food items are abundant and there might be less competition for food or space, as well as lower risk of predation (Marshall et al., 2007). Independent of the season of the year it may be that embryonic diapause has evolved as a mechanism that guarantees that parturition will occur when newborns are more likely to succeed through the early stages of life and reach maturity. While these authors have speculated on various reasons for diapause in elasmobranchs, there has been no rigorous testing of these hypotheses.

Embryonic diapause in *R. taylori* has possibly evolved along with a reduction in the size at birth and a relative increase in litter size (Simpfendorfer, 1992). In essence, a trade off between the size at birth and the litter size – probably imposed by the restricted space within the mother during internal development – could have allowed a reduction in the developmental time and thus allowing for a period of embryonic diapause. Simpfendorfer (1992) found evidence of other possible advantages of diapause by looking at trends in litter size and birth size of carcharhinid sharks (Figure 2.4). *R. taylori* appeared to be an outlier among the family; young were proportionally smaller, and litter size was proportionally higher than average. Embryonic diapause may therefore relax some of the constraints of the elasmobranch reproductive system and allow species to pursue alternative strategies. However, this pattern does not match that observed in *T. personata* (White et al., 2002), as this species gives birth to a single and relatively large young. Therefore, it is possible that this species has evolved a larger young, as opposed to large litter size, in order to reduce predation.

Hypotheses in the literature have looked at embryonic diapause from the aspect of how the timing of birth will benefit the young (e.g. more food and less predators; Marshall et al., 2007) and sometimes in terms of life history traits (e.g. increased litter size in the *R. taylori*; Simpfendorfer, 1992). However, the evolution of a period of embryonic diapause in elasmobranchs from the perspective of mothers' physiology has not yet been considered. It is important for females to have the breeding cycle at a favourable time of the year (Sandell, 1990); a delay in the embryonic development could possibly allow females to restore /meet their energy requirements after events of birth, ovulation and mating. Sandell (1990) hypothesises that a delay in seasonally breading mammals has evolved to maximize male competition or female choice. In this hypothesis, mating season would coincide with a seasonal gathering of otherwise vagrant females and at a time with high quality resources easily available. In certain situations, these conditions are met by mating early and a delay in development would increase female fitness (Sandell, 1990).

Simpfendorfer (1992) proposed that other alternate strategies adopted by viviparous elasmobranchs are functionally similar to embryonic diapause (i.e. they provide a shorter development period in a annual reproductive cycle). Delayed fertilization (e.g. *Chiloscyllium plagiosum*; Chen and Liu, 2006) and reduced gestation period (e.g.

S. tiburo; Manire et al., 1995) respectively, separate mating from the beginning of embryogenesis and parturition from mating. The reasons why embryonic diapause have evolved as opposed to other alternate modes is unknown. It is possible that diapause is involved with the timing of these events (embryogenesis, mating and parturition) and provides some behavioural or physiological advantages for individuals, especially females. In some species, aggregation of males and females only occurs during short periods of mating events and embryonic diapause could have evolved to extend gestation, allowing parturition and mating to be synchronized (Kyne and Bennett, 2002). However, results reported by Kume et al. (2009) do not provide any evidence of sex segregation in the population of *Rhinobatos hinnycephalus* in Japan. Although many hypotheses have been raised regarding the benefits a temporary arrest of embryonic development might confer to the species, none of them has been tested. Moreover, limited information makes it difficult to evaluate how this trait actually benefits the species in which it occurs.

2.6. Future research directions

Current hypotheses on why embryonic diapause has evolved and what determines the optimal timing for reproduction require rigorous testing while the life-history parameters remain to be determined. Since the advantages of diapause are difficult to pinpoint, and likely to be species specific, there is also the need to better understand the basic biology of these animals. A number of sharks and rays have been described with this reproductive trait, however further studies are needed to confirm the absence of embryonic development during extended diapause periods. Very little is known about the control mechanisms of diapausing species. The correlation of the levels of circulating hormones and reproductive structure throughout the reproductive cycle will provide a better understanding of this trait among elasmobranchs.

A complete understanding of reproductive events is crucial to assist in the formulation of management strategies, therefore such knowledge will be particularly important for species taken in fisheries (e.g. *Rhinobatos horkelii*, IBAMA, 2008; *P. nudipinnis* and *Trygonoptera fasciata*, Bensley et al., 2009; *R. taylori*, Harry et al., 2011). Moreover, understanding the benefits and uses of each reproductive trait is an important step towards a more realistic evaluation of elasmobranch populations.

2.7. Conclusion

Embryonic diapause is a reproductive strategy widespread among vertebrate taxa and adopted by at least two species of elasmobranchs, and likely present in at least a fourteen others. The reproductive cycles and the mechanisms involved in the control of the reproductive trait are poorly understood in elasmobranchs. However, as in other vertebrates, it appears that by delaying development and extending the gestation period, diapause allows the young to be born in the most favourable conditions for survival or enhances reproductive output. It is also possible that a period of embryonic diapause will benefit species by allowing reproductive events to be synchronized (parturition and mating) and/or prolonged (fertilization and parturition). There are still many questions that remain unanswered concerning the knowledge on the biology of most diapausing species but it is clear that species benefit differently from this reproductive trait. As in other vertebrates, it is likely that environmental cues and hormones (especially progesterone and prolactin) are involved in the control of diapause in elasmobranchs, however rigorous testing of current hypothesis remains to be carried out.

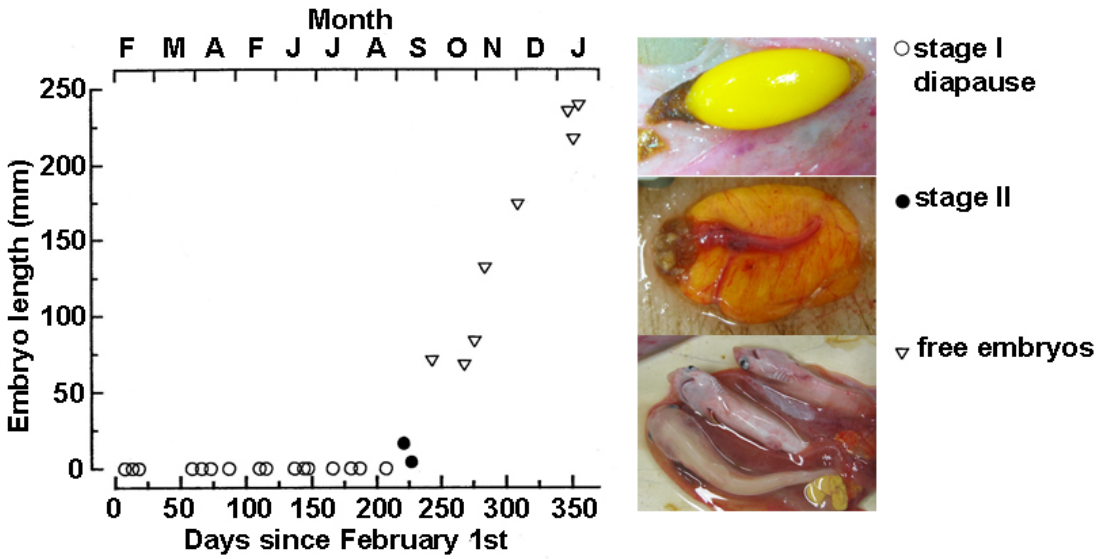

Figure 2.1: Relationship between embryo length and gestation time of Australian sharpnose shark *Rhizoprionodon taylori*. The stages of embryonic development are indicated as: stage I - diapausing fecundated oocytes; stage II - macroscopically visible embryo; and free embryos within the mother's uterus (adapted from Simpfendorfer, 1992)

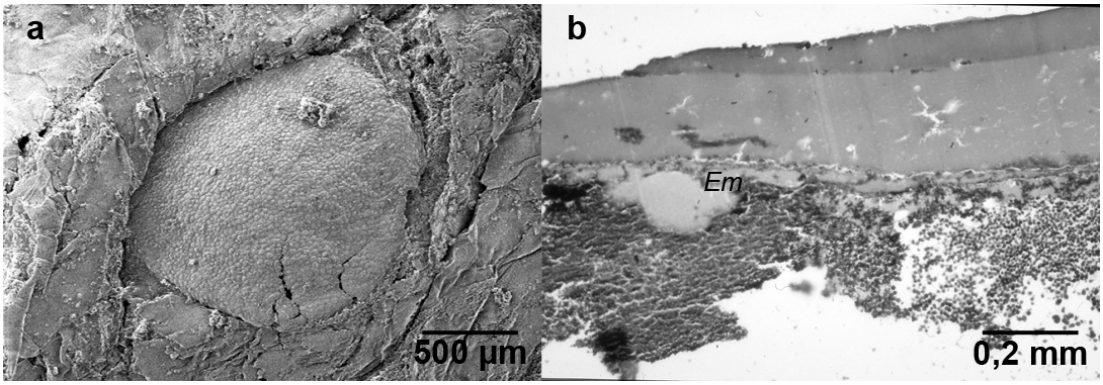

Figure 2.2: Sections through the diapausing blastodiscs of *Dasyatis say* (a) and *Rhizoprionodon taylori* (b). Unpublished photos by Wyffels (a) and Simpfendorfer (b)

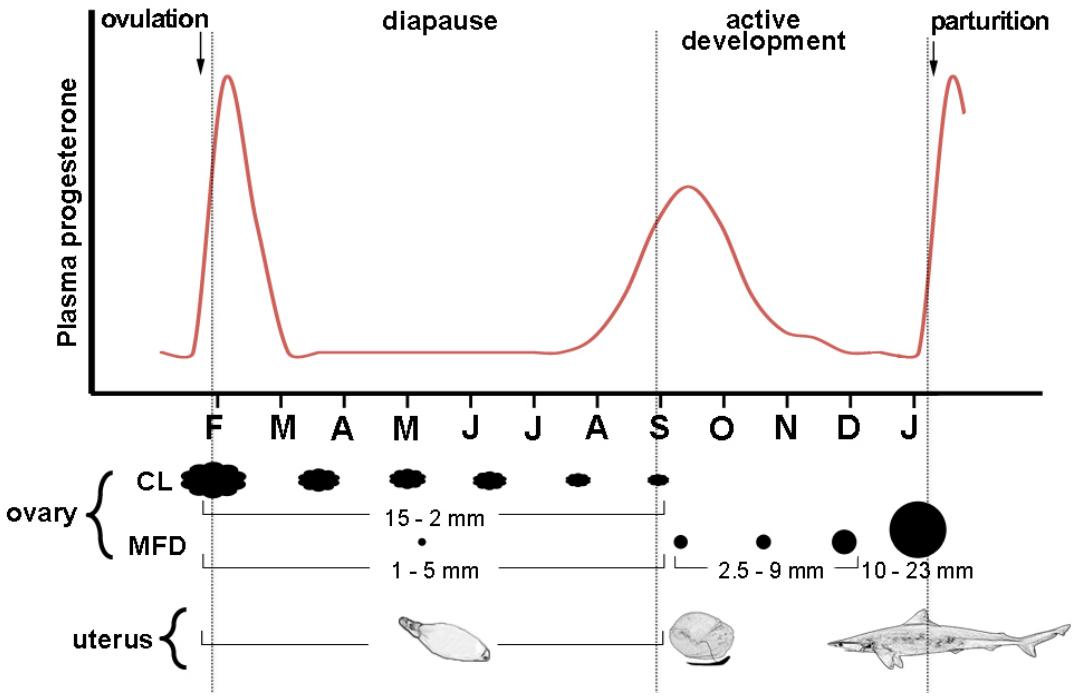

Figure 2.3: Proposed circulating levels of progesterone throughout the reproductive cycle of a female *Rhizoprionodon taylori*. Corpora lutea (CL) and the maximum follicular diameter (MFD) in the ovary and the embryonic stages of development are represented as observed by Simpfendorfer (1992) and Waltrick (unpublished data)

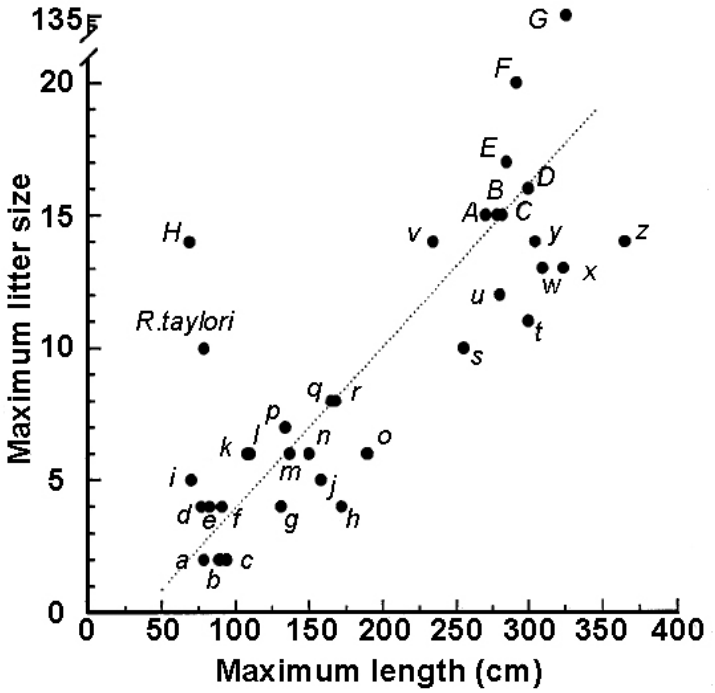

Figure 2.4: Relationship between maximum length and maximum litter size for species in the family Carcharhinidae. Data for *Rhizoprionodon taylori* are from Simpfendorfer (1992), all other data are from Compagno (1984). Dotted line is from Compagno's equation: litter size = -2.2 + (0.061 x maximum length). (a) *Rhizoprionodon taylori* (Compagno's estimate), (b) *Carcharhinus macloti*, (c) *C. sealei*, (d) *R. lalandii*, (e) *C. dussumieri*, (f) *Loxodon macrorhinus*, (g) *C. melanopterus*, (h) *C. wheeleri*, (i) *R. oligolinx*, (j) *Triaenodon obesus*, (k) *R. porosus*, (1) *R. terraenovae*, (m) *C. acronotus*, (n) *C. sorrah*, (0) *C. amblyrhynchos* and *C. isodon*, (p) *C. porosus*, (q) *R. acutus*, (r) *Lamiopsis temmincki*, (s) *C. limbatus*, (t) *C. albimarginatus*, (u) *C. signatus*, (v) *C. plumbeus*, (w) *Negaprion acutidens*, (x) *C. leucas*, (y) *C. falctformis*, (z) *C. obscurus*, (A) *C. longimanus*, (B) *C. brevipinna*, (C) *C. altimus*, (D) *C. galapagensis*, (E) *N. brevirostris*, (F) *C. brachyurus*, (G) *Prionace glauca*, (H) *Scoliodon laticaudus*. (adapted from Simpfendorfer 1992)

Table 2.1: Elasmobranch species in which embryonic diapause is classified as: confirmed (entire reproductive cycle have been studied, yolky ova have been observed for extended periods of time and histology was performed to confirm its developmental stage) or probable (although yolky ova have been observed during extended periods of time no confirmation technique was performed).

