Reproductive Biology and Steroidal Levels in Black Corals, *Antipathes curvata* in Hong Kong

LAU, Pui Ling

A Thesis submitted in Partial Fulfillment

of the Requirements for the Degree of

Master of Philosophy

in

Biology

The Chinese University of Hong Kong

September 2011


THE CHINESE UNIVERSITY OF HONG KONG
23 JAN 2013

Thesis/Assessment Committee

Professor Norman YS WOO (Chair)
Professor Put ANG, Jr (Thesis Supervisor)
Professor W GE (Committee Member)
Professor CF CHANG (External Examiner)

Abstract

Antipatharians are black corals which can be found worldwide, especially in tropical and subtropical waters. They have been harvested for centuries by trawling or by divers for use in the jewelry industry. Thus, together with red and blue corals, they are considered as one of the precious corals. All antipatharians have been listed in CITES II since 1981 as they were over-harvested in some regions over time.

Black corals are ahermatypic (non-reef building) and lack symbiotic algae. Thus most of them appear below the euphotic zone from 200m to 6000m depth. Some could be found in shallower region, at less than 100m depth. Due to their inaccessibility, their reproductive biology and life cycle are hardly known. It would thus be essential to understand the basic biology of these antipatharians as this information will be critical and helpful in setting up strategic management protocols for their conservation.

Interestingly, black corals can be found in shallow water at a depth of 6 m in Hong Kong waters. They are therefore readily accessible for studies. A total of 38 *Antipathes curvata* colonies that ranged from 31 to 110cm in heights at 9 to 11m below Chart Datum (C.D.) were haphazardly tagged in September 2008 in Lan Guo Shui, Tung Ping Chau Marine Park. From September 2008 to March 2010, samples were collected from these colonies once a month for sexual reproductive study and endogenous sex hormone quantification.

Based on histological examination, *A. curvata* is a gonochoric broadcaster. Gametes were found in both male and female colonies from September to November 2008 and from June to October 2009. The development of oocytes was asynchronuos within and

between different female colonies at the beginning of reproductive cycle but became more synchronized as reproductive period proceeded. On the other hand, spermatogenesis among different male colonies was more synchronized throughout the whole period.

Mean (± S.D.) geometric diameter of oocytes and spermaries collected from tagged colonies in November 2008, which was one month before spawning month, was 70.9 ± 13.2 μm and 83.9 ± 8.1 μm respectively. Since no gametes could be found in December 2008, spawning of *A. curvata* was likely to have occurred between November and December 2008. Gametogenesis in both male and female colonies started again around May or June 2009. In June 2009 samples, the minimum size of oocytes and spermaries being 25.2 μm and 28.4 μm respectively. Mean (± S.D.) geometric diameter of oocytes and spermaries in October 2009 reached 88.7 ± 5.9 μm and 109.3 ± 11.0 μm respectively. In November 2009, no relic gametes could be found in any tagged colonies, suggesting that spawning took place sometime between October and November 2009.

Estrogen was first detected in hard coral tissues in 1992 during a mass spawning event in Australia. They were subsequently discovered in other hard corals, soft corals and gorgonians. Moreover, the levels of sex steroids in cnidarians varied significantly throughout the reproductive cycle and some were found to be sex-related. Therefore, it is believed that besides environmental factors like seawater temperature, tidal level and lunar cycle, endogenous factors such as the level of sex hormones in coral tissue is also one of the many regulating-factors in mass spawning and gametogenesis in corals.

Levels of vertebrate-type sex steroid, 17β-estradiol (E2), in *A. curvata* tissues were quantified from October 2008 to February 2010 in this study. No E2 was detected in unsexable colonies (i.e. colonies with no gonads over time) throughout the whole sampling period. E2 was observed to be sex-related and its levels were higher among female colonies than in males. E2 concentrations in female colonies in October, November and December 2008 were 7.22 ± 2.01, 8.74 ± 2.84 and 6.38 ± 1.92 pg/g tissue respectively, whereas E2 was undetectable among male colonies in that same period. E2 became undetectable in both sexes in January and February 2009 right after spawning in December 2008, during which time no gametes were found in the corals. E2 started to appear in females from March, and continued to be detected till November 2009 and from March to October 2009 in male colonies.

E2 in female colonies reached a peak at 65.13 ± 9.5 pg/g tissue in April 2009 while E2 in male colonies peaked at 7.41 ± 1.31 ng/g tissues in May 2009. The presence of E2 peaks before the onset of gametogenesis in both female and male colonies supports its role in initiating gametogenesis. E2 levels among female colonies remained high throughout summer and early autumn during which oogenesis was active. This strongly supports its role especially in oogenic maturation and in continuously triggering some minor spawnings to release some mature gametes that developed asynchronously. No such pattern was found among male colonies, suggesting that some other sex hormones might be responsible for spermatogenic maturation and spawning. E2 could no longer be detected after all colonies had spawned. Spearman Rank Correlation Analysis indicated that warming of seawater temperature was correlated with the increment in E2 level in *A. curvata* samples, which in turn was correlated with the onset of oogenesis.

From this study, the effect of E2 in initiating gametogenesis was more prominent among female colonies than among males, consistent with the basic, fundamental role of E2 as being a female sex hormone.

The present study is a pioneering work in investigating the annual reproductive cycle of black corals in the South China Sea and Southeast Asia regions. It provides baseline information on the basic biology of the antipatharian *A. curvata* in Hong Kong waters. Future works can focus on understanding the roles of endogenous sex hormone E2 as bioregulatory molecules in simple organisms like black corals that do not have endocrine system. The conserved roles of such vertebrate-type sex hormone E2 in regulating sexual reproduction across different organisms, from simple invertebrates such as corals to vertebrates, have an evolutionary implication. Finally, increasing threat of exogenous estrogenic compounds from human wastes on the marine environment cannot be underestimated. Many of these compounds would disrupt the reproduction patterns of many marine organisms. Thus, information from this study will be useful as a baseline in comparing with studies looking into these effects and contribute towards a better understanding of the biology and thus, the conservation of these corals.