Taxon	Species	Diapause Duration (months)	Active development (months)	Status	Reference
Order Myliobatiformes					
Dasyatidae	*Dasyatis say*	9 – 10	~ 2	Confirmed	Snelson et al. (1989); Morris (1999)
	Dasyastis brevis	9,5 – 10	2 – 2,5	Probable	Melendez (1997)
Myliobatidae	*Aetobatus flagellum*	9	~ 3	Probable	Yamaguchi (2006)
Urolophidae	*Trygonoptera personata*	5	5	Probable	White et al. (2002)
	Trygonoptera imitata	5 – 8	4 – 6	Probable	Trinnie et al.(2009)
Order Rhinobatiformes					
Rhinobatidae	*Rhinobatos cemiculus*	~ 4	~8	Probable	Seck et al. (2004)
	Rhinobatos horkelli	~ 9	3	Probable	Lessa (1982)
	Rhinobatos hynnicephalus	9 – 10	2 – 3	Probable	Kume, (2009); Wenbin & Shuyuan (1993)
	Rhinobatos percellens	4	8	Probable	Grijalba-Bendeck et al. (2008)
	Rhinobatos productus	~ 8,5	3 – 4	Probable	Hoffmann (2007); Márquez-Farías (2007)
	Rhinobatos rhinobatos	8 – 9	3 – 4	Probable	Enajar et al. (2008); Abdel-Aziz et al.(1993)
	Zapteryx exasperata	6	5	Probable	Blanco-Parra et al. (2009)
	Trygonorrhina dumerilii	7 – 8	4 – 5	Probable	Marshall et al. (2007)
Platyrhinidae	*Platyrhinidae sinensis*	?	?	Probable	Yamaguchi and Kume (2009)
Order Carcharhiniformes					
Carcharhinidae	*Rhizoprionodon taylori*	7	~ 5	Confirmed	Simpfendorfer (1992)
Order Pristiophoriformes					
Pristiophoridae	*Pristiophorus nudipinnis*	7-10	12 – 15	Probable	Walker & Hudson (1999)

Chapter 3 Morphology of pre- and postovulatory follicles in Chondrychthyes: a case study on the ovarian follicles of *Rhizoprionodon taylori*

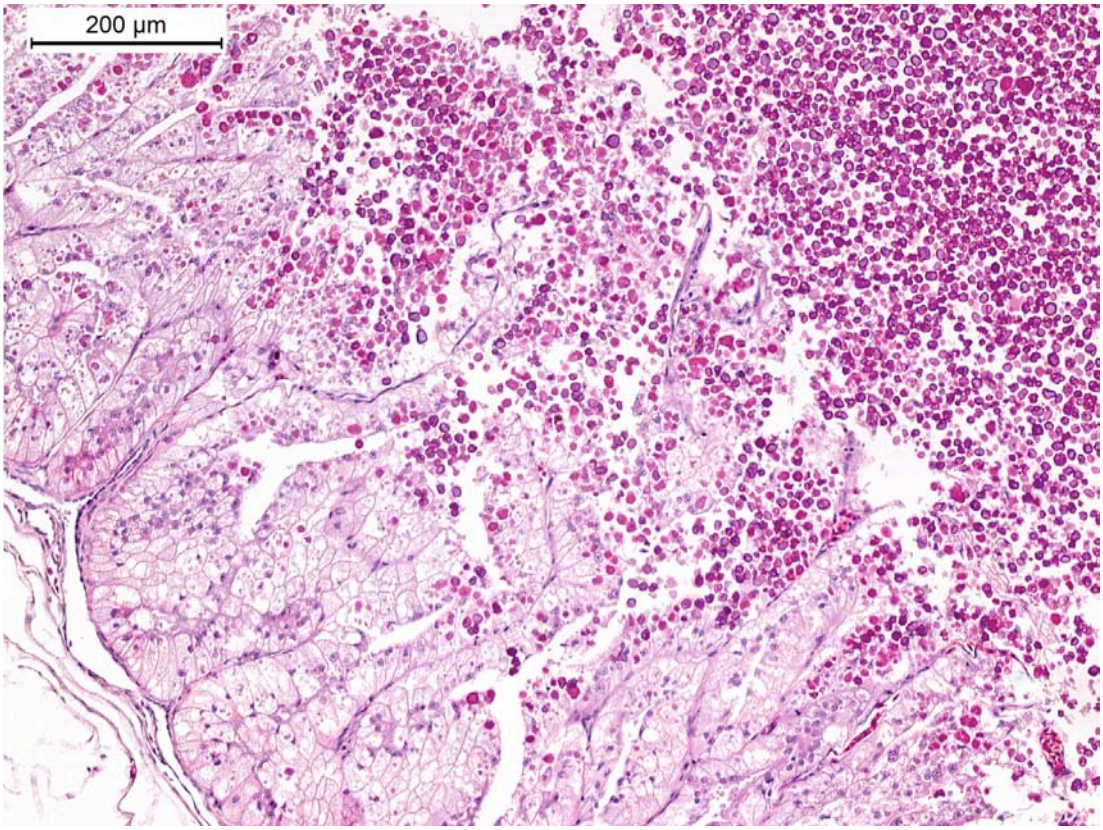

Plate 3: Granulosa cells actively ingesting abundant yolk present in the central cavity of a Stage 1 atretic follicle of *Rhizoprionodon taylori*

Waltrick, D.S., Awruch, C.A. & Simpfendorfer, C.A. **In prep**. Morphology of pre- and postovulatory follicles in Chondrychthyes: a case study on the ovarian follicles of *Rhizoprionodon taylori*.

3.1. Introduction

The ovary of all vertebrates is structurally similar and always functions as both gametogenic and as an endocrine organ (Dodd, 1972a; Dodd, 1972b). The formation of female reproductive cells (oogenesis) and their maturation process (folliculogenesis) occur within the ovary where, at every reproductive cycle, a number of primordial germinal cells will undergo a series of meiotic divisions and transformations culminating at ovulation (Zuckerman et al., 1977). Generally, the remnants of ovulated follicles remain in the ovary where they undergo luteinisation and develop into transient endocrine glands, the corpus luteum (Harrison, 1948). In mammals the corpora lutea becomes a temporary source of steroids hormones, mainly progesterone (P_4), necessary for the maintenance of pregnancy (Zuckerman et al., 1977; Norris, 2007)

The formation of structures homologous to the mammalian luteogenic tissue has been extensively reported in the ovary of Chondrichthyes (e.g. Lance and Callard, 1969; Tewinkel, 1972; Chieffi Baccari et al., 1992). Such structures are formed in two distinct processes: (I) from the remnants of an ovulated follicle, referred to as postovulatory follicle (POF) or corpora lutea; and (II) from the atresia of follicles at any stage of folliculogenesis without ever discharging the ova, called preovulatory corpora lutea, corpora atretica or atretic follicles (AF) (Hisaw Jr. and Hisaw, 1959; Chieffi Baccari et al., 1992). However, distinguishing between AF and POF in chondrichthyans can be challenging (Hisaw Jr. and Hisaw, 1959; Callard et al., 1989) and has led to apparent confusion in the literature.

Problems identifying ovarian structures in chondrichthyans mainly arise from the similarities among these structures, but also from their variable morphology among different species (Hisaw Jr. and Hisaw, 1959; Callard et al., 1989). With some exceptions in species where these follicles are strikingly different (*Cetorhinus maximus*, Matthews, 1950; *Mustelus canis*, Tewinkel, 1972), AF and POF share a number of morphological characteristics (e.g. colour, shape, texture) that make their macroscopic identification challenging (Hisaw Jr. and Hisaw, 1959; Teshima, 1981; Callard et al., 1989). Such similarities have hindered a recent attempt to establish a field identification of POF in *Mustelus antacticus* (Storrie, 2004). In addition, coloration (Tovar-Ávila et al., 2007) and size (Çek et al., 2009) alone have recently been used as the base for the identification of

such ovarian structures, increasing a margin for misidentification and adding to the confuse literature.

Another problem with the identification of chondrichtnyan ovarian structures in the literature is believed to arise from the casual use of terms and detailed descriptions mostly derived from mammalian studies (Storrie, 2004). The first description of POF in a chondrichthyan (*Pteromylaeus bovinus*) was based in the mammalian 'corpora lutea' (Giacomini, 1896). Since then, numerous morphological and histochemical studies have reported the presence of both AF and POF in the ovary of at least 25 Chondrichthyes (Table 3.1). These structures are often referred to as preovulatory 'corpora lutea' (AF) and postovulatory 'corpora lutea' (POF) due to their obscure homology to the mammalian temporary endocrine structure. The use of these terms is not discussed here and the reader is referred to further readings on this topic (Hisaw Jr. and Hisaw, 1959; Hoar, 1965; Chieffi and Botte, 1970; Dodd, 1972a).

The function and morphology of ovarian follicles are a matter of ongoing debate in the chondrichthyans. Therefore, the purpose of this work was to synthesise information available on the histology of AF and POF, highlighting their known morphology and steroidogenic capacity in Chondrichthyes. In addition, using the Australian sharpnose shark *Rhizoprionodon taylori* as a case study, this information was then applied in an attempt to identify ovarian follicles according to their pre- or postovulatory origin based on the information compiled from the available literature. The interesting reproductive strategy adopted by this species – embryonic diapause – places embryonic development seven months apart from fertilization, possibly extending the life of ovarian structures and providing a useful model to understand the topic.

3.1.1. Ovarian follicles

Ovarian follicles are formed by the germinal epithelium. They develop in the mature ovary to sizes that vary from 1-100 mm, depending on the species (Wourms, 1977; Dodd et al., 1983). The general composition of the follicular wall is retained during oocyte development and its later development into AF and POF.

The follicular wall surrounding the ooplasm consists of the basement membrane, granulosa layer, zona radiata, and theca layer (Figure 3.1). The initially single layered granulosa epithelium may either become multilayered (Giacomini, 1896; Dodd et al., 1983; Chieffi Baccari et al.; Hamlett et al., 1999), pseudostratified (Storrie, 2004; Awruch et al., 2008b) or maintain its single layer characteristic (Wallace, 1904) during the follicular development. However it always becomes single-layered when the follicle reach the mature stage and vitellogenesis is complete (Dodd, 1983; Dodd et al., 1983; Chieffi Baccari et al., 1992).

Two acellular membranes also compose the follicular wall. A basement membrane separates the theca and granulosa layers, while the zona radiata separates the granulosa layer from the ooplasm. This initially thick membrane narrows down as vitellogenesis proceeds (Tewinkel, 1972; Chieffi Baccari et al., 1992; Storrie, 2004; Lutton et al., 2005). Finally, a well-vascularised network of connective tissue stroma, the theca layer, surrounds the follicles of all sizes holding them together (Chieffi and Botte, 1961; Lance and Callard, 1969; Dodd, 1983).

At ovulation, mature ovarian follicles (graafian follicles) rupture releasing the oocyte to be fertilized. The remnants of these follicles stay within the ovary and may develop into postovulatory structures. However, not all follicles have the same fate and some undergo a degenerative process (atresia) without ever having discharged their ova to develop into atretic follicles.

3.1.2. Atretic vitellogenic follicles (AF)

At any stage during follicular development, a degenerative process may be triggered at preovulatory follicles and give rise to AF (Callard et al., 1989; Chieffi Baccari et al., 1992). Few studies have reported the longevity of these structures and therefore comparison among species is not possible. Nevertheless, data on two species suggest AF can persist from several months in *Mustelus canis* (Tewinkel, 1972) to approximately one year in *Torpedo marmorata* (Chieffi and Gualà, 1959). Visual inspections of recently formed AF describe these structures as wrinkled looking bodies (Chieffi and Gualà, 1959) resembling deflated balloons (Callard et al., 1989). Aging follicles undergo a gradual reduction in size (Chieffi and Gualà, 1959), while the coloration ranges from opaque

yellow to whitish-yellow in recently formed AF (Chieffi and Gualà, 1959; Callard et al., 1989; Tovar-Ávila et al., 2007) and can become dark-brown in advanced stages in some species (Chieffi and Gualà, 1959).

Changes in the microscopic morphology of AF have been described in a number of Chondrichthyes (Chieffi and Gualà, 1959; Chieffi and Botte, 1961; Chieffi, 1962; Lance and Callard, 1969; Tewinkel, 1972; Chieffi Baccari et al., 1992; Chatchavalvanich and Visuttipat, 1997). However, the sequence of events from the onset of atresia to degeneration of AF is highly variable among species. Differences such as the amount of yolk observed within the early stages of AF is directly dependent on when atresia started during the follicle development and the time elapsed since its onset (Hamlett and Koob, 1999). Thus, only a generalised description is presented here.

At the beginning of atresia, the follicle looses its zona radiata and basement membrane. The granulosa layer folds forming villi, while small granulosa cells grow in length and large cells degenerate (Chieffi and Gualà, 1959; Hisaw Jr. and Hisaw, 1959; Chieffi and Botte, 1961; Chieffi, 1962; Tewinkel, 1972; Chieffi Baccari et al., 1992). The theca layer, formed by theca cells, connective tissue and blood vessels, intrude on these villosities forming a well-vascularised stroma (Tewinkel, 1972; Chieffi Baccari et al., 1992).

At the centre of the follicle, adjacent to the granulosa layer, lays a conspicuous cavity filled with yolk granules. Shortly after the formation of the AF, the granulosa cells start an active process of yolk phagocytosis (Chieffi and Gualà, 1959; Hisaw Jr. and Hisaw, 1959; Lance and Callard, 1969; Chieffi Baccari et al., 1992; Chatchavalvanich and Visuttipat, 1997). The villi continue to lengthen and anastomose, resulting in a reduction of the central cavity (Lance and Callard, 1969; Chatchavalvanich and Visuttipat, 1997).

When phagocytosis is complete the nuclei of granulosa cells migrate from the basal to apical pole of the cells. This nuclear shift indicates a change in cell function from phagocytic to secretory (Chieffi and Gualà, 1959; Chieffi, 1962; Tewinkel, 1972; Chieffi Baccari et al., 1992). At this stage, the cytoplasm of the granulosa cells may also present small granulation and some vacuoles (Chieffi and Gualà, 1959; Chieffi and Botte, 1961; Chieffi Baccari et al., 1992).

The final stage of the AF is characterized by involution and sclerosis (Chieffi and Gualà, 1959; Chieffi and Botte, 1961). Granules and vacuoles become numerous in the granulosa and theca cells. The villi's vascular net undergoes sclerosis, resulting in pigmentary degeneration of the follicle (Chieffi and Gualà, 1959; Chieffi, 1962; Chieffi Baccari et al., 1992). At a more advanced stage, the granulosa cells become uncommon and dispersed among the theca and eventually the cavity disappears (Tewinkel, 1972; Chatchavalvanich and Visuttipat, 1997). In Mustelus, the granulosa cells disappear and a large number of "blood spaces" are left in the connective tissue of the follicle (Chatchavalvanich and Visuttipat, 1997).

3.1.3. Postovulatory follicles (POF)

At ovulation, the graafian follicle rupture and release the ovum through the ovary's outer layer (Hisaw Jr. and Hisaw, 1959; Callard et al., 1989; Hamlett and Koob, 1999). The remaining follicular wall collapses to form the POF (Callard et al., 1989; Hamlett and Koob, 1999). Postovulatory follicles have been reported as large yellow shrunken bodies (Hisaw and Albert, 1947; Callard et al., 1989; Girard and Du Buit, 1999; Storrie, 2004) in a biconvex (lenticular) shape (Matthews, 1950; Dodd, 1983).

The size of the POF in its early stages of development varies greatly (from 12 to 20 mm) (Giacomini, 1896; Samuel, 1946; Hisaw and Albert, 1947; Tsang and Callard, 1987b), probably due to the highly variable oocyte sizes among species in this group (Lutton et al., 2005). After full development, the POF undergo degeneration, resulting in small compact yellow masses ranging between two and three millimetres in size (Samuel, 1943; Samuel, 1946; Hisaw and Albert, 1947; Tsang and Callard, 1987b). Postovulatory follicles usually persist through pregnancy and therefore longevity of these structures vary from three months (e.g. Babel, 1966; Tewinkel, 1972) to almost two years (Hisaw and Albert, 1947).

The general microscopic morphology of POF is similar to that of the AF, except for the lack of yolk granules. In some species, the distinction between POF and AF after the completion of yolk phagocytosis may be impossible (e.g. 5 elasmobranch species, Hisaw Jr. and Hisaw, 1959; *Mustelus manazo* and *M. griseus*, Teshima, 1981). Yet, the classification of this distinct structure is possible in other Chondrichthyes (e.g. *Squalus*

acanthias, Hisaw and Albert, 1947; *M. antarcticus*, Chieffi and Botte, 1961; *M. canis*, Tewinkel, 1972) and its characteristics have been summarized below.

Postovulatory follicles are composed by a conspicuous central cavity surrounded by a granulosa layer that is thrown into folds and is invaginated towards the centre of the follicular cavity (Giacomini, 1896; Samuel, 1943; Samuel, 1946; Hisaw and Albert, 1947; Lance and Callard, 1969; Tewinkel, 1972; Callard et al., 1989; Chatchavalvanich and Visuttipat, 1997). Theca cells and connective tissue compose the follicular framework, which infiltrates on the folds supporting a generous supply of blood vessels through the structures (Wallace, 1904; Samuel, 1946; Hisaw and Albert, 1947; Tewinkel, 1972; Callard et al., 1989).

Initially, the granulosa cells hypertrophy and become elongated and columnar in shape. Vacuoles have been reported in the cytoplasm of granulosa of some species (Samuel, 1946; Hisaw and Albert, 1947; Chieffi and Botte, 1961). Although in some areas the granulosa appears to be multilayered, no mitosis can be observed and therefore this must result from the follicle contraction after the expulsion of the oocyte (Wallace, 1904; Samuel, 1946). Where yolk and cell debris have been observed in the cavity, granulosa cells have been reported to actively ingest these structures (Samuel, 1943; Hisaw Jr. and Hisaw, 1959; Chatchavalvanich and Visuttipat, 1997). The theca layer, composed by small oval cells, remains distinctively separated from the granulosa layer (Samuel, 1946; Matthews, 1950; Chieffi and Botte, 1961), which becomes thicker and extends into the lengthening folds of the granulosa providing an increased vascularisation (Samuel, 1943; Samuel, 1946; Hisaw and Albert, 1947; Tewinkel, 1972).

As the POF age, granulosa cells tend to become more spherical or oval and undergo a reduction in size. Signs of the degeneration, indicated by the presence of large vacuoles and picnotic nuclei, are observed throughout the follicle. Free nuclei can also be observed at the follicular cavity (Wallace, 1904; Samuel, 1943; Samuel; Hisaw and Albert, 1947; Chatchavalvanich and Visuttipat, 1997). The central cavity of some species is reported to be taken over by granulosa cells and disappear due to the invasion of these cells and overall reduction of follicle size (Chieffi and Botte, 1961; Lance and Callard, 1969; Chatchavalvanich and Visuttipat, 1997). The theca layer carries more fibrous material and

connective tissues, the outer zone is invaded by blood vessels and the cells may become flattened (Wallace, 1904; Samuel, 1943; Matthews, 1950).

3.1.4. Steroidogenic potential

The use of histochemical and in vitro techniques in the chondrichthyan AF and POF are very limited (Table 1) and the results are rather variable. Thus, the steroidogenic capacity of these follicles still remains unclear for many species. An early study of the AF and POF of five elasmobranchs by Hisaw Jr. and Hisaw (1959) concluded that these structures were devices for the removal of yolk and cellular debris after ovulation rather than having an endocrine function. However, a number of histochemical and biochemical examinations have identified the presence of steroid hormones (e.g. testosterone, progesterone and 17ß-estradiol) or enzymes necessary for their production in ovarian follicles (Lance and Callard, 1969; Tewinkel, 1972; Tsang and Callard, 1992).

There seem to exist no consistency on the steroidogenic capacity of the chondrichthyan ovarian structures in regards to reproductive mode. Although AF occur in all species studied to date, the transformation of these structures into temporary endocrine glands is not a rule. The secretory capacity of such structures has only been demonstrated in two placental viviparous (*Scoliodon laticaudus*, Guraya, 1972; *M. canis*, Tewinkel, 1972), and two aplacental viviparous species (*Torpedo torpedo*, Chieffi, 1962; Chieffi Baccari et al., 1992; and *T. marmorata*, Fasano et al., 1992).

With the exception of *M. canis*, in which weak signs of steroidogenesis were also observed in the POF (Tewinkel, 1972), it remains unclear why the luteinisation only occurs in the AF of these species but not in the POF as in mammals. Chieffi Baccari (1992) argues that this could be an adaptation "related to the preparation of the reproductive organs, such as the uteri, for gestation". Similarly, the luteinisation process in POF has only been reported in two oviparous, (*Scyliorhinus stellaris*, Chieffi and Botte, 1961; and *S. canicula*, Chieffi, 1962), one placental viviparous (*M. canis*, Tewinkel, 1972) and two aplacental viviparous sharks (*S. acanthias*, Lance and Callard, 1969; *M. antarcticus*, Storrie, 2004).

3.2. Case study

This study aimed to differentiate ovarian follicles of the Australian sharpnose shark, *R. taylori* according to their pre or postovulatory origin. The investigation was based on: (1) macroscopic and microscopic morphology of ovarian structures of *R. taylori* throughout the year; (2) current knowledge of this species' reproductive cycle; and (3) the existing literature of the chondrichthyan, especially elasmobranchs, ovarian structures.

Rhizoprionodon taylori is an annual viviparous elasmobranch with synchronous uterine and ovarian cycles. This species displays a gestation period of 11.5 months, which includes a 7 months period of embryonic diapause during which development of the embryo and ovarian follicles is arrested (Simpfendorfer, 1992). The ovarian cycle occurs concurrently with the 4.5 months period of active gestation and is concluded shortly after parturition and mating (Jan – Feb), when ovulation occurs (Figure 3.2).

3.3. Materials and methods

3.3.1. Animal and tissue sampling

Female *R. taylori* were collected monthly from February 2010 to February 2012 using 10 cm stretched mesh monofilament gillnets in Cleveland Bay, north Queensland, Australia (19º14'S, 146º48'E). Animals were measured and euthanized shortly after capture and preserved in ice for dissection. All females with ovarian follicles and uterine eggs or embryos were considered mature. From these specimens, ovaries were extracted and weighted to the nearest gram and the largest follicle diameter was measured. Deflated (Callard et al., 1989) or wrinkled looking bodies (Chieffi and Gualà, 1959) in the ovary were quantified and measured to the nearest millimetre before preserving in 10% neutral buffered formalin.

3.3.2. Structural examination

Macroscopic characteristics (i.e. coloration and shape) of AF and POF were recorded on fresh samples throughout the ovarian cycle. After excision, samples were dehydrated through a graded series of ethanol baths, infiltrated and embedded in paraffin using an automatic tissue processing (Shandon Hypercentre). Blocks were then sectioned at 5 -

10µm on a manual rotary microtome. Representative sections for each month were manually stained with Mayer's haematoxylin and Young's eosin (H&E). This stain has affinity to and dye basophilic tissue (including nuclei) blue and acidophilic tissues (including cytoplasm and connective tissue) pink to orange.

Selected sections of AF from stages 2 to 4 where post fixed in Bouin's fixative and manually stained using Martius yellow, Scarlet and Blue (MSB) technique (Bancroft and Gamble, 2008), which stains fibrin in red (early may stain yellow and very old blue), in order to detect tissue damage. Alcian Blue and Periodic Acid Schiff (AB/PAS) technique (Bancroft and Gamble, 2008) for mucossubstances was used to verify the composition of developing follicle, where acid glycoproteins stained blue and neutral glycoproteins stain purple. All sections were mounted with BDH DPX, covered with glass microscope coverslips and examined using a Leica DFC 420C digital camera attached to a Leica DM2500 microscope.

3.4. Results

Histological sections were performed in selected follicles from 53 ovaries of mature female *R. taylori*. Developing follicles, POF and two types of atretic follicles (non vitellogenic and vitellogenic preovulatory) were identified in the ovary throughout the year. Previtellogenic atretic follicles differed significantly from the remaining follicles macroscopically by their white coloration and larger size compared to AF and POF when they coexisted in the ovary. However, due to the external similarities of AF and POF, discerning one from the other solely by their macroscopic morphology was unreliable during part of the diapausing period and during early active embryonic development. Both AF and POF usually display an oval-flattened shape with a wrinkled surface. The coloration of these structures varies from dark yellow to pale-white (rarely brown) regardless of stage or microscopic morphology and therefore it was not considered a reliable characteristic for their identification. Size was the only distinguishing feature allowing the distinction of AF (1 – 26 mm) and POF (1 – 5 mm), which differed for a short period after ovulation.

During most of the period of embryonic diapause, a single distinctively larger "corpus" (also present during the two months preceding ovulation) and several smaller ones could

be easily distinguished in the ovary of *R. taylori*. Through histology these large "corpora" were identified as AF, and the smaller ones as POF (microscopic morphology below; Table 3.2). At later stages however, macroscopically distinguishing between ovarian follicles was impossible due to the similar size of the then very compact structures. The total number of these "corpora" (AF and POF) displayed a weak positive correlation with litter size (P=0.025, rho=0.296). This correlation became slightly stronger if the larger bodies (AF) were removed from the analysis (P=0.009, rho=0.3145).

3.4.1. Follicle development and maturation

From early to late development, follicles of *R. taylori* always display the same wall composition. The most external layer (theca layer) is rich in blood vessels, connective tissue and theca cells. The granulosa layer lies among two membranes, the basement membrane, and a zona radiata, which surrounds the ooplasm (Figure 3.3 A-C).

In follicles approximately 1 mm wide a thick zona radiata, composed of neutral and acid mucopolysaccharides (Figure 3.3A) surrounded the yolk-free ooplasm. The granulosa is single layered and composed of cubic cells displaying a clear cytoplasm. As follicles grow and yolk starts to accumulate in the ooplasm (≥ 4mm), the zona radiata becomes thinner. The granulosa layer, with elongated cells and an eosinophilic cytoplasm, becomes pseudostratified, while more blood capillaries infiltrate the theca layer (Figure 3.3 B). The granulosa of fully developed follicles returned to the initial single layered condition. At this stage granulosa cells were longer with the nuclei positioned on the base of the cell. A much thinner zona radiata separates the granulosa layer from the ooplasm, now filled with large yolk platelets (Figure 3.3 C).

Atresia can start at any time during the follicular development and give rise to two morphologically distinct structures. The first, much smaller (up to 4 mm), occurred more frequently during early folliculogenesis, prior to the onset of vitellogenesis (November). Histology of these follicles showed a central cavity filled with an ooplasm-like material, but never yolk. The second type of atretic follicles was larger (3 to 26 mm) and occurred later in the reproductive cycle, from late active pregnancy (December) to the commencement of subsequent ovarian cycle (September). Histology of recently formed follicles displayed abundant yolk in the central cavity. The distinct characteristics (size,

timing of occurrence and presence or absence of yolk) among these follicles suggest these structures are formed by the atresia of follicles at distinct stage of follicular development.

3.4.2. Atresia of previtellogenic follicles

Atretic non-vitellogenic follicles, usually white shrunken bodies, occurred more frequently from September to December and therefore did not coin cide with the occurrence of early POF. The timing of occurrence, smaller size and evidence of ooplasm-like material within the central cavity indicate that these differentiated follicles are formed from previtellogenic follicles. Atresia of previtellogenic follicles commenced with the multiplication of granulosa cells, which are then taken into folds towards the ooplasm-filled central cavity supported by an intrusion of richly vascularised theca layer (Figure 3.4 A). The initially small, round granulosa cells with clear cytoplasm become elongated while some eosinophilic material is deposited at the apical pole of the cell together with the nucleus. It appears that the granulosa cells ingest the ooplasm and cellular debris as it lengthens taking up the central cavity in a maze-like appearance (Figure 3.4 B).

As the follicles age, degeneration occurs unevenly around the granulosa layer resulting in reduction of follicle size. Cellular breakage possibly commences in areas where the ingestion of cell debris and ooplasm was complete earlier. Follicles in advanced atresia are compact structures with a conspicuous cavity containing little debris (Figure 3.4 C).

3.4.3. Atresia of vitellogenic follicles (AF)

The development of the AF was divided into at least 4 stages (Figure 3.5 A-D). MSB staining of AF sections were positive, showing an increased intensity of red from stages 2 to 4, indicating an increased accumulation of fibrin and therefore the aging of the follicles. Stage 1 occurs in *R. taylori* from late active gestation (December) until shortly after ovulation, when the maximum observed size (26 mm) was observed. Recently formed AF display an intense folding of the granulosa layer throughout the follicle, especially around its extremities. Granulosa cells are mostly elongated with nuclei centrally positioned and displaying a vacuolated cytoplasm while actively ingesting yolk from the central cavity. The theca layer, carrying abundant blood vessels, infiltrates

through the folds of the granulosa. No acellular membranes were observed in any section indicating they quickly degenerated at the early stages of atresia (Figure 3.5 A).

Stage 2 AF are approximately 10 mm (Figure 3.5 B). No yolk is left within the follicle. The theca further infiltrates the granulosa folds extending the septa and overtaking the lumen, resulting in little free space among them. The granulosa, which retains its elongated shape, no longer show vacuolation of the cytoplasm. The nuclei and eosinophilic material are positioned at the apices of these cells. Some cell division is observed among the granulosa.

Stage 3 constitutes the early degeneration of the AF. Continued intrusion and thickening of the connective tissue appears to force the granulosa into lobes, resulting in further reduction of follicular space. The granulosa cells become spherical and highly vacuolated. Degeneration of cells occurs in small isolated areas of the follicle, adjacent to the thecal extensions (Figure 3.5 C).

Stage 4 AF displays a reduction in size of the granulosa cells, which contain small to medium vacuoles and some granules through the cytoplasm. Degeneration is now widespread and open spaces observed in this layer are probably a result from the breakage of theca cells and theca extensions. The theca layer is limited to a few thick intrusions and remains thick and well vascularised around the follicle (Figure 3.5 D).

Later stages could not be identified due to the reduced size (1 - 2 mm) and morphological similarities shared between AF and POF, thus classifying these stages was not possible.

3.4.4. Postovulatory follicles (POF)

After ovulation, three to ten POF measuring up to 5 mm occur in the ovary of *R. taylori*. Based on their morphology, these structures were divided into four stages as follows (Figure 3.6 A-D):

Stage 1 follicles are composed by small lobes of granulosa cells, mostly arranged around the extremities of the follicle. The granulosa looks rather unorganized with broken and loosened cells vastly taking the central cavity. No blood clots or yolk are present. The

cells of the granulosa are small, filled with eosinophilic material and with the nucleus facing the closest connective tissue. The thick theca layer enveloping the follicle somewhat stretches into the granulosa layer (Figure 3.6 A).

Stage 2 POF are completely filled up by spherical granulosa cells organized into lobes by a further intrusion of the theca (Figure 3.6 B). Granulosa cells contain small vacuoles and exhibit signs of breakage in isolated parts of the follicles only. Blood vessels are not as abundant as in AF and mainly occur around the thick connective tissue surrounding the corpora. Follicles at this stage resemble Stage 3 AF.

Stage 3 has the same overall structure observed in stage 2 POF (Figure 3.6 C). At this stage, vacuoles are larger and granulosa cell breakage is more frequently observed on the boundaries between granulosa and theca layers. The theca layer thickens and carries a large number of free nuclei.

At stage 4 the theca layer further thickens, causing the lobes of GCs to become smaller. Blood vessels are abundant all through this layer. Granulosa cells are much reduced and filled with granules (Figure 3.6 D).

3.5. Discussion

Atretic and POF occur in the ovary of *R. taylori*. The development of temporary ovarian structures originated by means of atresia and ovulation of ripe follicles is not a rule in Chondrichthyes as some have been described to quickly degenerate (Chieffi and Gualà, 1959; Teshima, 1981). From the present study it is clear that in *R. taylori* the process of atresia and ovulation of follicles may develop temporary structures that maintain the same general constitution as the follicles they originate from. Although the presence of hormones was not investigated, the longevity of these follicles, their morphology and similarities with other taxa could indicate some function in the reproductive cycle of this species.

The follicular constitution in *R. taylori* is similar to that of other chondrichthyans with few remarks. As in *M. antarcticus* (Storrie, 2004) and *Cephaloscyllium laticeps* (Awruch et al., 2008b), the granulosa layer undergoes a period of pseudostratification during the

vitellogenenic process. The presence of neutral and acid mucopolysaccharides within the vitelline membrane, indicated by the positive histochemical reaction to AB and PAS, in the present study has only been previously demonstrated in two *Squalus* species (*S. acuta*, Díaz-Andrade et al., 2009; *S. bonaparti*, Díaz-Andrade et al., 2011). The presence of neutral mucossubstances alone, however, has been demonstrated in several chondrichthyans (*S.canicula*, Dodd and Sumpter, 1984; *M. antarcticus*, Storrie, 2004; Barone et al., 2007). The function of the basement membrane remains unknown in Chondrichthyes. It has been suggested, however, that, as in other vertebrates, the basement membrane may function in acrosomic reactions, prevention of polyspermy, and may be pertinent to embryo survival (Storrie, 2004; Díaz-Andrade et al., 2011).

While developing follicles are easily identified in the ovary, the ovarian follicles derived from them may be very similar to each other and therefore difficult to identify. Macroscopic distinction among ovarian structures of *R. taylori* is possible during most of the diapausing period but should be done with caution. Although coloration and external morphology of AF and POF are basically identical, the relatively larger size sustained by the former compared to the latter allows the distinction of these two structures with some confidence during most of the seven months of embryonic diapause. However, the extrapolation of morphological characteristics of this to other species is not encouraged because of the relatively unique period of embryonic diapause. Comparisons with other diapausing species could also be of interest.

Atresia occurs at any time during the folliculogenesis in *R. taylori*. Although this condition has been previously reported in Chondrichtyes, the occurrence of atretic previtellogenic follicle has only been described in few species (e.g. *S. laticaudus*, Guraya, 1972; *M. canis*, Tewinkel, 1972; *Dasyatis bleekeri*, Chatchavalvanich and Visuttipat, 1997). The atretic previtellogenic follicles of *R. taylori* resemble those of *M. canis* (Tewinkel, 1972) in regards to the granulosa arrangement, scarcity of granules and presence of distinct thecal tubes. Described as special atretic follicles by TeWinkel (1972), these structures were reported to have strong indication of steroidogenesis and the same may apply to *R. taylori*.

After the complete absorption of the ooplasm of atretic previtellogenic follicles of *R. taylori* they structurally resembles a stage 2 AF reported by Chatchavalvanich and

Visuttipat (1997) in *D. bleekeri*. Given these findings were based on a small sample size (n=5) obtained at one given point of the reproductive cycle of this species, this information should be treated with caution. Therefore further studies on the ovarian follicles of *D. bleekeri* may be necessary to understand their development and progression.

The onset of atresia in larger follicles, in which yolk has been deposited, generates follicles with a remarkably different microscopic morphology. The early stages of AF of *R. taylori* are similar to those described for some aplacental viviparous (*T. marmorata*, Chieffi and Gualà, 1959; *S. acanthias*, Lance and Callard, 1969; Chieffi Baccari et al., 1992), placental viviparous (*M. canis*, Tewinkel, 1972) and oviparous (*S. stellaris*, Chieffi, 1962) elasmobranchs. As in these species, the granulosa cells actively ingest the yolk abundantly present in the central cavity.

The change in polarity (nuclear migration from basal to apical) observed in the granulosa cells of stage 2 AF in the present study has been previously reported coinciding with the stage where AF obtain the aspect of true epithelial glands (Chieffi, 1962). In fact, histochemical and biochemical tests in AF of *M. antarcticus* (Chieffi and Botte, 1961), *M. canis* (Tewinkel, 1972) and *T. marmorata* (Chieffi Baccari et al., 1992), where a nuclear migration have been observed, indicated the presence of lipids and steroids. Therefore, Chieffi Baccari et al. (1992) have described such events as an indication of the change of cell function from phagocytic to secretory. Although the presence of lipids and steroids were not investigated in *R. taylori*, a change in polarity observed in stage 2 AF could indicate a temporary secretory activity in this ovarian structure.

The origin of POF is well recognized in the Chondrichthyes as developing from the remnants of ovulated follicles. Although matching numbers of POF and litter size have been previously reported (Wallace, 1904; Hisaw and Albert, 1947), most studies do not provide a correlation and limit to say multiple POF have been observed (Samuel, 1943; Matthews, 1950; Chieffi and Rattazzi, 1957; Chieffi and Gualà, 1959; Tewinkel, 1972). The weak correlation observed in the present study may be the reflection of misidentified structures (i.e. old atretic follicles identified as POF) since only few follicles within the same ovary were selected and identified through histology. However, it is also possible that a high number of ova are discharged and disposed without being fertilized.

Previous reports describe small holes in the surface of the ovary derived from the ovulation can indicate the position of the remnant follicle and thus an early POF (Chieffi and Gualà, 1959; Callard et al., 1989). Such rupture points were not observed in the present study neither on recently ovulated specimens or in one ovulating female. Therefore, the identification of such structures relied on the temporal microscopic changes and size observed in relation to reproductive stage and comparisons to the literature.