摘要

黑珊瑚遍佈於世界各地，尤其棲息於熱帶和亞熱帶水域。長久以來，黑珊瑚被人類以拖網或潛水方式用人手採集，加以打磨，造成首飾，來滿足珠寶界的需求。因此黑珊瑚就如紅珊瑚和藍珊瑚一樣，被譽爲寶石珊瑚。由於長期被過度採集，所有黑珊瑚在 1981 年被列入《瀕危野生動植物種國際貿易公約》附錄二。

黑珊瑚並非造礁珊瑚，體內缺乏蟲黃藻。由於牠們不需依靠蟲黃藻進行光合作用來製造養份，因此牠們可棲息於比真光層更深的水域，由 200 米至 6000 米不等，只有部份棲息在淺於 100 米水深的水域。由於黑珊瑚屬於深水珊瑚，全球只有少數關於黑珊瑚的繁殖和生命週期的研究。透過了解黑珊瑚的基本繁殖狀況，對日後制定保育黑珊瑚的管理措施有著重要的作用。

然而，香港水域內的黑珊瑚棲息於大約 6 米的深度，因此十分有利於進行黑珊瑚研究。自 2008 年 9 月，我們在東坪州海岸公園難過水 9 至 11 米深度爲 38 個 31 至 110 厘米高的 *Antipathes curvata* 黑珊瑚群落加上標籤，從 2008 年 9 月至 2010 年 3 月，每月從標籤了的黑珊瑚群落採集樣本一次，用作黑珊瑚有性繁殖和體內雌二醇定量的研究。

分析了組織學實驗結果後，我們確定 *A. curvata* 屬於雌雄異體而產卵時把配子撒播於體外的類型。2008 年 9 月至 2008 年 11 月及 2009 年 6 月至 2009 年 10 月，在成熟的雌、雄黑珊瑚體內均能發現配子。在生殖週期剛開始時，不同雌性珊瑚群體的發育過程並非同步發生，但隨著生殖週期，卵子的發育過程會越趨同步。相對地，雄性珊瑚群體的精子發育則明顯地比雌性卵子的發育更爲同步。

2008 年 11 月，在已被標籤的珊瑚體內找到的卵子直徑平均爲 70.9 ± 13.2 μm 而精巢直徑平均爲 83.9 ± 8.1 μm。所有卵子及精巢均在 2008 年 12 月消失，因此大規模的排放期很可能在 11 月至 12 月之間發生。新一階段的卵子及精巢生成期大約在 2009 年 5、6 月開始，由 6 月份的黑珊瑚樣本中發現最細小的卵子及精巢直徑分別是 25.2 μm 及 28.4 μm，經過四個月的發育到 10 月份的成熟期，成熟卵子和精巢的平均直徑分別大約錄得爲 88.7 ± 5.9 μm 和 109.3 ± 11.0 μm。由於雄和雌黑珊瑚的配子均在 2009 年 11 月消失，所以黑珊瑚 2009 年的排卵月份推測爲屆於 10 月至 11 月份之間。

1992 年，澳洲的海洋研究人員首度在石珊瑚排放期內，於其組織和海水中分離出與脊椎動物生殖相似的雌激素。後來其他硬珊瑚、軟珊瑚和柳珊瑚體內也分離出相類似的生殖激素。刺胞動物的生殖激素水平在生殖週期中有著重要的變化，而個別荷爾蒙被認爲是與性別有關的。因此，除了環境因素，如海水溫度、潮位和

月球週期之外，一些內源性因素，如在珊瑚組織內的生殖激素水平也是一種調節珊瑚配子發育及產卵期的重要因素。

此研究集中推測黑珊瑚 *A. curvata* 體內雌性荷爾蒙雌二醇(E2)的濃度對於配子生成期或大規模排放期潛在的影響。在未能分辨出性別的黑珊瑚體內均不能分離出任何雌性荷爾蒙雌二醇，另外在雌性珊瑚體分離的雌二醇濃度明顯比雄性個體的高。2008 年 10 月、11 月、12 月期間，雌二醇水平分別爲 7.22 ± 2.01, 8.74 ± 2.84 和 6.38 ± 1.92 pg/g，雄性黑珊瑚在同期內量度不到任何雌二醇水平。跟隨著 12 月份配子的消失，2009 年 1 月和 2 月份的雄或雌性黑珊瑚樣本再也發現不到雌性荷爾蒙雌二醇。2009 年 3 月至 11 月中，雌二醇再次發現於雌性黑珊瑚體內，而雄性黑珊瑚在同年 3 月至 10 月驗出雌二醇。

雌性和雄性黑珊瑚的體內雌二醇在配子生成期一至兩個月前達最高水平，這顯示雌二醇有著刺激生殖期的作用。雌性珊瑚體內的雌二醇水平在整個夏季及初秋也遍高，這顯然表示此荷爾蒙有趨化卵子成熟及刺激卵子小規模子排放的作用。然而，雄性珊瑚體內的雌二醇水平找不到相類似的起伏，這顯示有著其他荷爾蒙控制著精巢發育及精子散播。當配子消失後，黑珊瑚體內又不能分離出任何雌性荷爾蒙雌二醇。史比爾曼等級相關分析顯示，海水溫度變暖和珊瑚體內雌性荷爾蒙雌二醇上升互相有著影響，而雌二醇水平上升和卵子發育也互相有著影響。由於

雌二醇更能有效地刺激雌性黑珊瑚的配子生成期多於雄性珊瑚，此研究結果吻合了雌二醇本身作爲雌性荷爾蒙的作用。

繼 Parker 在新西蘭首項關於 *A. fiordensis* 的研究，本研究乃南中國海及東南亞地區首項關於黑珊瑚繁殖週期的研究。本研究對香港水域之黑珊瑚 *A. curvata* 的生殖生物學提供了基線資料，相信有助於未來制定保育黑珊瑚之措施。通過研究配子發育及內源性雌二醇的相關關係，對無脊椎動物的分泌功能提供了不可缺少的資料。此研究顯示，脊椎動物的性荷爾蒙在缺乏內分泌系統的無脊椎動物體內有相類似的生理調控作用，此發現在進化論上有著重要的啓示。最後，人類廢料中排放的外源性雌性荷爾蒙對海洋生物繁殖週期有著不可被忽視的影響。因此，此研究爲日後有關外源性雌性荷爾蒙對海洋生物影響的研究和對保育珊瑚提供了重要的對比基線資料。

Acknowledgments

Entering this marine ecology laboratory changed me in quite a lot of ways, in terms of academic aspect, physical fitness, mental power and personality. Now my project has almost come to an end and I would like to express my sincere thank to all those who have helped me in the past few years.

Firstly, I would like to thank my supervisor, Prof. P.O. Ang. As he was the one agreeing with this project on sex hormone in black corals when I first suggested to him, thanks for having trust in me and also thanks for the support over the whole research period. Moreover, I am grateful to have Prof. Y.S. Woo and Prof. G. Wei to be my thesis committee members. It is a delight to have Prof. C.F. Chang from Taiwan for being my external examiner as well.

My research could not be done without the logistic support by Captain Y.H. Yung who also gave us precious advice and knowledge on safety issues concerning SCUBA diving. Thanks to Mr. H.B. Yeung, our laboratory technician who has been helping us all the way in our research.

Last but not least, I would like to thank all my buddies for their endless support from diving to laboratory work: Grace S.M. Leung, Bug C.Y. Yeung, Ling Y.L. Hui, Apple P.Y. Chui, Chickee M.H. Chow, Kevin K.Y. Chu, Stella S.W. Chan, Jacky C.K. Kwok.