Early-formed POF of *R. taylori* could be easily distinguished microscopically from AF due to the lack of yolk and different arrangement of the granulosa layer. Postovulatory follicles did not present a folded granulosa layer, differing from most reports on the elasmobranch POF (e.g. Hisaw and Albert, 1947; Chatchavalvanich and Visuttipat, 1997). Similar to *S. stellaris* (Chieffi and Botte, 1961), *M. canis* (Tewinkel, 1972) and *C. maximus* (Matthews, 1950), it did however display a detachment of the thick granulosa layer from the theca and a cavity filled with cellular debris and blood cells. Therefore it is presumed that the initial stages shortly after the expulsion of the ova may have been missed in the present study.

The POF of *R. taylori* (Stage 3) resembled the general microscopic morphology of luteinizing POF reported in other Chondrichthyes (Chieffi and Botte, 1961; Lance and Callard, 1969; Storrie, 2004). The bulk of the follicle is composed of granulosa cells that are forced into bundles by the invasion of the theca layer concealing the central cavity. Although the persistence of the central cavity in mammals is related to the mode of reproduction and the relative size of oocytes they produce, Storrie (2004) found no relationship with size of follicles, parity or phylogeny in chondrichthyans.

Although this study did not look into the histochemistry of ovarian follicles, the development and persistence of both AF and POF for a period of seven to ten months may indicate a possible role of these structures in hormone production and hence the control of pregnancy of *R. taylori*. The identification of different ovarian follicles is possible in this species. However, there appears to exist an enormous morphological variation amongst these ovarian structures in comparison to different species. Whether these variations are related to evolutionary paths or mode of reproduction is unknown. Thus, identifying these structures remains a challenge and the generalisations must be

made with caution. Despite this, continued work to investigate the structure and function of the chondrichthyan AF and POF will be essential for understanding how reproduction and embryo development is controlled.

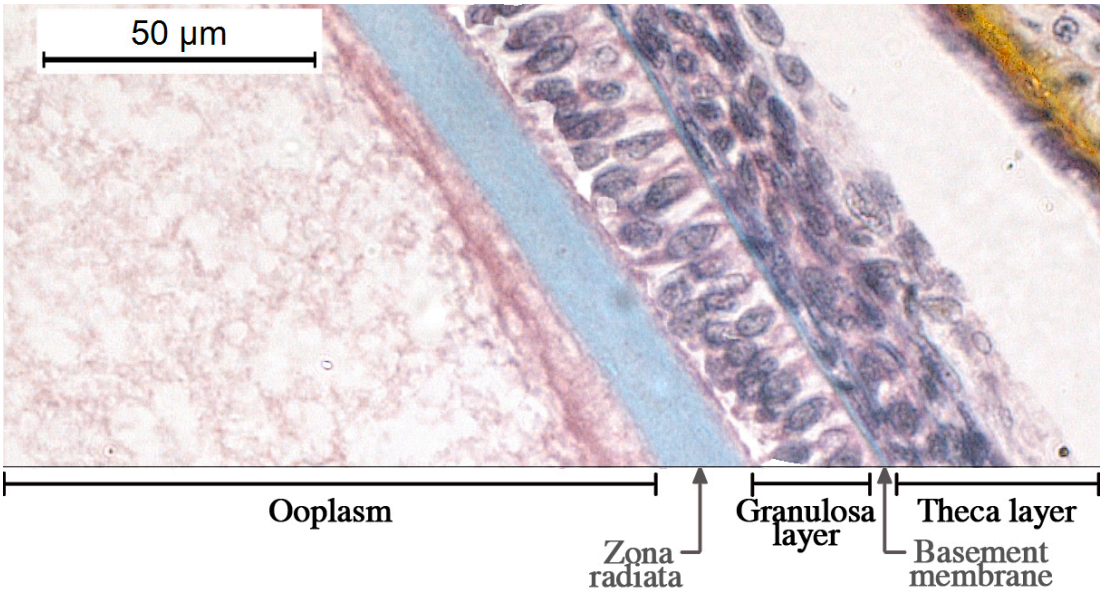

Figure 3.1: General composition of ovarian follicles

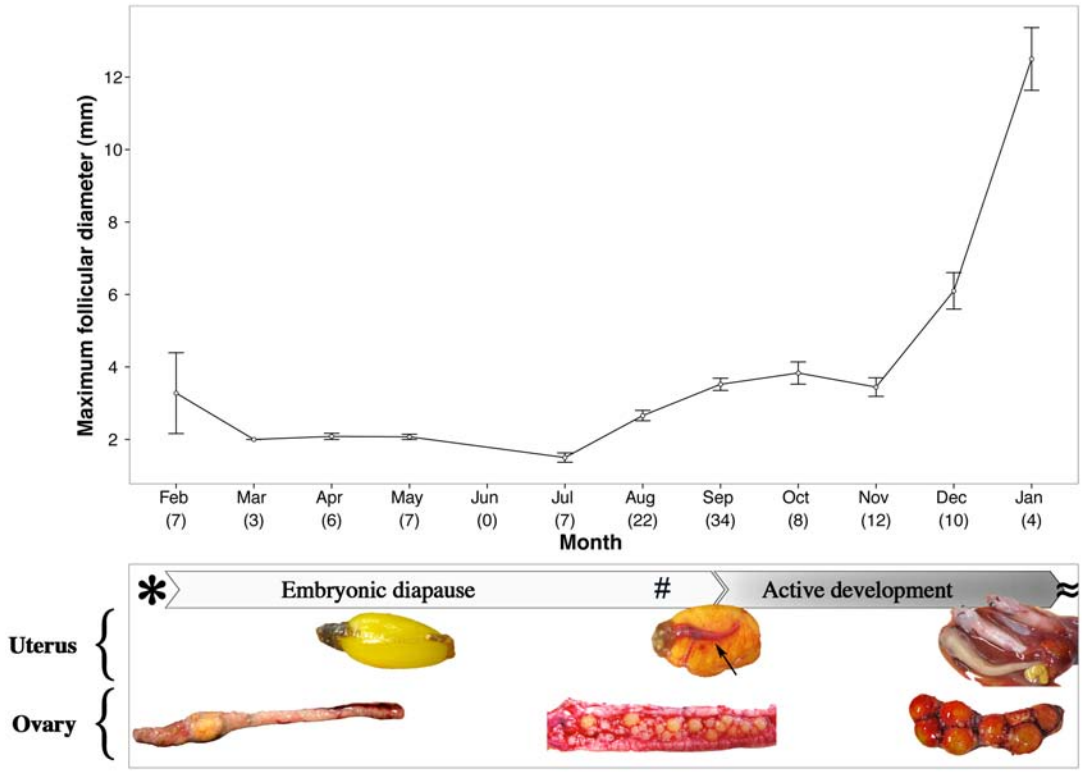

Figure 3.2: Monthly variation of maximum follicular size in the ovary of *Rhizoprionodon taylori*. The lower diagram shows the reproductive events throughout the year with representative photographs of embryonic development and ovarian condition. Symbols indicate: '*' ovulation, '#' initiation of oogenesis and '≈' parturition.

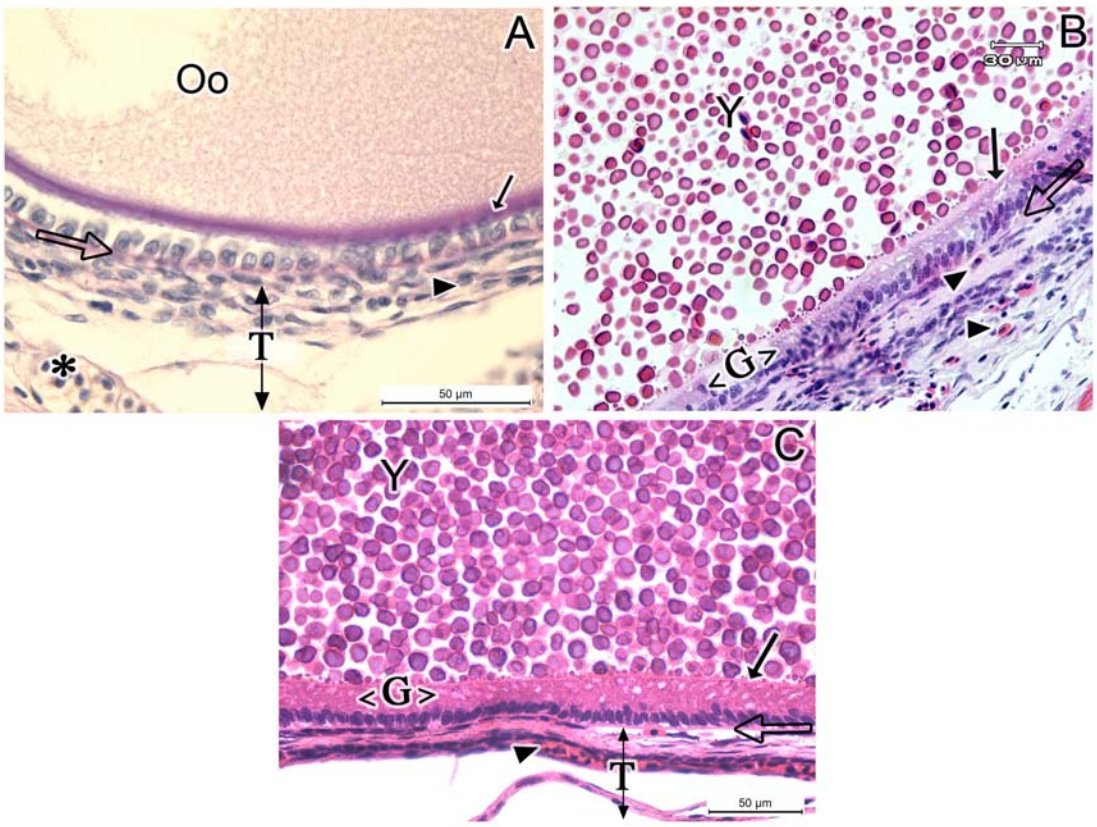

Figure 3.3: Organization of *Rhizoprionodon taylori* oocytes at different sizes: A) 1 mm. AB/PAS; B) 7 mm. H&E; and C) 14 mm. H&E. Arrows indicate the zona radiata; open arrows, basement membrane; arrow heads, blood cells; '*', blood vessels; G, granulosa layer; T, theca layer; Oo, ooplasm; Y, yolk.

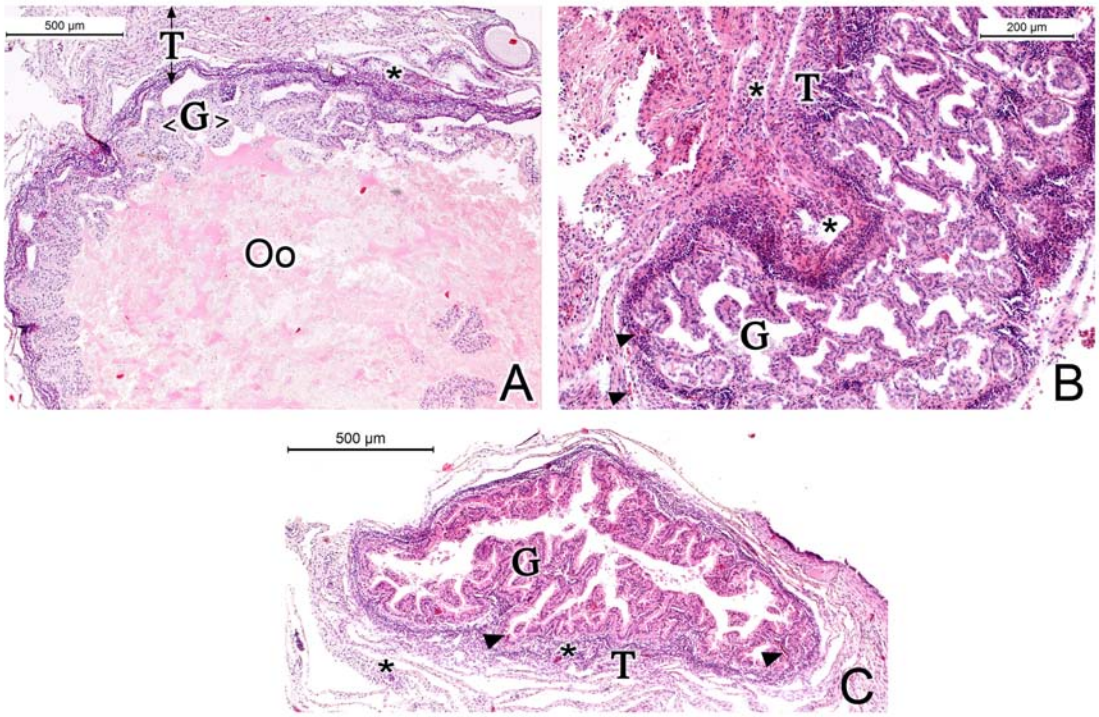

Figure 3.4: Organization of *Rhizoprionodon taylori* previtellogenic atretic follicles: (A) commencement of atresia. Note the presence of ooplasm within the follicle. H&E. (B) an older follicle where the ooplasm has been reabsorbed. Granulosa villi have extended and take over the centre of the follicle. H&E. (C) Very reduced and compact follicle. H&E.

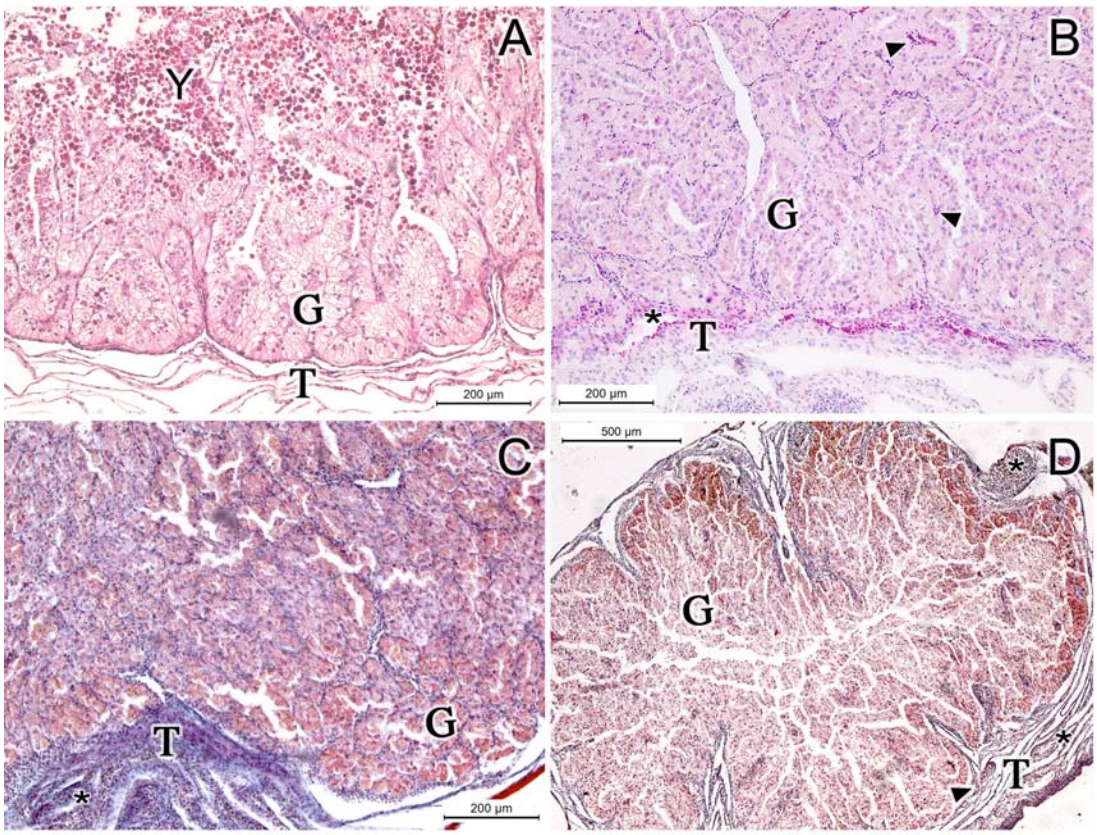

Figure 3.5: Stages 1-4 Atretic follicles of *Rhizoprionodon taylori* (A) Stage 1 is recently formed. Note the folded granulosa and the presence of yolk. H&E (B) Stage 2 slightly older, this follicle does not contain yolk. The granulosa cells have lost their vacuoles and are now arranged in long villi. The theca is well vascularised. H&E. (C) In stage 3 the granulosa is now arranged in lobes due to further intrusion of theca and connective tissue. Note some degeneration in the granulosa layer. MSB. (D) Stage 4 display open spaces possibly due to the degeneration of the theca layer. MSB.