Being a member of the marine laboratory is something I am most glad of as it is a place where team spirit is thriving. We used sweat and shudder in exchange of data about marine ecology in Hong Kong. Yet, we do believe it is worth doing.

Contents

Chapter 2 Reproductive Biology of Antipatharian Black Coral, *Antipathes curvata* in Lan Guo Shui, Hong Kong

List of Figures

from December 2008 to May 2009 (samples in April 2009 were missed), and from November 2009 to February 2010.

Chapter 1 General Introduction

1.1 Regulations of gametogenesis and mass spawning in corals

With more than 6000 species worldwide, anthozoans belong to the largest class of organisms in the phylum Cnidaria. Their body structure is simple, with diploblastic tissue comprising of epiderm and gastroderm. Circulation of materials within tissues can be made via diffusion. Despite their simple body structure and organization, anthozoans exhibit reproductive strategies that include larval brooding or broadcasting of gametes in a mass spawning. Three-quarters of all corals, mostly hermatypic (reef-building) and some ahermatypic ones, adopt mass spawning as their reproductive strategy in which millions of gametes are released into water column within hours for external fertilization. Mass spawning facilitates the dispersal of coral larvae over long distance and therefore creates genetic links between different coral reefs or communities. Such synchrony in releasing gametes helps to maximize the rate of successful fertilization.

The exact cues triggering such synchronized annual mass spawning event in corals remain unknown. Yet it is believed that among these different species of broadcasting corals, fecundity, gametogenic cycles, date and time of mass spawning are determined by both endogenous (Slattery *et al.*, 1999; Tarrant *et al.*, 1999; Twan *et al.*, 2003, 2005) and environmental factors (Korringa, 1947; Giese and Pearse, 1974; Babcock *et al.*, 1986; Mendes *et al.*, 2002). Thus, under the influence of various external factors, a

single species of coral may have different reproductive modes in different geographic locations.

1.1.1 Endogenous cues

In vertebrates, androgens and estrogens play important roles in regulating gametogenesis and mass spawning. Sex steroids were also found in invertebrates like mollusks (Reis-Hendriques *et al.*, 1990), crustaceans (Jeng *et al.*,1978) and echinoderms (Voogt and Dielemen, 1984). Estradiol (E2) was detected in both coral eggs and seawater during mass spawning in Australia in 1992 (Atkinson and Atkinson, 1992). Thus, it was hypothesized that "endocrine system" might be one of the endogenous factors that regulates coral gametogenesis and mass spawning. In later studies, estrone (E1) and estradiol (E2) were also detected in the tissues of scleractinian coral, *Montipora verrucosa* (Tarrant *et al.*, 1999). Estradiol peaked prior to spawning and estrone peaked during spawning, indicating that they may have different roles in late gametogenesis and spawning. Progesterone (P), testosterone (T) and estradiol (E2) were found in soft coral, *Sinularia polydactyla* (Slattery *et al.*, 1999). Testosterone levels in *S. polydactyla* significantly increased just prior to spawning while the levels of estradiol were much more ambiguous. The latter might be due to the presence of multiple cohorts of oocytes. All these studies suggested that sex hormones, long thought to be feature of vertebrates, are also present in simple organisms like cnidarian and their roles in regulating reproduction might be conserved.

1.1.2 Environmental cues

Simpson (1985) suggested that seasonal changes in seawater temperature did not control critical reproduction events in corals because both timing of gametogenesis and spawning differed between populations of the same species on the east and west coasts of Australia, with one spawning in spring and the other spawning in autumn respectively. Thus, scleractinian corals in different geographical locations showed mixed reproductive traits and more than one environmental factor must be regulating their reproductive cycle. This suggested that there is certain plasticity in coral reproductive strategy that can be shaped by the environment (Harrison, 1985; Harrison and Wallace, 1990). These exogenous factors interact with endogenous biorhythms, which are genetically programmed inside corals, to cause shifting in reproductive strategies (Giese and Pearse, 1974).

It was shown that the population of colonial coral *Montastrea annularis* in shallow water became sexually mature at a smaller size than the population in deeper water in the Caribbean (Van Veghel, 1994; Van Veghel *et al.*, 1994). Kramarsky-Winter and Loya (1998) investigated two closely related fungiid corals in the Red Sea, *Fungia scutaria* and *F. granulose*, which were found in shallow (0.5-5 m) and deep water (5-25 m) respectively. Though both of them were broadcasters and spawned in summer, they differed in synchrony, timing of spawning as well as maturity size. Similarly, *F. scutaria* in shallow water reached maturity at smaller size, had repeated synchronous spawning and prolonged spawning season than their deeper water counterparts. These

reproductive features might help them to overcome the unpredictable environmental conditions or some sporadic catastrophes in shallow water habitat (Jokiel *et al.*, 1993).

The timing of spawning was thought to be affected by numerous environmental factors, including annual temperature variation, lunar cycle, diurnal light cycle, rainfall, the time of sunset, among others (Korringa, 1947; Giese and Pearse, 1974; Babcock *et al.*, 1986; Mendes *et al.*, 2002). Though broadcasting corals lack any organs to detect any of the above environmental factors, they can still release their gametes precisely at about the same time. Babcock *et al.* (1986) found that on the Great Barrier Reef, these spawning-initiating factors function at a much finer time scale. For example, seawater temperature controls the time of year for spawning, monthly lunar cycle controls the time of month, while the diurnal light cycle controls the hour of spawning. The principle underlying the timing signal of seawater temperature is that it provided sufficient time for gonad maturation (Oliver *et al.*, 1988). On the other hand, the factor underlying the lunar or tidal cycles that spawning is likely to occur at low amplitude neap tide is to increase the probability of fertilization at a period of low water motion and low water volume. This explains why most mass spawnings occur after sunset, a few days after full moon when the moon is rising late at night. Finally, the ultimate factor underlying diurnal light cycles is to reduce the exposure of gametes to predators in order to increase their survival rate.

Achieving this synchrony has the advantage of increasing the chance of fertilization as fertilization rate could be very low due to gamete dilution and drift. Being synchronized in spawning could result in higher genetic mixing between colonies and in reducing overall predation (Oliver *et al.*, 1988). The disadvantages of mass spawning

include the possible formation of non-viable, interspecies hybrids during the period of fertilization (Willis *et al.*, 1985; Oliver *et al.*, 1988), the exposure of all gametes to adverse environment at the surface, and the transportation of gametes and larvae away from their reef of origin by strong surface currents (Veron 1995; Wolanski and Sarsenski, 1997). Nonetheless, the mechanism(s) involved in achieving this synchrony and the linkage between environmental factors and endogenous biorhythm remain unclear.