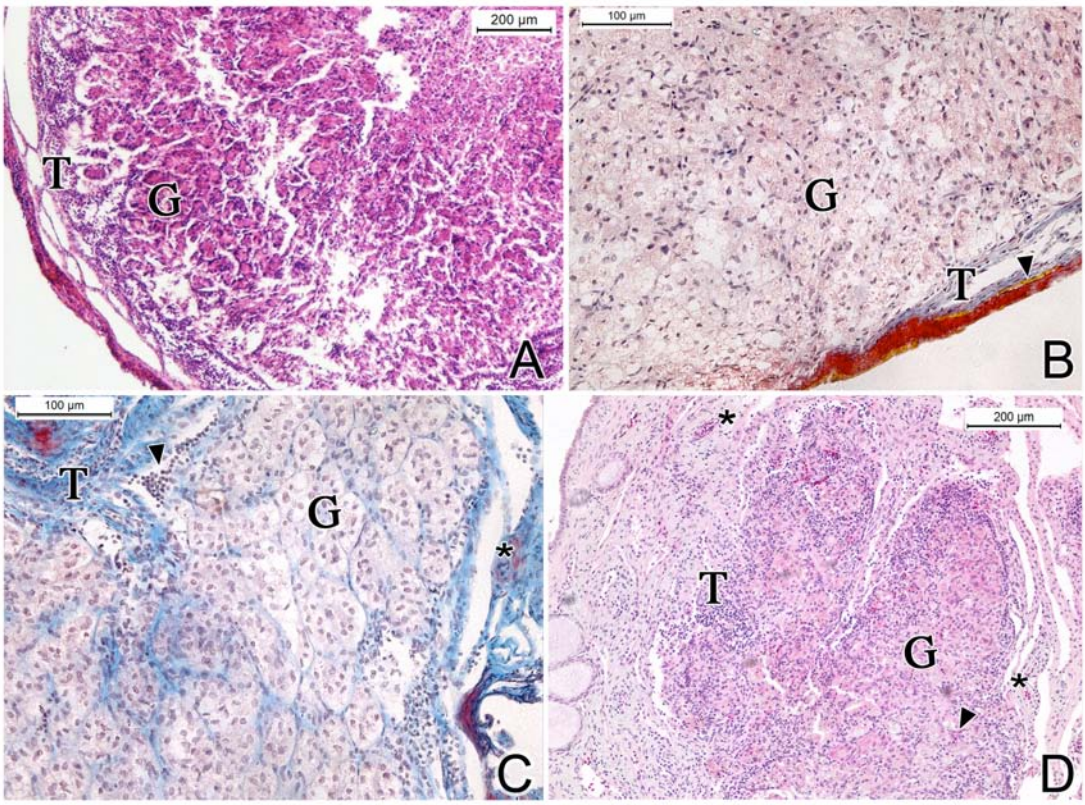

Figure 3.6: Stages 1-4 postovulatory follicles of *Rhizoprionodon taylori.* (A) Stage 1 sampled shortly after ovulation. A thick theca layer surrounds a very disorganized granulosa layer is roughly organized in lobes. Note the presence of cellular debris at the central cavity. H&E. (B) Stage 2 POF are mainly composed by granulosa cells which now compose the bulk of the follicle. Thin theca intrusions are seen all through the follicle. MSB. (C) Stage 3 the theca intrusions have thickened, forcing the granulosa into lobes. AB/PAS. (D) Stage 4 shows a further thickening and infiltration of the theca and a reduction in granulosa cells size. H&E.

Table 3.1: Chondrichthyan species in which atretic (AF) and postovulatory follicles (POF) have been studied. Histologically studied structures are marked with "Y", whereas those in which histological or histochemical studied have shown positive (#) or negative (x) signs of steroidogenic function are represented accordingly.

Taxon	Family	Species	Mode of reproduction	Structure		Reference
				POF	AF	
Subclass Elasmobranchii						
Subdivision Selachii						
Superorder Galeomorphi						
Heterodontiformes	Heterodontidae	*Heterodontus portusjacksoni*	Oviparous	Y	Y	Tovar-Ávila et al. (2007)
Orectolobiformes	Hemiscyllidae	*Chiloscyllium griseum*	Oviparous	Y	-	Samuel (1946)
Lamniformes	Cetorhinidae	*Cetorhinus maximus*	Aplac viviparous	Y	Y	Mattheus (1950)
Carcharhiniformes	Triakidae	*Mustelus canis*	Viviparous	Y	Y	Hisaw Jr & Hisaw (1959)
				Y #	Y #	Tewinkel (1972)
		Mustelus manaza	Viviparous	Y	Y	Teshima (1981)
		Mustelus griseus	Apl. Viviparous	Y	Y	Teshima (1981)
		Mustelus antarcticus	Apl. Viviparous	Y #	Y x	Chieffi & Botte (1961)
				Y #	-	Storrie (2004)
	Scyliorhinidae	*Scyliorhinus stellaris*	Oviparous	Y #	Y x	Chieffi (1962)
		Scyliorhinus canicula	Oviparous	Y #	-	Chieffi & Rattazzi (1957)
				Y #	Y x	Chieffi (1962)
	Carcharhinidae	*Scoliodon laticaudus*	Viviparous	-	Y #	Guraya (1972)
		Rhizoprionodon taylori	Viviparous	Y	Y	Present study
Superorder Squalomorphi						
Squaliformes	Squalidae	*Squalus acanthias*	Aplac viviparous	Y	-	Hisaw & Albert (1947)
				Y	Y	Hisaw Jr & Hisaw (1959)
				Y #	Y x	Lance & Callard (1969)
	Somniosidae	*Centroscymnus coelolepis*	Viviparous	Y	-	Girard & Du Buit (1999)
		Centrophorus squamosus	Viviparous	Y	-	Girard & Du Buit (1999)
		Squalus sucklei	Aplac viviparous	Y	Y	Hisaw Jr & Hisaw (1959)
	Etmopteridae	*Etmopterus spinax*	Aplac viviparous	Y	-	Walace (1904)
Superorder Batoidea						
Torpediniformes	Torpedinidae	*Torpedo marmorata*	Aplac viviparous	Y #	-	Chieffi & Rattazzi (1957)
				Y x	Y #	Chieffi & Gualá (1959)
				Y x	Y #	Chieffi (1962)
				-	Y #	Chieffi Baccari (1992)

				-	Y [#]	Fasano et al. (1992)
		Torpedo torpedo	Aplac viviparous	Y [x]	Y [#]	Chieffi (1962)
Rajiformes	Rhinobatidae	*Rhinobatus granulatus*	Aplac viviparous	Y	-	Samuel (1943)
		Rhinobatos Rhinobatos	Aplac Viviparous	Y	Y	Çek et al (2009)
	Rajidae	*Raja binoculata*	Oviparous	Y	Y	Hisaw Jr & Hisaw (1959)
		Leucoraja erinacea	Oviparous	Y	Y	Hisaw Jr & Hisaw (1959)
	Urotygonidae	*Urobatis halleri*	Viviparous	Y	-	Babel (1966)
	Dasyatidae	*Himantura bleekeri*	Aplac viviparous	Y	Y	Chatchavalvanich & Vesuttipat (1997)
Myliobatiformes	Myliobatidae	*Pteromylaeus bovinus*	Aplac viviparous?	Y	?	Giacomini (1896)
Subclass Holocephali						
Chimaeriformes	Chimaeridae	*Hydrolagus colliei*	Oviparous	Y	Y	Hisaw Jr & Hisaw (1959)

Table 3.2: Period of occurrence of atretic (AF) and postovulatory follicles (POF) in *Rhizoprionodon taylori*

	AF	**POF**
Stage 1	Dec – Feb	Feb
Stage 2	Mar	Feb
Stage 3	Apr – May	Mar – May
Stage 4	Jul – Sep	July – Sep

Chapter 4 Endocrine control of embryonic diapause and reproductive envents in *Rhizoprionodon taylori*

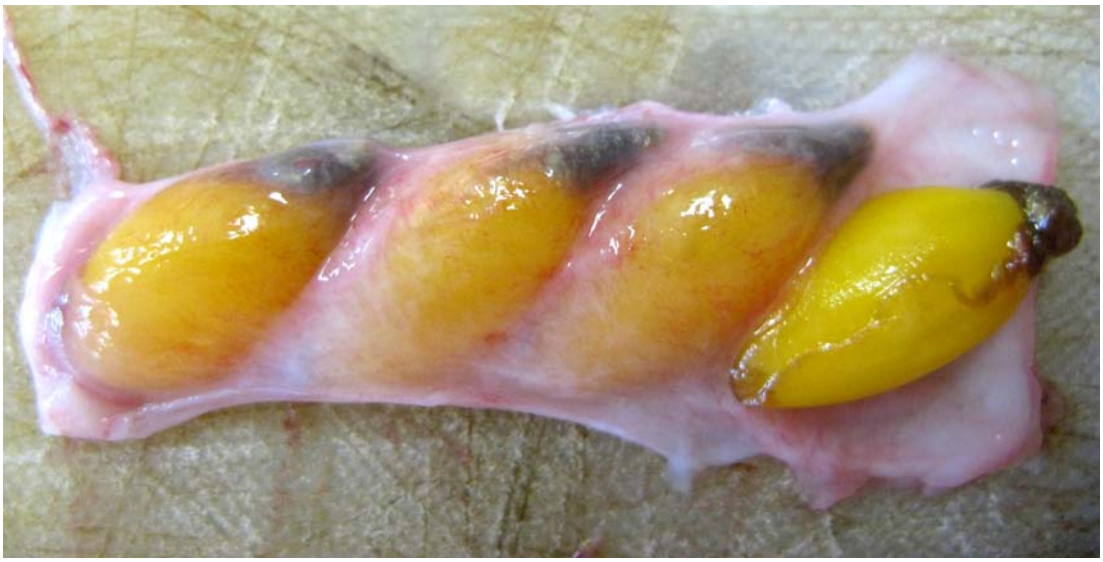

Plate 4: Uterus of female *Rhizoprionodon taylori* containing 4 diapausing uterine ova.

Waltrick, D.S., Awruch, C.A., Jones, S.M. & Simpfendorfer, C.A. **In prep.** Endocrine control of embryonic diapause and reproductive events in *Rhizoprionodon taylori.*

4.1. Introduction

The ability to evolve and adapt reproductive strategies in response to changes in environmental conditions ensures the evolutionary success of any species (Norris, 2007). As such, it has been postulated that habitat seasonality allows for the adjustment of reproductive events to occur over the most favourable period of the year (Angelini and Ghiara, 1984; Hastings et al., 2006). Seasonal breeders utilize predictable environmental fluctuations to maximize survival and reproductive success (Jacobs and Wingfield, 2000). By anticipating less favourable conditions, individuals are able to modify their morphology, physiology and/or behaviour by means of endocrine secretions (Jacobs and Wingfield, 2000). Among seasonal breeders, several mechanisms have evolved to cope with unfavourable conditions and reduce energy waste, including embryonic diapause (Angelini and Ghiara, 1984).

Embryonic diapause is the temporary ceasing or retardation of development during any stage of embryogenesis (Mead, 1993; Renfree and Shaw, 2000). Hypothetically, delaying gestation allows parturition to occur at the time of the most favourable environment that would enhance survival of young (Simpfendorfer, 1992; Marshall et al., 2007), or would allow females to restore their energetic reserves after events of birth, ovulation and mating, without compromising the success of the following gestation period (Chapter 2). Although this trait is believed to have evolved independently in at least 16 elasmobranch species displaying three different types of viviparity, the selective pressures that lead to embryonic diapause, as well as its benefits to the species, remain largely unknown (see review in Chapter 2).

Investigation of diapausing uterine eggs using histology and scanning electron microscopy have confirmed this trait exists in two species (*Rhizoprionodon taylori*, Simpfendorfer, 1992; *Dasyatis say*, Morris, 1999), while others remain unconfirmed. Aside from the occasional description of embryonic diapause in a number of elasmobranchs, there has been no systematic study of this phenomenon. Investigations have generally been limited to the observation of uterine eggs in the uteri for extended periods (e.g. Yamaguchi, 2006; Marshall et al., 2007). However, the physiological

mechanisms controlling this process have never been investigated within this group of vertebrates.

As in other vertebrates, reproductive events are associated with periodic cycles of steroid hormones and therefore, understanding elasmobranch endocrinology is important to accurately delineate reproductive processes (Tricas et al., 2000; Callard et al., 2005; Sulikowski et al., 2005; Awruch et al., 2008a). Testosterone (T), 17β-estradiol (E_2) and progesterone (P_4) are the main steroid hormones produced by the ovary (Gelsleichter, 2004) and are involved in controlling the reproductive cycle of female elasmobranchs. Estradiol is known to play a role in vitellogenesis and follicular development (Craik, 1978b; Callard et al., 1991), while P_4 can cause an antagonistic effect on E_2 (Perez and Callard, 1993). Other roles attributed to P_4 are a regulation of ovulatory events (Manire et al., 1995; Snelson et al., 1997; Tricas et al., 2000), parturition (Rasmussen and Gruber, 1993; Snelson et al., 1997; Tricas et al., 2000), implantation and the inhibition of myometrial contractions (Callard et al., 1992; Manire et al., 1995). The roles of T are less clear as it shows no distinct patterns among species and therefore it has been associated with several reproductive events (e.g. sperm storage, courtship, ovulation) (Rasmussen and Gruber, 1990; Rasmussen and Murru, 1992; Manire et al., 1995). Although previous investigation of these three key hormones have lead to a better understanding of the physiology of female elasmobranchs, their role in controlling embryonic diapause in this group remains unknown.

The endocrine control of embryonic diapause has been well studied in mammals (Lopes et al., 2004) and reptiles (Ewert, 2004). In these groups, concentrations of luteal P_4 are directly associated with the onset, maintenance and termination of this reproductive trait (Renfree and Shaw, 2000). However, an environmental cue, usually photoperiod, is necessary to trigger the endocrine pathways necessary to regulate the development of the corpora lutea (Curlewis, 1992), a temporary endocrine gland capable of synthesizing significant amounts of P_4 (Callard et al., 1989; Callard et al., 1992). For example, in mustelid carnivores, increasing day lengths increase the production of prolactin (a peptide hormone), which induces the development of the corpora lutea and production of luteal P_4 necessary for the regulation of embryonic diapause (Renfree and Shaw, 2000; Lopes et al., 2004). In mammals, P_4 is required to maintain pregnancy and the suppression of its production induces diapause. However, in many cases the administration of P_4 alone does

not induce the termination of diapause. Investigations in mustelids and rodents have shown that a combination of P_4 with some other(s) unknown luteal protein(s) (Foresman and Mead, 1978; Murphy et al., 1983) or estrogen (Dey et al., 2004), respectively, is necessary to induce the termination of diapause. There is no evidence in the literature for the involvement of T in the control of embryonic diapause in other vertebrate/ mammalian groups.

In this context, the aim of this study was to investigate the role of reproductive hormones, and their correlation with abiotic factors, in the control of diapause and reproductive events in the Australian sharpnose shark *R. taylori*. This species is a viviparous carcharhinid shark endemic to waters across northern Australia and southern Papua New Guinea (Last and Stevens, 2009). *R. taylori* is an annual reproducer with a gestation period of 11.5 months during which embryonic development is halted at blastodisc stage for a seven-month period (between February and September) of embryonic diapause (Simpfendorfer, 1992). This study describes the circulating steroid hormone levels of E_2, P_4 and T throughout the reproductive cycle of *R. taylori* in relation to changes in gonadal morphology and environmental parameters. This is the first time that steroid hormone levels are described in a diapausing elasmobranch species.

4.2. Materials and methods

4.2.1. Sampling

Female *R. taylori* were collected at night using 10 cm stretched mesh monofilament gillnets in Cleveland Bay, north Queensland (19º14’S, 146º48’E), monthly from February 2010 to February 2012. The nets were checked at 15-minute intervals. Shortly after capture, blood samples (3 ml) were collected from each specimen through caudal venipuncture using pre-heparinised syringes fitted with 22 gauges needles. Blood samples were preserved on ice for no longer than five hours and then centrifuged for five minutes at 1000 x g. The separated plasma was stored at -15ºC until analysed for hormone levels through radioimmunoassay.

A total of 152 females were captured. From these, 29 did not survive capture and were retained for examination of the reproductive organs. Blood samples were collected from

the remaining 123 animals, from which 23 were released back to the water. Two immature females measuring 510 and 593mm and six males ranging from 488 to 620 mm were captured during the sampling efforts but disregarded from all analyses.

All retained animals with vitellogenic follicles in the ovary or embryos or ova in the uterus were considered mature. Their total stretched length (TL; mm) was measured and total weigh (g) and liver weight (g) obtained in order to calculate the hepatosomic index (HIS = liver mass/ body mass X 100). For the analysis of reproductive organs, ovaries were extracted, weighted and the diameter of the largest follicle (MFD) measured to the nearest millimetre; the total number of ova or embryos in each uterus was counted, and total uterus weight (g) recorded, and the ova or embryos weighted (g) and measured to the nearest millimetre. Presence of atretic previtellogenic follicles (AF) and postovulatory follicles (POF) were recorded, however only AF was used in the statistical analysis due to the lack of data on POF. Females were classified into five reproductive stages (Table 4.1).

Environmental data for the study site was obtained from open online databases. Daily mean water temperature and air pressure were obtained from the Australian Institute of Marine Sciences (AIMS, 2012), and data on day length throughout the study period was obtained from U.S. Naval Observatory Astronomical Applications Department (USNO, 2011).

4.2.2. Radioimmunoassay

Plasma levels of P_4, T and E_2 were determined by radioimmunoassay (RIA) in 123 plasma samples. Plasma aliquots of 100µl were extracted with ethyl acetate (1 ml) and 100µl aliquots were then analysed.

17β-Estradiol and P_4 antiserum and E_2 and P_4 [1,2,6,7-^{3}H] were purchased from Sigma-Aldrich (Australia). The E_2 and P_4 antiserum were reconstituted by adding 5 ml of Tris buffer (pH 8, 0.1m HCl) and 50 µl of [1,2,6,7-^{3}H] E_2 and P_4 were diluted in 5ml of 100% ethanol and kept as stock for the assay. Duplicate standards (0–800 pg/tube authentic E_2 and P_4 in ethanol) and sample extracts were dried down and 200 µl of the reconstituted antiserum, diluted 1:10 in assay buffer (containing 0.1 % of gelatin and 0.01% of Thimerosal), and 100ul of the E_2 and P_4 stock, diluted 1:9 in assay buffer, added to each

tube. Samples were placed in a bath at 37°C for an hour. Bound and free fractions were then separated using dextran-coated charcoal and aliquots of the supernatants counted in a Beckman LS 5801 liquid scintillation counter. All assays were validated by the evaluation of the slope of serially diluted extractions of plasma against the assay standards. Extraction efficiency was determined from recovery of ^{3}H–labelled steroid added to pooled aliquots of plasma and assay values corrected accordingly. Extraction efficiency was 86% (E_2) and 90% (P_4). The detection limit for all assays was 0.02 $ng.ml^{-1}$. Intra and interassay variability was determined by including in each assay replicates of three levels of commercially available human control serum (CON 4, CON5, and CON 6 DPC). Interassay variability was 7% (E_2) and 9% (P_4) and intrassay variability less than 5%.