1.2 Studies on black corals

1.2.1 Introduction to black corals

Black corals (Antipatharians) belong to Class Anthozoa in Phylum Cnidaria. They were grouped under subclass Hexacorallia (Brugler *et al.*, 2007; Lapian *et al.*, 2007) based on phylogenetic analyses of multiple protein-coding genes, instead of being under the subclass Ceriantipatharia. Thus they are in the same subclass as hard corals and sea anemones, both of which have the number of tentacles in multiples of 6, as opposed to those under subclass Octocorallia (soft corals, gorgonians and sea pen) that have the number of tentacles in multiples of 8. Black corals are colonial, with growth forms that can be branching (bushy, feathery, fan-shaped, bottlebrush-shaped) or wire-like without branching (such as whip corals) (Grange, 1988; Montgomery, 2002). Each colony (Fig. 1.1) can have hundreds or over thousands of polyps that are white, yellow, orange, red

or green in colors. Each polyp has six unbranched, non-retractile tentacles with nematocysts.

By convention, hard corals are also known as hermatypic corals as they are reef-building with their calcium carbonate exoskeleton, while black corals and gorgonians are examples of ahermatypic corals as they do not deposit calcium carbonate as their skeleton. Black corals on the other hand, possess spiny axial skeleton with concentric hollow core that is composed of chitin and proteins (Grigg and Opresko, 1977; Montgomery, 2002). Unlike hard corals, black corals are deep-water corals and do not contain zooxanthellae in their tissues. Their range of distribution is not limited by the presence of light. Therefore, most antipatharians live at a depth of 20m to 8000m in tropical and sub-tropical waters. They are carnivorous. Together with their six non-retractile tentacles, mucus secretion, ciliary currents and mesentery filaments, they capture and ingest amphipods, copepods, chaetognaths and other zooplankton (Lewis, 1973). Among the major groups of Anthozoans, black corals are the least studied with respect to their general and reproductive biology.

1.2.2 Black coral harvesting

Occasional collection of broken black coral branches recovered by the entangled lines of fishermen in Lahaina, Hawaii, led to the exploration and discovery of a large bed of black corals of commercial quality between 35-100m depth off Lahaina in Maui, Hawaii by Ackerman and Windley in 1958. Three species were discovered, namely *Antipathes griggi, A. grandis* and *A. ulex.* Earlier, some other black coral species had

been discovered in Hawaii by the Challenger Expedition (1872-76) and the Albatross Expedition (1902). However, all of these species were too small to be of commercial value. Among the 150 species of black corals known worldwide (Grigg and Opresko, 1977), less than 6 of them are large enough to be suitable for cutting, carving and polishing into black coral jewelry and dense enough for commercial exploitation.

Upon the discovery of the Maui bed, Ackerman and Windley set up a small company called Maui Divers of Hawaii, hiring some divers to harvest black corals in the Auau Channel off Lahaina, Maui (Grigg, 2001). Harvest rate remained low for the next 10 years with less than 3,000 kg/yr (Poh, 1971). This industry was considered to be economically profitable and thus, had room for growth. In the mid-sixties, five other small companies started up on Maui or Oahu.

Prior to 1978, management of fishery resources in the US Pacific Islands was plagued by many problems, such as the incursions by Japanese and Taiwanese fishermen who harvested large beds of black corals intensively using destructive tangle gear. These destructive fishery methods exploited many year classes at the same time. Decades of standing stock were collected within a short duration of intensive fishing. This practice led to a pattern of discovery, heavy exploitation, followed by depletion. Those suspected poaching in the US waters by Japanese and Taiwanese fishermen as well as the expansion of black coral fishery in Hawaii from Maui to Kauai and to the big island of Hawaii were some of the major reasons why management of precious coral fishery became urgently needed to achieve sustainable selective harvesting.

After a series of ecological researches on black corals, the State of Hawaii and the Western Pacific Regional Fishery Management Council (WPRFMC) set up guidelines

on black coral fishery. Fishermen are required to accurately report their annual harvest to fully abide by the 48-inch size limit. By following these guidelines, black coral fishery and its industry continue to thrive after 50 years of operation. The industry has grown to have an annual value of over US$ 50 million and employs more than 1000 people. Since 1981, all antipatharians are listed in the CITES Appendix II, which means they are not necessarily threatened to extinction but may become so if trade is not closely controlled.

1.2.3 Biodiversity and distribution of black corals in Chinese and Hong Kong waters

Only very few studies on black coral ecology and reproduction have ever been carried out worldwide. In South China Sea, Zou and Zhou (1984) conducted a series of expeditions in the 1980s in which black coral colonies were trawled from sea bottom beyond 50m. From these expeditions, 49 species of black corals in 10 genera were recorded. This constitutes about one-fifth of all black coral species described worldwide. This indicates the high diversity of black coral fauna in the Chinese waters. In Hong Kong, Zou and Zhou (1984) reported 6 species of *Antipathes* and *Cirrhipathes* collected between 10 to 20 m from its northeastern waters. More recently, a comprehensive survey of black coral distribution was conducted throughout northeastern to southwestern waters of Hong Kong between 2006 to 2008 (Ang *et al.*, 2010). Six species of black corals in two genera, *Antipathes* and *Cirrhipathes*, were identified.

Ang *et al.* (2010) found that even if both *Antipathes* and *Cirrhipathes* co-exist in the same site in Hong Kong waters, their relative abundance differ quite significantly. Genus *Antipathes* was more commonly found in shallow waters, between 8-10m while genus *Cirrhipathes* was more commonly found at relatively greater depth. The most common black coral species in each genus are *Antipathes curvata* and *Cirrhipathes sinensis*. *Antipathes* spp. are branching in form. The largest colony found in Hong Kong waters was in Moon Island, with the height reaching 150 cm. On the other hand, *Cirrhipathes* spp. are wire-like and unbranched. The tallest colony found was 133.40 cm. In southeastern Hong Kong waters, highest density of black corals, being around 18 colonies/m^2, was found in Beaufort Island and Po Toi at depths greater than 20 m. It is likely that extensive healthy beds of black corals could be found in greater depths in this area, especially in the deep channel between the two islands.

Monospecific beds of *Antipathes* spp. were found in a number of sampling sites in the north-eastern region of Hong Kong, such as Fung Wong Wat, Cheuk Chau and Moon Island (Ang *et al.* 2010). Beds of black corals in shallow waters < 15 m are quite uncommon in other parts of the world. This provides a unique opportunity to study these black corals.

1.2.4 Threats to black corals

Hard corals in Hong Kong are subject to anthropogenic and natural threats and black corals are no exception. Among the anthropogenic factors, pollution is a major issue.

Pollution problems are contributed by coastal reclamation, dredging, sewage efflux and marine dumping. All these increase the sedimentation rate that in turn poses serious threats to the corals. It is unlikely that black coral colonies could easily recover from destructions given that they have slow growth rate, mature at older age and have long life span (Bo *et al.*, 2009; Parker *et al.*, 1997). Growth rates differ among different black coral species. *Antipathes dichotoma* collected at 50m depth off Lahaina, Maui, Hawaii yielded radial growth rates that ranged between 130-1140 μm/year and was thought to become sexually-matured at the age of 12 to 13 (Roark *et al.*, 2006). Bo *et al.* (2009) found that unbranched black corals in Celebes Sea are among the fastest growing colonial organisms, that the longitudinal growth rates of *Cirrhipathes* cfr. *anguina* and *Stichopathes* cfr. *maldivensis* were 13.25 and 1.3 cm/month respectively. Sherwood *et al.* (2009) used growth ring counting method to calculate growth rate of axial skeleton of deep sea antipatharians, *Stauropathes arctica*, at depths of between 400 to 900m from the continental slope of Newfoundland and Labrador. *S. arctica* had the lowest radial growth rate that ranged between 33 ± 11 to 75 ± 11 μm/year. The minimum sexually-mature age and size of *A. fiordensis* in New Zealand waters was 31 years old and 70 cm tall respectively (Parker *et al.*, 1997). Deep-sea antipatharian, *Leiopathes* sp., found at depths between 300 to 500 m in Hawaiian waters, suggested radial growth rate as low as 4 to 13 μm/year. By using radiocarbon dating, the oldest colony was found to be at the age of 4200 ± 70 calendar years B.P. (Roark *et al.*, 2009).

Black corals, together with blue and red corals, are known as precious corals as they are raw materials for the jewelry industry in many parts of the world. In Nan Ao, Guangdong, in Southern China, black coral skeletons are made into smoking pipes for

sale. These skeletons are usually part of the by catch of trawl fishing. There are no known protection measurements for black corals in China. With an almost complete lack of information on the basic biology of black corals worldwide, not to mention about knowledge of their reproductive biology, it would be difficult to develop any conservation strategy for them.

1.3 Significance and objectives of the present study

Despite its worldwide distribution, only one study has ever been carried out to monitor annual reproductive cycle of black coral reproduction (Parker *et al.*, 1997). Parker *et al.* (1997) showed the spawning period of *Antipathes fiordensis* in New Zealand to be in March, during the mid- and late-summer. A few studies were done on the morphology of sperms and oocytes of antipartharians. Gaino *et al.* (2008) showed the arrangement of oocytes in polyps of *Cirrhipathes* cfr. *anguina* sampled from Siladen Island in the Bunaken Marine Park (North Sulawesi, Indonesia) in August 2007. Final stage of oocytes with the largest size measured 125 μm in diameter were found among August sample. Gaino *et al.* (2008) used SEM and TEM methods to investigate the ultrastructural organization of sperms in *Cirrhipathes* sp. from Bunaken Marine Park, Indonesia. A series of electron-dense vesicles were found in the apical nuclear region. A cup-like electron-dense body, edged with electron-dense granules, was interposed between the nucleus and the tail. The lack of studies on black coral biology worldwide may be due to most of them being found in deep-water habitat, making them not readily accessible for research study.

As black coral colonies are found commonly at shallow depth in Hong Kong waters, such as at 7m depth in Lan Guo Shui in Tung Ping Chau Marine Park, this provides an excellent opportunity for research to be carried out to study black coral biology. This thesis research therefore focuses on two aspects of the black coral biology: its reproductive seasonality and the potential role of both exogenous and endogenous cues, including sex steroids, in regulating this reproductive seasonality. Any understanding of the biology of black corals will be important in contributing to their future conservation.

1.3.1 Objectives

This thesis research has therefore the following objectives:

- To document the reproductive biology of *A. curvata* in Tung Ping Chau Marine Park, Hong Kong
- To quantify the seasonal levels of the sex steroid 17β-estradiol in this species of black coral
- To correlate the relationship between exogenous factors like seawater temperature and the endogenous factors like the tissue levels of 17β-estradiol and the gametogenic cycle of *A. curvata*

1.3.2 The target species, *Antipathes curvata* and the study site

Antipathes curvata is the most common species of black coral found in Hong Kong waters. It could be found in 42 out of 100 sampling sites surveyed in a recent study (Ang *et al.* 2010). It is therefore the target species for this thesis research.

Hong Kong is located in the southern coast of China at the mouth of Pearl River (Fig. 1). Tung Ping Chau Marine Park (TPCMP) is located in the northeastern part of the New Territories, Hong Kong SAR (22°33' N latitude and 114°26' E longtitude). The total area of the island is about 1.1 km^2, with some 2 km of coastline. TPCMP was designated as the fourth marine park of Hong Kong in November 2001. The northeastern part of the island is mainly of some sheltered sandy beaches with hard corals flourishing, while the southern and southwestern part is more exposed, composed of sedimentation rocks. Lan Guo Shui being the study site of this project, is in the southeastern region of the island, with cliffs running down into the sea. Its substratum is of large boulders.

A large bed of black coral, *A. curvata* could be found in Lan Guo Shui. *A. curvata* starts to aggregate on rocky substratum at a depth of 7 m below Chart Datum (C.D.) in this site. On average, these black coral colonies are 1 to 2 m apart from each other in shallower water and become more densely populated with increasing depth.

Colonies of *A. curvata* (Fig. 1.1) are distinctly but irregularly branched to become tree-like in appearance. Moreover, they have a ramified base. The smallest branchlets are arranged in different directions from the large branch. Their skeletons are chitin and protein in nature, thus they appear black in color. The colors of the living polyps are

either pale yellow or white. Antipatharians are ahermatypic corals, they do not support symbiotic zooxanthellae in the tissues of their polyps.

1.4 Thesis Outline

This thesis is arranged in the following structure:

Chapter 1 General Introduction

This chapter gives a general introduction on the biology of black corals and their distribution in Hong Kong waters. Some harvesting history of black corals in the west, especially in Hawaii, is also given. The need to understand black coral reproductive biology is elaborated, followed by the description of the objectives, the target species and the study site of this thesis research.

Chapter 2 Reproductive Biology of Antipatharian Black Coral, *Antipathes curvata* in Lan Guo Shui, Hong Kong

The annual reproductive cycle of *Antipathes curvata* in Lan Guo Shui, Tung Ping Chau Marine Park was monitored from September 2008 to February 2010, in a total of 18 months. Their gametogenic cycles were described based on the development of their oocytes and spermaries. Mass spawning was thought to occur in December 2008 and November 2009, based on indirect evidence of the disappearance of all oocytes and

spermaries among all tagged colonies. The two mass spawning events, was separated by 12 lunar months.