The T antiserum was Sirosera C-6050 (Bioquest) and [1,2,6,7-^{3}H] T was bought at Sigma-Aldrich (Australia). Duplicate standards (0–800 pg/tube authentic testosterone in ethanol) and sample extracts were dried down and the assay protocol used was as described by Nicol *et al.* (2005). The assay was validated by the evaluation of the slope of serially diluted extractions of plasma against the assay standards. The detection limit for the assay was 0.02 $ng.ml^{-1}$. Extraction efficiency was 90%, interassay coefficient of variation was 11% and intrassay variability was less than 4%.

4.2.3. Statistics

All statistical analysis were conducted using R system 2.15.0 (R Development Core Team, 2012) at a critical probability level of 0.05. Hormone concentrations failed the Kolmogorov-Smirnov test for normality and therefore nonparametric statistic tests were used. Differences in samples medians (steroid hormone, TL and HSI) over time and different stages were evaluated by Kruskal-Wallis one-way ANOVA on ranks followed by a pairwise Wilcoxon rank sum tests with Bonferroni adjustment. The Spearman correlation (Savicky, 2009) was used to determine the existence of associations between hormone levels, environmental parameters and morphometric parameters.

4.3. Results

4.3.1. Reproductive cycle

Females ranging from 517 to 975 mm TL (688 ± 4 mm; mean±SE) were captured in all months of the year, except in June. Mean TL did not differ significantly between months (Kruskal Wallis, rho = 15.48, df = 10).

The average litter size per female was 5.29 ± 0.18 embryos (range: 2 - 10). Diapausing ova, observed in the uterus from mid February to mid September, were bright-yellow dense yolk masses concealed in brown egg cases measuring 23.85 ± 0.27 mm (range: 21 – 29 mm). Upon termination of diapause (stage 1), uterine ova became flaccid and slightly increased in size (29 ± 0.18 mm, range: 25 – 32 mm). At this stage, from mid September, embryos from 15 to 17 mm were first visible. Embryos grew continuously to size up to 212 mm in January, when parturition occurs (Figure 4.1).

The development of ovarian follicles commenced in late diapause (August). This initially slow process sped up on the two months prior to parturition (December to January; Figure 4.2), when the largest embryonic size was recorded. Independent of the litter size, a single AF was present in the ovary throughout the diapausing period. The maximum size of these structures (26 mm) was observed in postovulatory females, after which it continuously reduced in size. The identification of AF at the end of the diapausing period (August – September) was difficult and sometimes not possible due to its small size (similar to POF) and atresia of previtellogenic follicles (1-2 mm).

The HSI showed a clear pattern throughout the reproductive cycle, ranging from 1.2% in *postpartum* females, to 9.6% in late diapause females (Figure 4.3). In fact, statistical analysis showed a significant difference in median HSI among the reproductive stages ($\chi^2[5]=36.341$, $p<0.001$).

4.3.2. Serum steroid analysis

All reproductive hormones showed distinct cycles throughout the year (Figure 4.4), however statistical analysis showed no significant correlation amongst them indicating that levels changed independently (P_4 vs. E_2: $r_s[123]= -0.110$, $p = 0.23$; P_4 vs. T: $r_s[123]= -$

0.011, p = 0.90; E_2 vs. T: r_s[123]= 0.150, p = 0.10). Restricted sample sizes of different stages in January and February did not allow statistical determination of median differences amongst steroids at different stages over this period. However, differences in hormone levels between stages at each given month were examined when necessary. None of the steroids showed a significant correlation to liver weight (E_2: r_s[98]= -118, p = 0.25; P_4: r_s[98]= 0.097, p = 0.34; T: r_s[98]= 0.113, p = 0.27).

4.3.3. 17ß-Estradiol

Concentrations of E_2 ranged from 0.03 to 6.2 ng.ml^{-1} (1.42 ± 0.13) throughout the year (Figure 4.4). Plasma levels remained low through most of the diapausing period, starting to increase at the beginning of follicular development. Levels gradually increased to a peak at late stage 2 and postpartum females before declining prior to ovulation (Figure 4.5). A Kruskal Wallis test revealed significant differences between E_2 levels at different reproductive stages (χ^2[5]=46.52, p<0.01). A post-hoc test using pairwise Wilcoxon rank test with Bonferroni correction showed significant differences between levels of this hormone in early diapause and late diapause, stage 2 and stage 3 respectively.

Estradiol levels were significantly correlated to ovarian structures. This association was positive with MFD (r_s[100]= 0.658, p<0.001; Figure 4.6A) and negative when correlated with the size of AF observed during embryonic diapause, from February to October (r_s[54]= -0.479, p<0.001; Figure 4.6B).

4.3.4. Progesterone

Serum levels of P_4 (0.900±0.082; range: 0.02 – 5.49 ng.ml^{-1}) in female *R. taylori* remained relatively low and not significantly different throughout the year (Figure 4.4). However, animals at different reproductive stages in January and February showed distinct differences in P_4 concentration. The only stage 2 female captured in January showed P_4 level almost undetectable (0.02 ng.ml^{-1}), being much lower than the three postpartum females (1.81 ng.ml^{-1} ± 0.53, range: 0.97 – 2.80 ng.ml^{-1}). Progesterone levels then increased in February to a peak (3.55ng.ml^{-1}) in one ovulating female before a sharp decrease observed in six females in early diapause stage (0.43 ng.ml^{-1} ± 0.17, range: 0.02 – 0.85 ng.ml^{-1}). Thus, in general, P_4 levels increased from late pregnancy to a peak at ovulation before returning to basal levels at early gestation (Figure 4.5). There was no

significant correlation of this hormone with the female's reproductive stage (χ^2[5] = 9.329, p = 0.10), AF size (r_s[95] = 0.147, p = 0.15) or MFD (r_s[100] = -0.014, p = 0.89).

4.3.5. Testosterone

Plasma T levels (1.86 ± 0.17, range: 0.02 – 7.20 ng.ml^{-1}) varied widely throughout the year (Figure 4.4). In January and February, where sample sizes of different stages were too low to allow statistical comparisons some distinct variations were observed. In January, a rapid increase in T level was observed from one stage 2 female (0.33 ng.ml^{-1}) compared to three *postpartum* females (2.01 ± 0.62; range: 0.81 – 2.84 ng.ml^{-1}), before returning to basal levels in February at ovulation and early diapause (0.68 ± 0.13; range: 0.06 – 1.13 ng.ml^{-1}). Therefore, it appears that T levels may undergo a small ephemeral *postpartum* peak before reducing at ovulation.

Levels of T varied significantly throughout different reproductive stages (χ^2[5]=29.87, p<0.01; Figure 4.5). The Wilcoxon rank test with Bonferroni correction showed significant differences between levels of this hormone in late diapause and stages 2 and 3. However, it is important to notice that high temporal variability of T concentrations during each given reproductive stage influenced this result. In fact, T concentrations remained elevated through most of embryonic diapause, after which two distinctive peaks were observed (Figure 4.4). The first one, in September, was analysed using a Kruskal-Wallis test, which revealed a significant difference in plasma T levels at different stages occurring in September (χ^2[2]=13.9, p<0.01). A post-hoc test showed significant differences in T in late diapausing females (1.09 ± 0.20; range: 0.39 – 2.93 ng.ml^{-1}, n=11) compared to those at active development stages 1 and 2 (3.46 ± 0.33; range: 0.53 – 7.09 ng.ml^{-1}, n=23). This suggests a possible role for T in the termination of diapause. The second peak occurred at Stage 2, in November, and was not correlated to any observed morphological change. Testosterone levels showed a weak positive correlation to MFD (r_s[100]= 0.22, p=0.027) and negative to AF size (r_s[95]= -0.24, p=0.018; Figure 4.7).

4.3.6. Abiotic factors and reproduction

The water temperature in Cleveland Bay varied in close association with day length (Figure 4.8). Maximum seawater temperature of 30.1°C was recorded in February 2010

and January 2011 and after this peak, temperature dropped gradually to a minimum of 20.8°C and 19.6°C during winter in July 2010 and June 2011 respectively. Day length at the study site latitude varied from 13.3 h in December to 10.9 h in June. Statistical analysis showed significant correlation between the environmental variables and E_2 (day length: $r_s[123]= 0.487$, $p<0.001$, Figure 4.9A; water temperature: $r_s[127]=0.360$, $p<0.001$, Figure 4.9B), but not with T or P_4. Amongst the measured biological parameters, environmental parameters have also shown a significant correlation with HSI (day length: $r_s[98]= -0.557$, $p< 0.001$; and water temperature: $r_s[96]= -0.664$ $p <0.001$) and MFD (day length: $r_s[100]= 0.656$, $p< 0.001$; and water temperature: $r_s[98]= 0.559$, $p <0.001$).

4.4. Discussion

This is the first assessment of hormone levels of a diapausing elasmobranch providing quantitative description of the main circulating reproductive hormones throughout the annual reproductive cycle. The amplitude of hormonal change in female *R. taylori* in association to reproductive events suggests some involvement of E_2, T and P_4 in the regulation of these reproductive events. The results of this study indicate that, unlike mammals and reptiles, T and P_4 (to a lesser degree) are possibly involved in the control of embryonic diapause, but only T seems to be required at the release from this reproductive trait. Although E_2 does not appear to have a direct role in controlling diapause, evidence suggests that photoperiod influences levels of this hormone, which could possibly be involved in the regulation and synchronization of reproductive events.

4.4.1. Reproductive biology

Rhizoprionodon taylori displays little variation in reproductive characteristics across its geographical range in Australia (Stevens and McLoughlin, 1991; Simpfendorfer, 1992). The smallest pregnant female recorded in this study (454 mm TL) was significantly larger than previously reported by Simpfendorfer (1992) in the same location, but similar to those reported by Stevens and Mcloughlin (1991) for samples caught in Northern Australia. Populations are highly synchronous and reproduce every year, with a gestation period of 11.5 months, which includes a period of seven months of embryonic diapause. As previously shown by Simpfendorfer (1992) shortly after fertilization, in February, the

embryonic development was arrested and uterine eggs showed no changes in weight or size until September.

It is well known that the liver is an important energetic reservoir, utilized in the production and maturation of large oocytes and the nourishment of the developing embryos (Craik, 1978a; Lucifora et al., 2002). Commonly, mating and fertilization quickly follow parturition in annual breeders with concurrent ovarian and gestation cycles, such as *R. taylori* (Simpfendorfer, 1992; Carrier et al., 2004). Nonetheless, all seasonal breeders with consecutive ovarian cycle and gestation period (*Carcharhinus plumbeus*, Cliff et al., 1988; *C. isodon*, Castro, 1993) require a 'resting stage', usually one year, in order to recover their hepatic reserves and produce large vitellogenic follicles in preparation for the following gestation (Castro, 1993; Carrier et al., 2004). This study indicates that a diapausing period, as the resting stage, could allow females to restore energetic reserves in preparation for the subsequent ovarian cycle and embryonic nourishment, once development is restarted. Although more studies are required to confirm this, it seems that embryonic diapause allows females to restore their fitness between gestations without undergoing a biennial cycle.

4.4.2. Maintenance of diapause

Moderate levels of P_4 in *R. taylori* throughout most of the diapausing period (except in May) indicate a possible role of this steroid in maintaining this phase of arrested development. Progesterone is closely related with the vertebrate gestation and is generally required to allow the embryonic development of mammalians. In fact, where diapause occurs within this group, P_4 production is inhibited during the period of arrested development, only allowing enough secretion to maintain the viability of the embryos (Mead, 1993; Desmarais et al., 2004). However, this steroid hormone seems to have evolved different roles among elasmobranchs as only few species have been shown to elevate P_4 levels through the gestation period (e.g. *Squalus acanthias*, Tsang and Callard, 1987a; Tsang and Callard, 1987b; *Sphyrna tiburo*, Manire et al., 1995). The role of P_4 in *R. taylori* seems to be diametrically opposed to mammals and remained at moderate levels through most of diapause. The role of P_4 working as an inhibitor to the embryonic development should be considered for this species in future research.

Nevertheless, it is also possible that P_4 does not play a direct role in the inhibition of active embryonic development. Another role attributed to P_4 in elasmobranchs is the inhibition of the spontaneous uterine contractions, maintaining a quiescent uterus for recently formed embryos in *S. acanthias* (Sorbera and Callard, 1995). Sorbera and Callard (1995) suggested that uterine contractions allow the flushing of seawater into the uterine cavity, providing oxygenated water and the removal of waste materials. Assuming that, as in other vertebrates, diapausing embryos of *R. taylori* have very low metabolic rates (Renfree and Shaw, 2000; Ptak et al., 2012), the exchange of water and removal of waste material is unnecessary and could potentially damage the delicate embryos. Therefore it seems possible that, as in *S. acanthias*, sustained moderate P_4 levels in diapausing *R. taylori* could play a role in the inhibition of uterine contractions, providing embryos with a quiescent uterus through this period.

The major site of P_4 synthesis in elasmobranchs has been postulated to be the CL, which can be formed after ovulation from the remnants of postovulatory follicles or from atretic preovulatory follicles (Lutton et al., 2005). The presence of both POF and AF persisting in the ovary through most of the diapausing period suggests these structures could be responsible for the observed sustained P_4 levels during embryonic diapause. The lack of a significant correlation between AF size and levels of P_4 in *R. taylori* could indicate that the synthesis capacity of this temporary gland is not directly associated with its size. Alternatively, this could indicate that the POF or other structures could potentially be more important for the P_4 production in this species. Studies in reptiles have demonstrated the capacity of P_4 synthesis within the placental and ovarian tissues (Guarino et al., 1998; Girling and Jones, 2003).

Elevated T concentrations through most of the diapausing period (April to July) suggest this hormone may also function in the maintenance of embryonic diapause. Androgens have never been reported to have an involvement in the regulation of diapause of mammals or reptiles. Within non-diapausing elasmobranchs, concentrations of T are generally low during pregnancy and do not seem to have a specific role during this period. The exception is *Dasyatis sabina* (Snelson et al., 1997) where T was associated with changes in uterine events (embryonic nourishment). Histotroph secretion (uterine nourishing secretions) in *R. taylori* only starts at the release from embryonic diapause (Simpfendorfer, 1992), therefore increased T levels during this period could suggest this

androgen is required to maintain the embryo viability while in the arrested development period.

Contrary to P_4 and T, which seem to be associated with the regulation of some reproductive events, including embryonic diapause, the major role of E_2 seems to be with follicular growth and maturation. The present data demonstrated a strong linkage between increasing levels of E_2 and vitellogenesis, which increase concomitantly from late diapause to a maximum in *postpartum* animals. Similar patterns have been reported for several elasmobranchs studied to date (Callard et al.; Rasmussen and Murru, 1992; Snelson et al., 1997; Koob and Callard, 1999; Tricas et al., 2000), corroborating with earlier *in vitro* studies where the E_2 synthesis capacity by the elasmobranch ovarian follicles have been demonstrated (Fileti & Callard 1990 apud Tsang and Callard, 1987b; Callard et al., 1993). Once synthesised and secreted, E_2 has been shown to induce the liver to produce vitellogenin, the main yolk precursor (Koob and Callard, 1999; Gelsleichter, 2004). Thus, the strong correlation between E_2 levels and MFD observed in this study would reflect this vitellogenin production by the liver (Craik, 1978a; Koob and Callard, 1999). Therefore, it seems that this sex steroid is not directly involved in the regulation of embryonic diapause but, as in other elasmobranchs, it is essential for the control of ovarian events of *R. taylori*.

4.4.3. Termination of diapause and active development

Amongst the measured hormones, the termination of embryonic diapause and reactivation of embryonic development in *R. taylori* possibly only requires T presence. Unlike previous hypotheses that, as in other diapausing vertebrates, P_4 would play an important role at the reactivation of normal development in elasmobranchs (Chapter 2), the data in the present study do not show a peak in P_4 prior to or at the release from embryonic diapause in September. Instead, a 3-fold increase in levels of T was observed in females from late diapause to stages one and two, suggesting this hormone is required for the reactivation of embryonic development.

A further decline in P_4 levels following the reactivation of active development supports the aforementioned theories that this sex steroid could be playing an inhibitory role in embryonic development and/or regulation of uterine contractions. Testosterone, on the

other hand, continued moderately elevated through the remaining part of gestation. A peak in November was not associated with any change in morphometric parameters, however previous observations by Simpfendorfer (1993b) suggest the timing could be associated with the transition of embryonic nourishment (from histotroph to placenta). Testosterone has been previously associated to changes in embryonic nutrition in at least one elasmobranch. Embryos of *D. sabina* are nourished by yolk followed by uterine secretions and this transition is associated with a peak in T (Snelson et al., 1997). Thus, T could possibly play a role in the uterine events related to the embryonic nutrition.