Chapter 3 Detection of the Sex Steroid 17β-estradiol and its Possible Roles on Gametogenesis in Black Corals *Antipathes curvata* from Hong Kong

The levels of E2 in tissues of male, female and unsexable *A. curvata* colonies collected from Lan Guo Shui on a monthly basis from October 2008 to February 2010 were examined. The levels of E2 were more prominent among females than among males and were never found in unsexable colonies. E2 level in female peaked 2 months before the start of oogenesis and was higher than its basal level by 15 folds. The increment of endogenous E2 level was correlated with the onset of oogenesis. This pattern was less clear with the spermatogenesis in male colonies.

Chapter 4 Summary and Perspectives

This chapter summarizes the experimental findings in this thesis research. These results provide fundamental knowledge on the sexual reproduction of antipatharians, *A. curvata*, and help to fill the knowledge gap about antipatharians. Results on the endogenous E2 level of black corals were a pioneering work. It suggested the conserved role of E2, being a female sex hormone, even in simple organisms like cnidarian that are without an endocrine system. This study can provide some clue to the study of the evolution of regulatory molecules from simpler to higher animals in the future.

Fig. 1.1 An underwater picture of an *Antipathes curvata* colony in Lan Guo Shui, Tung Ping Chau Marine Park. The colony is about 110 cm tall.

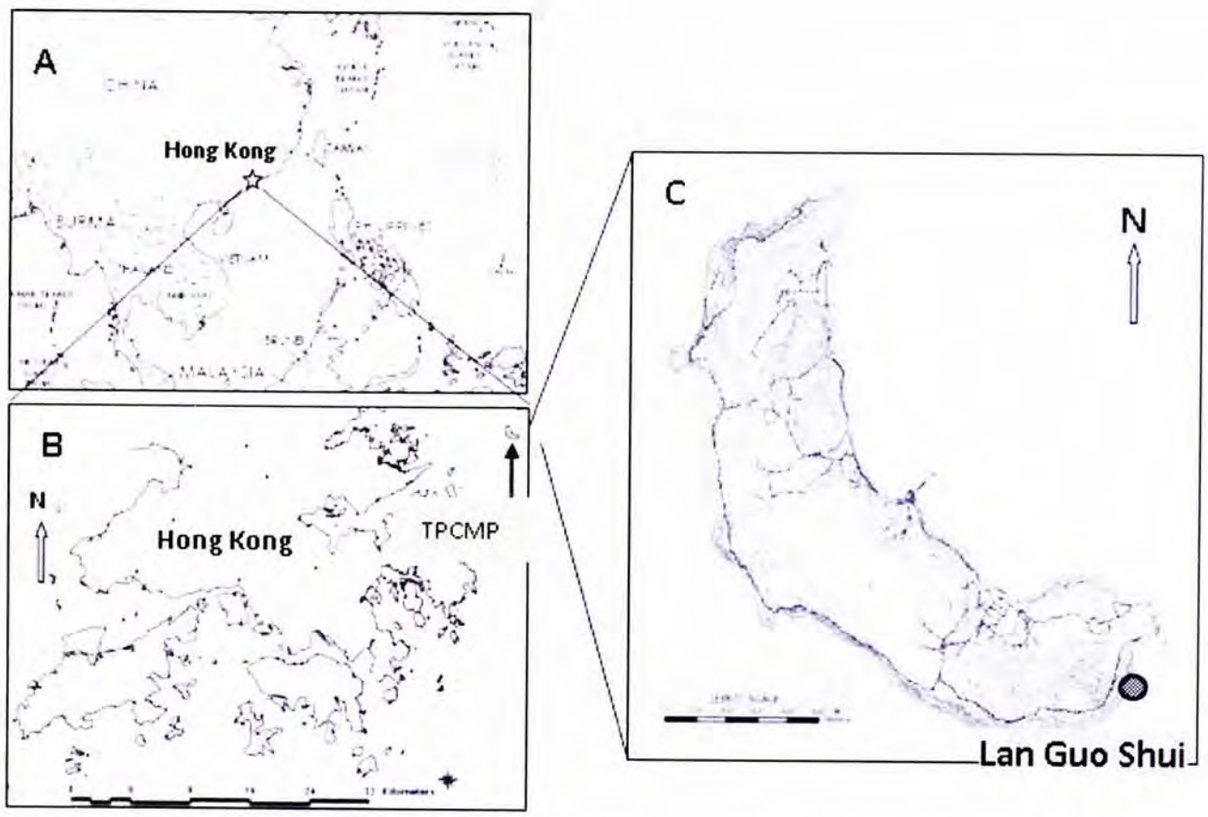

Fig. 1.2 A. Map of East Asia showing the location of Hong Kong and B. Map of Hong Kong showing the location of Tung Ping Chau Marine Park (TPCMP). The study site Lan Guo Shui is located in southeastern part of the marine park (C).

Chapter 2 Reproductive Biology of Antipatharian Black Coral, *Antipathes curvata* in Lan Guo Shui, Hong Kong

2.1 Introduction

Understanding the reproductive pattern of corals is crucial for their conservation. Even though Hong Kong is geographically located in the marginal limits of many scleractinian corals, more than 84 species could still be found in Hong Kong waters. Most of the coral reproductive studies in Hong Kong were focused on hard corals as they are readily found in very shallow water less than 5m deep (Ang *et al.*, 2006) where regular sampling is not a problem. On the other hand, soft corals, gorgornians and black corals which inhabit at greater depth (>5m), were less focused in research in Hong Kong. The first study on reproduction of octocorals in Hong Kong waters was on *Lobophytum sarcophytoides* in Lan Guo Shui, Tung Ping Chau Marine Park (Yeung and Ang, 2010). This species was found in 2007 to spawn in early summer between June and July. This coincided with spawning period recorded for other hard coral species in Hong Kong, such as *Platygyra sinensis* in May (Liu and Ang, 2002), *Favia speciosa* and *Favites adbita* from May to June and *Leptastrea purpurea* from June to July (Lin, 2003).

There are about 240 recognized species of Antipatharians black corals worldwide (Opresko, 2001, 2002, 2003, 2004; Bisby *et al.*, 2008), and they are widely distributed in tropical and subtropical oceans (Davidoff, 1908; Grigg, 1965; Olsen and Wood, 1980; Grange, 1997; Sanchez *et al.*, 1998; Tsounis *et al.*, 2010). Most of them are found at depths of 20m or greater, with some even to the depth of 8000m (Grigg, 1993; Sanchez *et al.*, 1998; Lumsden *et al.*, 2007; Bruckner *et al.*, 2008). Perhaps because of their great bathymetric distribution, they are the least known of the azooxanthellate corals (Opresko, 1972; Warner, 1981; Parker *et al.*, 1997; Montgomery, 2002). In spite of this, they have been harvested for centuries as medicine and were believed to have the abilities to ward off evil spirits. Moreover, they are highly valued as jewelry in many places or have been made into other commodities like cigarette pipe (e.g. in Nan Ao, Southern China). Due to the fact that many black coral populations have been exploited extensively, all species under the genus *Antipathes* were listed in CITES Appendix II since 1981.

Six species of Antipatharians, three under the genus *Antipathes* and three under *Cirrhipathes*, were recorded in Hong Kong waters with the branching *Antipathes curvata* being the most common (Ang *et al.*, 2010). Many populations of this species can start to be found at a depth of 6m below Chart Datum (C.D.), a depth that is much shallower than many other Antipatharians recorded elsewhere (Grigg, 1965; Opresko, 1972, 1976; Grigg and Opresko, 1977; Warner, 1981). Such shallow populations of black corals offer an excellent opportunity for more regular sampling and systematic research into their reproduction biology.

2.1.1 Coral reproduction

Corals are ancient organisms that consist of a free-living planular and a sedentary adult stages. They exhibit a diverse range of reproductive patterns including asexual reproduction (via fragmentation, budding or parthenogenesis) and sexual reproduction (via internal brooding or broadcast spawning) (Stoddart *et al.*, 1983; Harrison and Wallace, 1990; Sherman *et al.*, 2006). The products of these two reproductive processes differ physically and ecologically as asexual reproduction will be used to maintain population within the parental habitat (Ayre, 1995; Richmond, 1997) while sexual reproduction can produce a large number of energetically cheaper but genetically diverse offsprings in uncertain environment which are able to colonize distant habitats (Bengtsson and Ceplitis, 2000; Sherman *et al.*, 2006). Thus, corals often exhibit mixed life strategies.

In their sexual reproduction, gametogenesis takes place in gonads present on mesenteries that radiate inward from the layer of tissue lining the gut cavity. Colonies can be gonochoric, i.e. with sexes being in separate colonies, or hermaphroditic, having both male and female gonads within the same colony. Corals exhibit various modes of reproduction, as brooders that incubate the planula larvae or as broadcasters that release their gametes into the open water.

Brooding corals can store unfertilized eggs for weeks, undergo internal fertilization within the polyps and brood their larvae thereafter. Larvae are then released, settle onto suitable substratum and grow into new colonies. Gametogenesis in these brooding corals is less synchronous as synchrony in the release of gametes is not essential for

successful fertilization. Thus, these corals usually have several reproductive cycles within a year (Harrison and Wallace, 1990; Richmond and Hunter, 1990). On the other hand, broadcasting corals carry out mass spawning in which both sperms and eggs are released into the water simultaneously for external fertilization. These externally-fertilized eggs develop while drifting in the water column. After a few days, fertilized eggs become free-swimming larvae which will then settle on any favorable substrata. This spawning strategy allows larvae to be dispersed over longer distances and helps to establish genetic links between reefs.

2.1.2 Sexual reproduction in black corals

In spite of the fact that Antipatharians are extensively being harvested, and perhaps partly due to their inaccessibility, very little is known about their reproductive biology. This is in sharp contrast with other groups of hexacorallia, such as scleractinian corals, whose reproductive biology has been extensively studied (Kojis and Quinn, 1982; Fadlallah, 1983; Harrison and Wallace, 1990; Richmond and Hunter, 1990; Richmond, 1997; Guest *et al.*, 2002, 2005; Baird *et al.*, 2009; Harrison, 2011). They remain one of the least known cnidarians (Gaino *et al.*, 2008).

Some black coral species can also cover a considerable proportion of the marine benthic substratum in temperate waters. Fjords in New Zealand are of glacial origin and were last filled with ice some 20,000 years ago (Grange, 1985b; Miller, 1996). Endemic black corals belonging to *Antipathes fiordensis* (Grange, 1986, 1990; Parker *et al.*, 1997) dominantly colonized the shallow region of rock walls of the fjords between depth of 10

to 35m. The discovery of this population provided a unique chance to study black coral reproductive biology and ecology. This species was found to be dioecious in which only male or female gonads could be found in polyps of a colony (Parker *et al.*, 1997). Moreover, this species was found to be spawning in March, which corresponds to the mid- to late-summer season when water temperatures are at their maximum (Grange, 1988). On average, it took more than 2 weeks for them to complete their spawning.

Molodtsova (2006) investigated the dimorphic polyps of the two new species in the genus *Heliopathes*, *H. hivaensis* from Marquesas Islands and *H. alis* from Fiji. Both species were found on slope at depths of 400 to 430m. The vegetative polyps are located on the stem and lateral pinnules while the generative polyps are arranged on the subpinnules of the anterior row. Gonad-bearing polyps were found in different parts of the colony compared with those without gonads.

In 2008, morphology of spermatocytes and sperms in *Cirrhipathes anguina* collected along the coral reef of Siladen Island, Sulawesi, Indonesia, was investigated (Gaino *et al.*, 2008) to clarify the phylogenetic relationships of this black coral with other cnidarians. This ultrastructural study of sperms provides additional characteristics, other than morphological traits of the polyp and its skeletal organization, to be used in taxonomic work on Antipatharians. The fine organization of oocytes in this gonochoric, broadcasting species was also investigated (Gaino and Scoccia, 2009). No external morphological differences among male and female colonies of this species could be found to determine sex. However, during reproductive season, gravid female can easily be distinguished from the non-gravid ones owing to the bulging of the lateral tentacles of its polyps (Shick, 1991; Chadwick-Furman *et al.*, 2000). In contrast, the general

shape of fertile male polyps does not differ from that of the unfertile ones. From cross section at oral region, young gametes were located both at the emergence of the primary transverse mesenteries from the gastroderm lining the coelenterons, and where the mesenteries met the pharynx. At the basal level of pharyngeal cross-section, mesentery turned into mesenterial filaments, young gametes were only found in the initial part of the mesenteries.

Another black coral reproductive study was carried out in Ambon Island, Indonesia on *Cupressopathes pumila* (Gaino and Scoccia, 2009). These authors investigated the structural and ultrastructural morphology of the sperm clusters. From the longitudinal section passing through the mouth part of a gravid polyp, a spermatocyst was found at close proximity to the terminal region of the pharynx. In addition to this, numerous spheres which were sperm clusters were seen protruding from the mouth of polyps, in which gametes of various stages coexisted with the mature elements. Thus, gamete differentiation might continue after the release from the fecund polyps, increasing the total number of sperms and their activity over time. Those released spheres were actually spermatocysts bordered by cellular coat of varying thickness, confirming that they originated from the breakage of mesentery wall emerging from the pharynx.

So far, black coral studies were only limited to their basic ecology, the description of gamete structure and the seasonal reproductive cycle of only one species, *Antipathes fiordensis* (Parker *et al.,* 1997). My project would be the first in the South China Sea region where gametogenesis of *Antipathes curvata* was compared at the individual colony level in Hong Kong waters. It serves to fill the knowledge gap about the biology of Antipatharians in the South China Sea and the South-East Asian regions. Specific

aspects of the coral reproductive biology were examined, including the annual sexual reproductive cycle of female and male colonies of *A. curvata*, the fecundity, sex ratio, size of sexual maturity, and development of gametogenic stages in haphazardly tagged colonies. Ultimately, such information could serve as a reference for other reproductive studies on Antipatharians in the future as well as a basis for the development of scientifically based conservation strategies so that exploitation can be carried out within the sustainable range of their recovery. This is a first gametogenic study on black corals where the same colonies were tracked over time.