4.4.4. Late pregnancy and postpartum events

Although only small sample sizes were obtained from animals at the two stages occurring in both January (late stage 2 and *postpartum*) and February (ovulating and diapause), the patterns of hormonal change were similar to previously studied elasmobranchs, and therefore allows for some assumptions. Preovulatory peaks of E_2 and T levels followed by increments in P_4 concentrations at ovulation (when E_2 and T levels sharply drop), observed in *R. taylori*, have been previously reported in a number of oviparous (*Raja erinacea*, Koob et al., 1986; Koob and Callard, 1999) and viviparous elasmobranchs (*Negaprion brevirostris*, Rasmussen and Gruber, 1993; *S. tiburo*, Manire et al., 1995; *S. acanthias*, Koob and Callard, 1999). A surge of T during preovulatory events is commonly linked with reproductive behaviour, ovulation and courtship events (Rasmussen and Gruber, 1993; Manire et al., 1995; Snelson et al., 1997; Tricas et al., 2000), while a peak in P_4 is associated with the termination of the vitellogenic process. Therefore, it seems that in *R. taylori*, increased P_4 and T levels are necessary to induce ovulation and inhibit further enlargement of vitellogenic follicles stimulated by E_2.

4.4.5. Environmental factors

The present data indicated that abiotic factors could play a role as reproductive cues and synchronizing reproductive events in *R. taylori*. Strong relationships have been shown between the abiotic factors and MFD and HSI, which led to suggest that a certain level of dependency could exist between these variables and the changes in environmental factors. Additionally, the correlation of abiotic factors and E_2 support the idea of a possible dependency on environmental conditions to activate the synthesis of this hormone within this species.

Although the use of external factors as cues to regulate the timing of reproductive cycle is well known in other vertebrates (Lopes et al., 2004; Hastings et al., 2006), this strategy is not well documented in male and female elasmobranchs. While an association between abiotic factors and hormonal levels has been demonstrated in at least three elasmobranchs (*Scyliorhinus canicula*, Sumpter and Dodd, 1979; *Hemiscyllium ocellatum*, Heupel et al., 1999; *Urobatis halleri*, Mull et al., 2008; 2010), controlled experiments demonstrating such dependence has only been shown in male *U. halleri* (Mull et al., 2008), in which temperature has a role in elevating T levels.

Whether photoperiod or water temperature acts as reproductive cues controlling reproductive events (including embryonic diapause) in *R. taylori* and other elasmobranchs requires further investigation in controlled environments. Thus, it seems likely that a highly synchronous population, such as *R. taylori* in Cleveland Bay, would require a fairly stable element to allow changes in reproductive stages to occur a short period apart amongst individuals.

4.5. Conclusions

This study provides the profile of hormones throughout the reproductive cycle of a diapausing elasmobranch and suggests that *R. taylori* may have evolved distinct endocrine pathways from other studied diapausing vertebrates. The data in the present study suggests the involvement of P_4 and T in the control of embryonic development, while only T appears to be required for the reactivation of embryonic development, after diapause. As in other elasmobranchs, levels of E_2 were closely associated with ovarian follicles size. This investigation provides support for the regulation of reproductive events by environmental variables.

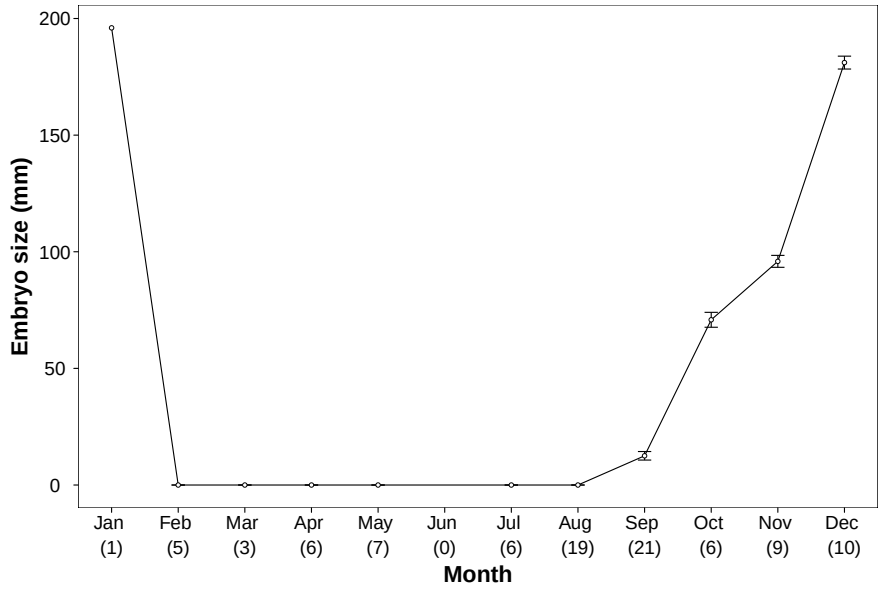

Figure 4.1: Embryo development of *Rhizoprionodon taylori* from Cleveland bay, QLD Australia. Values are mean embryo size (±SE). Numbers in brackets denote sample size

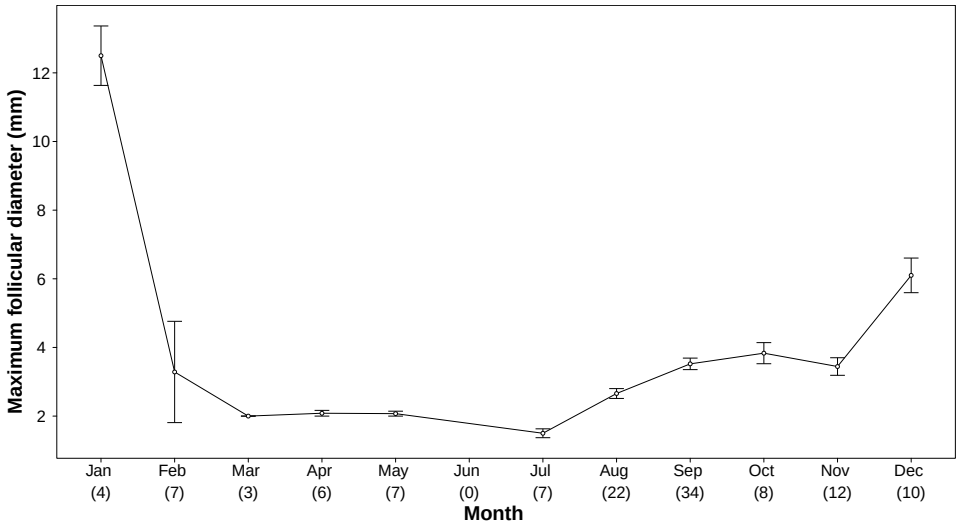

Figure 4.2: Average monthly size (±SE) of maximum ovarian follicles in *Rhizoprionodon taylori* from Cleveland bay, QLD Australia. Numbers in brackets denote sample size

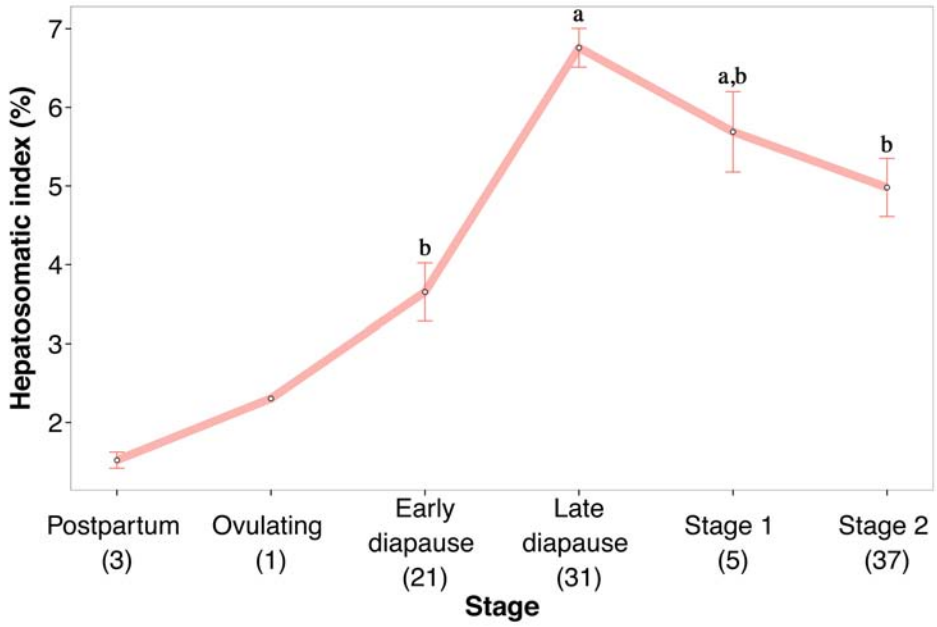

Figure 4.3: Hepatosomatic index variation throughout reproductive cycle in female *Rhizoprionodon taylori* from Cleveland bay, QLD Australia. Values are medians ± SE. Letters depict statistical similarity using pairwise Wilcox post-hoc test. Missing letters represent groups that are too small for statistical analysis. Numbers in brackets denote sample size

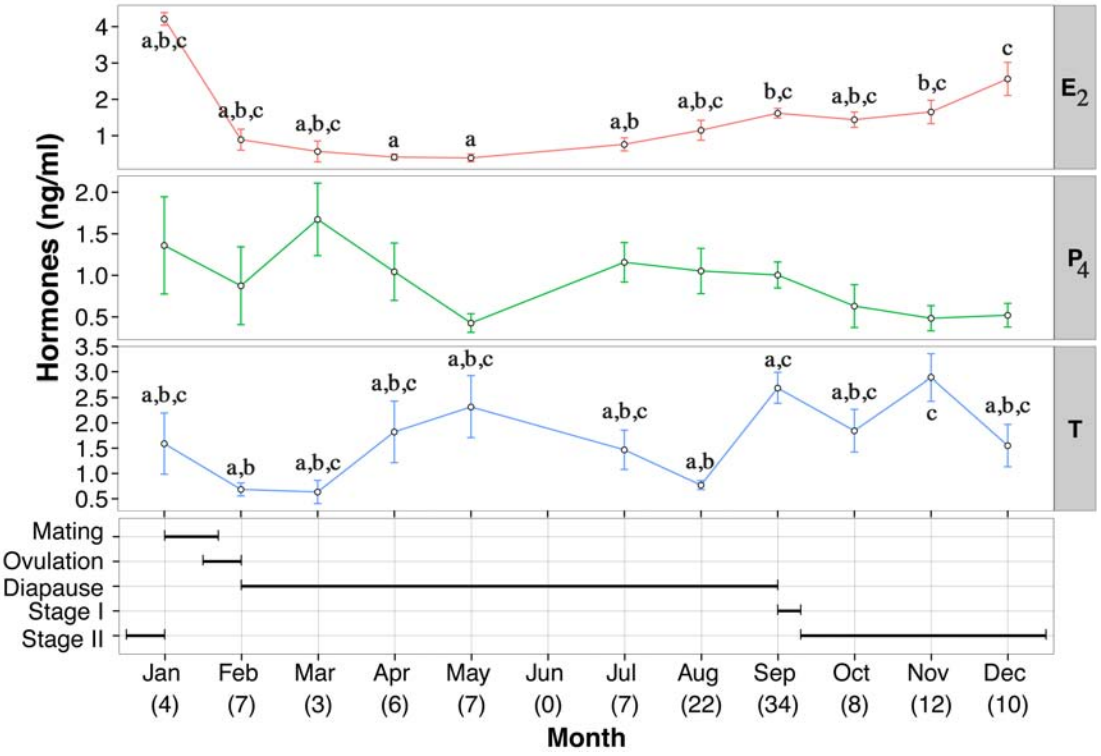

Figure 4.4: Seasonal variation of 17β-estradiol (E_2), progesterone (P_4) and testosterone (T) in *Rhizoprionodon taylori*. Letters depict statistical similarity using pairwise Wilcox post-hoc test. Progesterone levels were not significantly different throughout the year. The graph at the base of the figure indicates the timing of each reproductive event in females from Cleveland bay, QLD Australia and numbers in brackets denote sample size

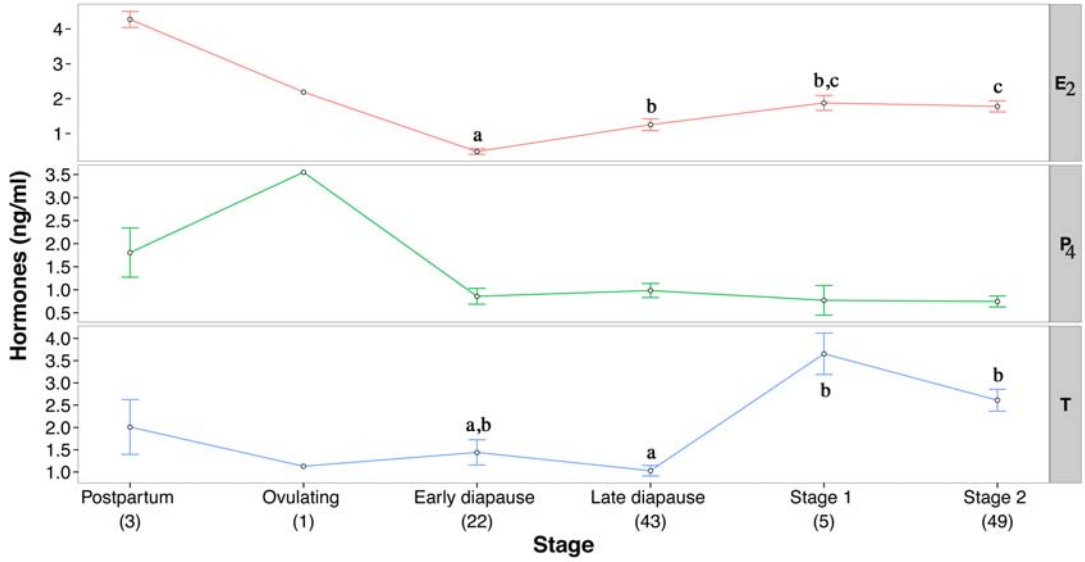

Figure 4.5: Mean plasma steroid hormone concentrations at different reproductive stages of female *Rhizoprionodon taylori* from Cleveland bay, QLD Australia. Letters depict statistical dissimilarity using pairwise Wilcox post-hoc test. Missing letters in E_2 and T graphs represent groups with sample sizes too small for statistical analysis. Progesterone levels were not significantly different among reproductive stages. Numbers in brackets denote sample size

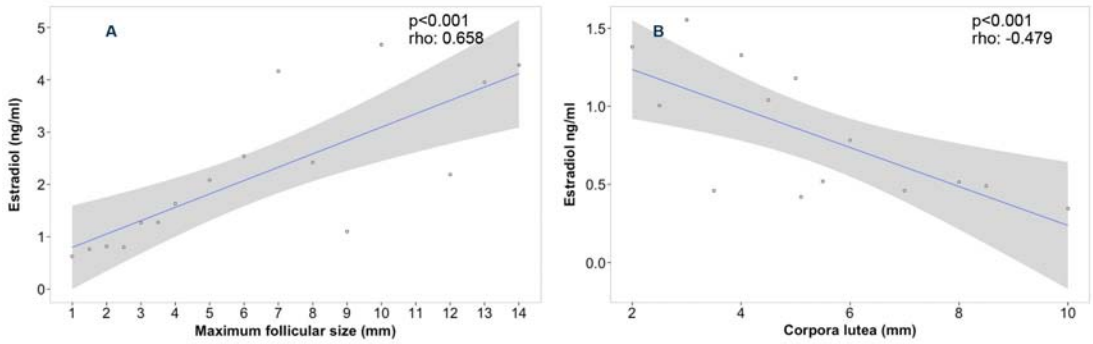

Figure 4.6: Relationship between plasma 17ß-estradiol (E_2) levels and ovarian follicles in *Rhizoprionodon taylori* from Cleveland Bay, QLD Australia. Changes were significantly correlated with (A) maximum follicular diameter, and (B) corpora lutea-like follicles observed from February to October. Graphs show the correlation, including regression line and confidence interval of 95% represented by the shaded area

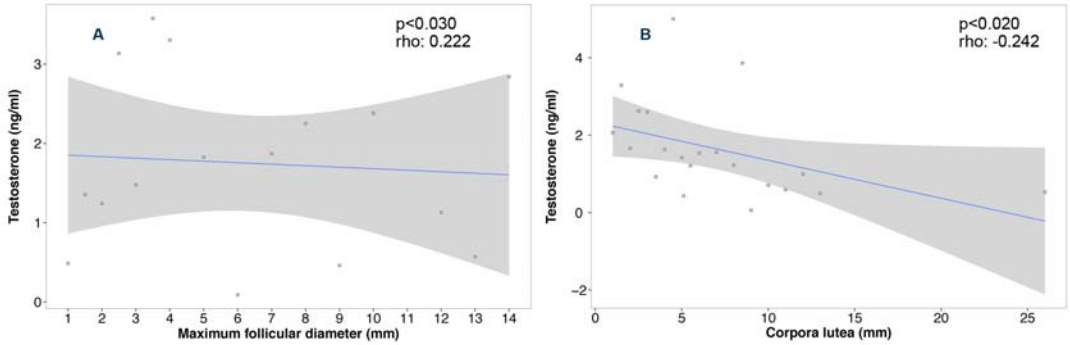

Figure 4.7: Relationship between testosterone (T) levels and ovarian follicles in *Rhizoprionodon taylori* from Cleveland bay, QLD Australia, demonstrated a weak correlation with changes in measured biometrical parameters. A weak correlation was observed between T and (A) maximum follicular diameter, and (B) corpora lutea-like follicles observed from February to October. Graphs show the correlation, including regression line and confidence interval of 95% represented by the shaded area

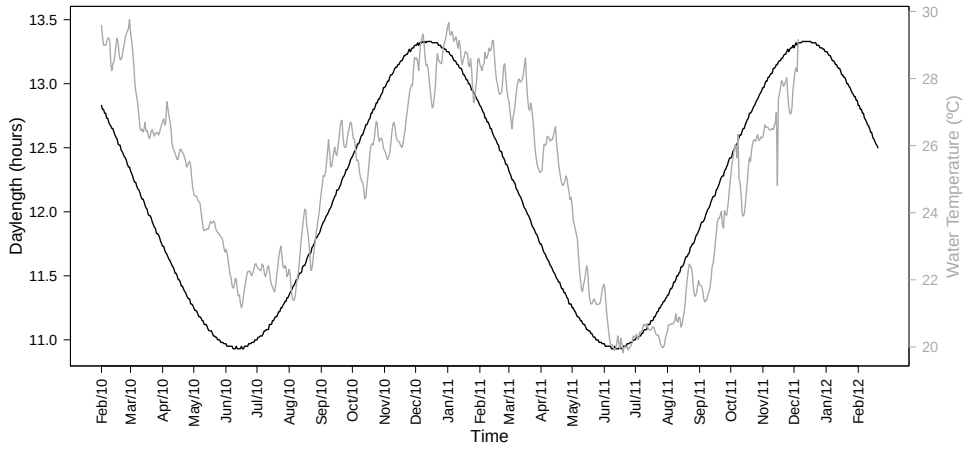

Figure 4.8: Monthly water temperature (ºC) and daylength (hours) in Cleveland bay, QLD Australia, from February 2010 to February 2012. Data obtained AIMS (2011) and USNO (2011)

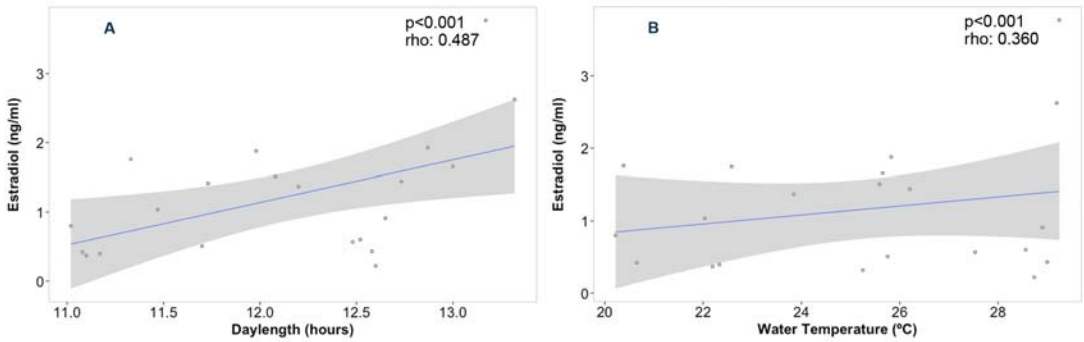

Figure 4.9: Seasonal changes in Estradiol (E_2) levels in *Rhizoprionodon taylori* were correlated with changes in environmental parameters. Changes in E_2 levels were positively correlated with (A) day length and (B) water temperature. Graphs show the correlation, including regression line and confidence interval of 95% represented by the shaded area

Table 4.1: Classification of reproductive stages for female *Rhizoprionodon taylori*. Numbers within the ovary column represent the range of maximum follicular size during each reproductive stage

Stage	Description	Ovary	Uteri
Postpartum	Not pregnant	Ripe follicles (10-14 mm)	Empty
Ovulating	Presence of ripe ova and uterine ova	Ripe follicles (~12.0 mm)	Ova half-way down the oviduct and/ or uterus
Diapause	No visible embryos	Only previtellogenic follicles (1-3 mm)	Presence of uterine ova
1	Active embryonic development	Developing follicles (3-5 mm)	Flaccid uterine ova (not attached to uterus) and visible embryo
2	Active embryonic development	Developing follicles (2.5-13 mm)	Visible embryo attached to the uterus

Chapter 5 General discussion

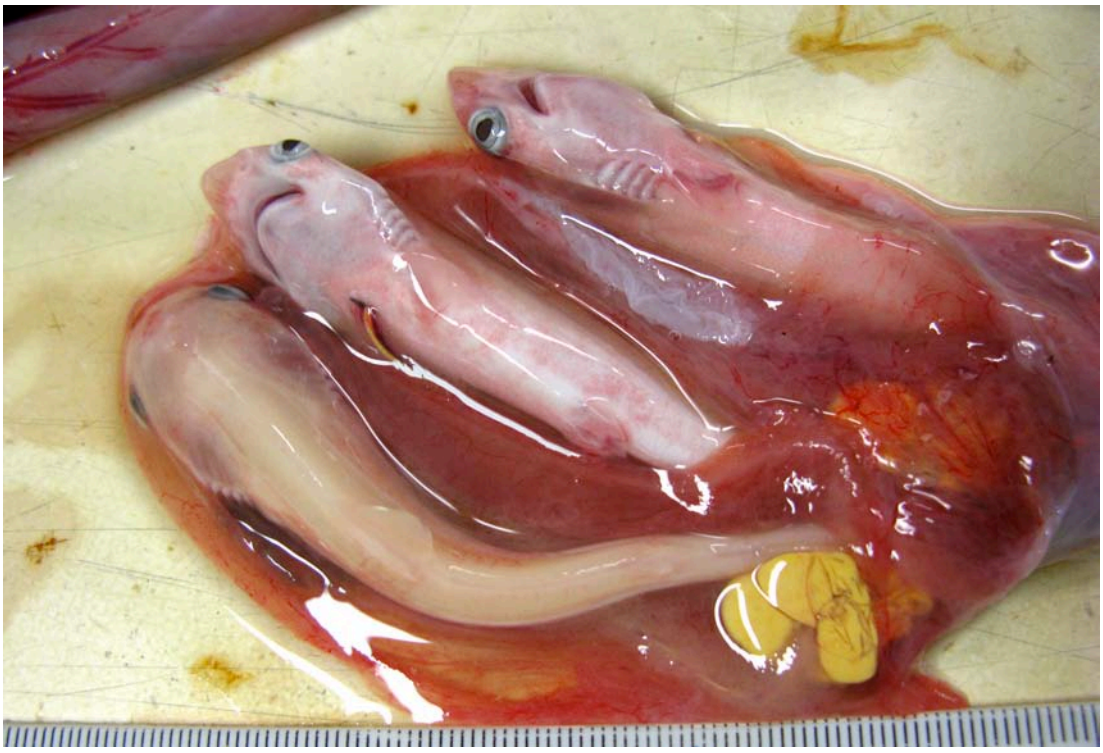

Plate 5: Uterus of a female *Rhizoprionodon taylori* containing three Stage 2 embryos.

The diversity of reproductive modes (from oviparity to viviparity) that evolved in elasmobranchs over hundreds of million years may have partially contributed for the resilience of this group (Carrier et al., 2004; Grogan and Lund, 2004). In particular, numerous reproductive specialisations originated within this group, possibly allowing species to cope with harsh ecological (biotic and abiotic) conditions while increasing reproductive success (Angelini and Ghiara, 1984) across a broad range of habitats. One such specialisation is embryonic diapause. This reproductive strategy is beneficial in situations when further embryonic development is risky due to external (e.g. adverse climates) or internal factors (e.g. maternally driven stimuli, such as during lactation in mammals) (Ptak et al., 2012). However, consideration of diapause in elasmobranchs to date has been purely to report its occurrence. In view of the importance of diapause as a reproductive strategy that enhances reproductive success, the occurrence of diapause was examined and compared among diapausing elasmobranchs and other vertebrates (Chapter 2). For the first time, the ovarian follicles (Chapter 3) and reproductive hormone levels (Chapter 4) of a diapausing elasmobranch were investigated in an effort to begin understanding the control mechanisms of diapause in elasmobranchs.

The current model on the control of diapause in vertebrates suggests that a suppression of the CL results in the arrest of embryonic development. The suppression of this ovarian gland leads to a reduction in the levels of P_4, a key hormone in controlling embryonic development in mammals (Mead, 1993; Lopes et al., 2004). Within this group, P_4 remains basal during the diapausing phase only increasing at the recommencement of normal embryonic development (Mead, 1993). Thus, the initial hypothesis for the elasmobranch endocrine control of diapause proposed in Chapter 2 suggested that a secondary source of P_4 would probably set in after the termination of diapause and degeneration of CL-like structures and provides levels high enough to support active embryonic development. However, results reported in Chapters 3 and 4 contradict this theory and provide new insights on the elasmobranch control of diapause (Figure 5.1).

Morphological investigations in Chapter 3 identified structures homologous to the CL (i.e. AF and POF) in the ovary of *Rhizoprionodon taylori* throughout the diapausing period, during which these follicles attain full morphological development and degenerate. At the termination of diapause and recommencement of active embryonic

development these structures are in advanced degenerative conditions and do not regenerate, which contrasts with reports in other vertebrates such as the mammalian mustelid mink (Murphy et al., 1981). Unlike other vertebrates, levels of P_4 remained moderate throughout most of the diapausing period. The recommencement of normal embryonic development associated with the pronounced reduction in circulating levels of P_4 indicates a possible role of this hormone in interrupting embryonic development in *R. taylori*. In addition, although the steroidogenic capacity of ovarian follicles was not assessed in the present study, the occurrence of AF and POF in parallel with elevated P_4 levels in this species suggests that these structures are probable sources of this steroid.

This is the first report in which an androgen has been associated with the control of embryonic diapause. The roles of T in the reproductive cycle of female vertebrates include the stimulation of sexual behaviour and suppression of ovulation in mammals (Krishna, 1996; Norris, 2007) and some role between vitellogenesis and mating in reptiles (Cree et al., 1992; Guillette Jr et al., 1997). However, the roles of T in elasmobranch are less clear. Elevations of this androgen have been previously associated with sperm storage, courtship and ovulation (Rasmussen and Gruber, 1990; Rasmussen and Murru, 1992; Manire et al., 1995). The observed T elevation concurrent with the termination of embryonic diapause and recommencement of active development in *R. taylori* in the present study provides the first evidence for the involvement of this hormone on control of embryonic diapause of a vertebrate. However, the specific role that T plays in the reactivation of active development in *R. taylori* remains unclear.

Considering the diversity of reproductive modes within elasmobranchs, the generalisation of hormonal control within species in this group is considered imprudent (Gelsleichter, 2004). Although the role of E_2 in the synthesis of vitellogenin is well established in elasmobranchs, the specific roles of other steroids such as P_4 and T are less understood and likely to vary among species. For instance, sustained increased P_4 levels during the embryonic development has only been observed in few elasmobranchs (Tsang and Callard, 1987b; Manire et al., 1995; Mull et al., 2010) while in others this hormone drops after fertilization and remains low during pregnancy (Rasmussen and Gruber, 1993; Snelson et al., 1997; Henningsen et al., 2008). Mull (2010) suggests the difference in reproductive cycle (e.g. annual, biennial) and different types of matrotrophy could reflect this variability of hormonal function within elasmobranchs. Similarly, species reported to

undergo diapause are mostly lecithotrophic or matrotrophic aplacental and only *R. taylori* is matrotrophic placental and thus are likely to display variability in the way they control events that regulate embryonic development and its arrest.

Estradiol does not seem to play a direct role in the control of embryonic diapause in *R. taylori*. However, a strong linkage between this hormone and both follicular growth and abiotic factors suggests that certain environmental conditions may activate the synthesis of this and possibly other steroid hormones. The use of environmental factors as triggers to the endocrine system is well known in other vertebrates. This mechanism however is less clear in elasmobranchs. Results from the present study suggest that extreme temperature or day length events (solstices) may function in the synchronization of the reproductive cycle and timing of reproductive events in *R. taylori* (including diapause). For instance, parturition and ovulation occur shortly after the summer solstice (maximum annual temperature and day length). The source of the peak in P_4 observed at ovulation is likely to be the AF, formed late in the previous gestation, while moderate levels sustained throughout most of the diapausing period are possibly synthesised by the AF and POF present in the ovary. Following the winter solstice, when temperature and day length start to increase, folliculogenesis is started and a peak in T is likely to induce the termination of diapause, concurrent with the final degeneration of AF and POF, reducing P_4 to basal levels (Figure 5.1).

5.1. Future research directions

A total of 88% of the elasmobranch species reported with diapause to date remains to be confirmed. Histological investigations accessing the developmental stage of embryos throughout the period where no macroscopic development was observed is required to confirm the absence of embryonic development in at least 14 elasmobranchs. There are still many questions remaining to be answered concerning the basic biology of most diapausing species and the benefits it provides them. With new species still being discovered and the increasing number of studies reporting the basic biology of elasmobranchs (White et al., 2012) the number of diapausing species is likely to increase.

There is a clear need for more endocrine studies in diapausing elasmobranchs in order to elucidate the specific roles and importance of each hormone associated with the

developmental arrest and their variation within this taxon. Investigation on the distribution of hormone receptors in diapausing females and their diapausing embryos will also assist on understanding the specific roles of each hormone in the endocrine control of development. Ultimately, experimental studies will be necessary to confirm these theories by experimentally inducing and terminating embryonic diapause under controlled environments.

Understanding diapause and reproductive control in elasmobranchs can contribute to the field of vertebrate endocrinology as a whole. Considering the conservation of endocrine systems through the evolutionary history, studies on the hormonal regulation in elasmobranchs can provide insights into the evolution of endocrine systems and contribute to a better understanding of the regulation of reproduction in other vertebrates (Dodd, 1972b; Callard et al., 1989; Gelsleichter, 2004). Moreover, in order to elucidate the steroidogenic potential of ovarian follicles of *R. taylori*, future researches should also take into consideration in vitro and histochemical investigations. Such investigations can indicate the possible sources of the observed steroid peaks during the reproductive cycle.

Furthermore, the diversity of reproductive strategies known in elasmobranchs offers an opportunity to assess specific endocrine roles in reproductive events. In particular the natural separation of events otherwise concurrent observed in some strategies allows a more precise correlation of hormonal variations to morphological changes. For instance long sperm storage in *Sphyrna tiburo* (Manire et al., 1995) and reduced gestation period in *Urobatis halleri* (Mull et al., 2010) separates mating from ovulation, and diapause in *R. taylori* separates fertilization from active development allowing these events to be assessed separately. Similarly, correlative studies in species undergoing consecutive ovarian and uterine cycles such as *Dasyatis sabina* (Snelson et al., 1997; Tricas et al., 2000) are likely to provide better insight on the endocrine control of the reproductive cycle due to the separation of folliculogenesis and gestation. Therefore investigations of “less common” reproductive modes and strategies are encouraged as well as comparative studies between them.

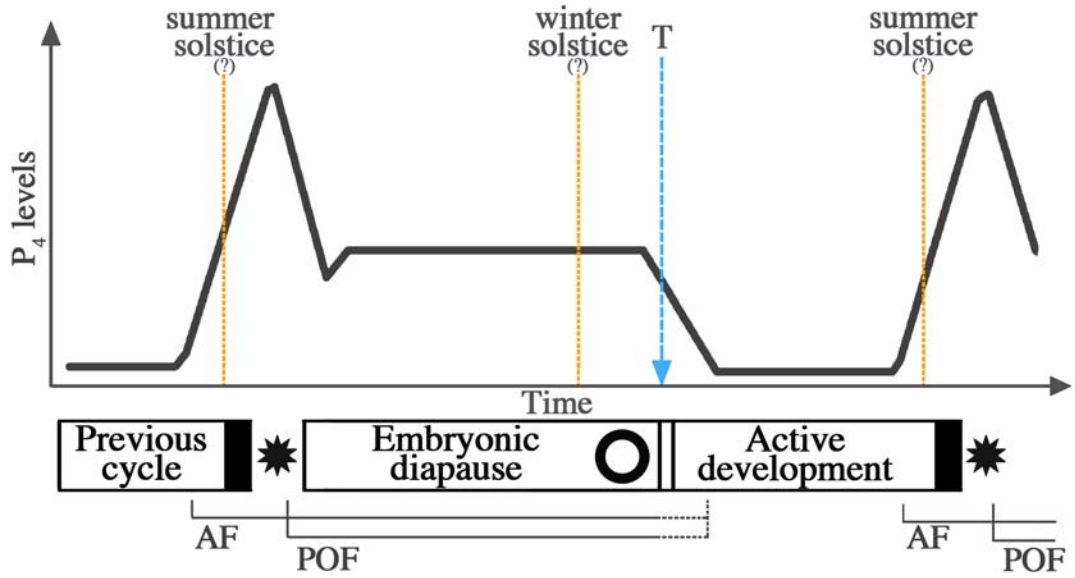

Figure 5.1: Schematic representation of the reproductive cycle and diapause in *R. taylori*. Environmental factors (day length and temperature) indicated by the dotted lines (solstices) possibly influence the timing of events. The lower diagram shows ovarian and uterine evens, where parturition (solid bars), ovulation (stars), commencement of folliculogenesis (open circles) and the transition between stages 1 and 2 at early active development (thin bars) are indicated. Progesterone (P_4) peaks at ovulation before declining at the onset of the diapausing period, during which moderate levels are sustained. The origin of this hormone is likely the ovary, which contains atretic (AF) and postovulatory follicles (POF) during all the diapausing period, which persist for some time after the recommencement of active development (length of complete degeneration is unknown and marked with intermittent lines). The recommencement of folliculogenesis shortly follows the winter solstice (minimum annual temperatures and day length), possibly activating endocrine pathways for the reactivation of embryonic development by testosterone (T; blue arrow). Estradiol does not seem to play a direct role in the control of diapause and is therefore not represented in this scheme.

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