2.2 Materials and Methods

2.2.1 Sample collections and pre-treatment

A total of 38 *A. curvata* colonies were tagged haphazardly at the depths between 9 to 11m below C.D. in Lan Guo Shui (See Chapter 1 Fig. 1.1) at the very beginning of the research period in September 2008. Monthly sampling was then carried out from September 2008 to February 2010 with collections made within one week before full moon. Since no reproductive studies on black corals were done before in the South China Sea region, it was not known if this species spawns before or after full moon. Therefore, collection carried out before full moon would ensure that oocytes or spermaries at their maximum sizes right before spawning would not be missed.

2.2.2 Histological processing

During each sampling, it was not possible to locate all the tagged colonies as the water was usually very turbid (visibility < 0.5 m). Nonetheless, as many tagged colonies would be located as possible. Upon finding each tagged colony, the terminal 5 cm of the branches collected were discarded since they were thought to be the sterile zone in many other corals (Wallace, 1985). The remaining 6 cm of the terminal branches would be collected for histological analysis.

All collected samples were preserved in 10% formalin in filtered seawater immediately upon return on board the boat to prevent tissue deterioration. After preserving in 10% filtered formalin for one week, the preserving medium would be changed to 75% ethanol thereafter for longer term preservation. Unlike scleractinian corals which have calcium carbonate skeleton, the skeleton of black corals was composed of chitin and protein. Thus, no decalcifying agent was needed to dissolve black coral skeleton.

Polyps collected from different colonies of *A. curvata* were processed in an Automated Vacuum Tissue Processor (Leica® TP 1050, Leica Instruments GmbH). A serial dehydration process with increasing ethanol concentration was carried out by the machine to remove any water content in the coral tissues. The processed samples were embedded in wax blocks with paraffin using THERMOLYNE ® histocentre. Blocks were then serially cut at cross-section at a thickness of 7μm. Since gonads were mainly located near the base of the polyp, more sections were cut at that region.

Those serial sections were mounted on glass slides and stained. There were four steps in the staining process: dewax, hydration, staining and dehydration. In the dewax process, slides were immersed in xylene to remove all the wax infiltrated into the coral tissues. This was followed by submerging the slides in a series of solutions with descending ethanol concentrations from 100% to 30%. This allows hydration to take place. Water-soluble stains i.e. haematoxylin and eosin, were used to stain those hydrated slides. Scott's tap water, a blueing agent, was used after haematoxylin to help stain the nuclei blue, while eosin stained the cytoplasm red. After staining, slides were put in a series of solutions with increasing ethanol concentrations from 75% to 100% to remove any water. All slides were then mounted with Permount® and placed horizontally overnight for complete drying.

2.2.3 Light microscopy

Mounted slides were examined under the light microscope for the presence of oocytes and spermaries, as well as their developmental stages. Developmental stages of the oocytes and spermaries were defined based on Parker's (1997) earlier study on the black coral *A. fiordensis* from New Zealand. Images of both the oocytes and spermaries were taken using a calibrated eyepiece micrometer on a light microscope.

2.2.4 Gametogenesis

The longest diameter and the corresponding longest diameter perpendicular to it of a minimum of 6 and a maximum of 20 largest oocytes and spermaries from each black coral colony were measured in a computer using the computer program, the Image-Pro Plus 5.0 (Media Cybernetics, Inc., Bethesda, MD, USA). The geometric diameter of each gamete was then calculated using the following equation (Wallace, 1985):

Geometric diameter = $\sqrt{}$ Maximum oocyte/ spermary diameter x Perpendicular diameter

Gametes of *A. curvata* were classified into different developmental stages and the number of gametes in each stage was expressed as a percentage of all the gametes encountered in the mounted slides for each colony. Changes in the proportion of different developmental stages of gametes over time were followed for individual tagged colony, as well as for the population of *A. curvata* in Lan Guo Shui. Synchrony of gamete development between colonies was assessed. Furthermore, density of oocytes and spermaries within the gonads along the polyp mesenteries in samples collected in September and October 09 was examined. Both male and female gametes were mostly mature within this period.

2.2.5 Environmental and statistical analysis

Our laboratory has been monitoring seasonal changes in various environmental parameters around TPCMP for the last 10 years. Among these parameters, seawater temperature shows a more consistent seasonal pattern. Seawater temperature was

measured at a depth of about 6m in A Ma Wan, about 800m away from Lan Guo Shui, by Minilog data loggers (VEMCO, AMIRIX Systems Inc., Canada). Spearman Rank Correlation was used to analyze the correlative relationship between seawater temperature and the pattern of change in the sizes of oocytes and spermaries of the black corals over time.

2.3 Results

2.3.1 General reproductive mode

No planulae were found inside the coelenterons of all the polyps examined. Moreover, no eggs and spermaries were simultaneously found in the same colony. This indicates that *A. curvata* is a gonochoric broadcaster.

2.3.2 Sex ratio, size at sexual maturity and density of gametes

Among all tagged colonies (n=38), 13 were females, 15 were males while the remaining 10 colonies were unsexable throughout the research period from October 2008 to March 2010. The sex ratio was approximately 1:1. The smallest sexually mature colony was 32 cm tall, whereas the largest immature colony was 50 cm tall. During September and October 09, the average density of oocytes was 256 ± 68 oocytes/mm^2 of polyp

transverse area while the density of spermaries was 165 ± 46 spermaries/mm^2 of polyp transverse area.

2.3.3 Characteristics of polyps and gametes of *A. curvata*

Polyps of *A. curvata* are distributed uniformly along and around the skeletal axis (Fig. 2.1A). For each polyp that is perpendicularly situated on the skeleton, there are six tentacles surrounding the central mouth. Two types of tentacles are present on each polyp: four shorter lateral tentacles and two longer sagittal tentacles. Any fecund polyp would be characterized by bulging at the base of lateral tentacles (Fig. 2.1B). The colors of fecund polyps were also less translucent at the basal part, compared with the non-fecund ones. Both of these were due to the presence of oocytes and spermaries inside the polyps (Fig. 2.1C).

From the sectioned images, gametes could only be observed at the lower part of lateral tentacles while they are absent in the sagittal ones (Fig. 2.2). From histological cross-sections at the oral level, young gametes could be found in the mesoglea of the mesentery. Specifically, they were present in the region where the mesentery originated from the gastroderm lining the wall of the body as well as at the point where it meets the pharynx (Fig. 2.3A). Some young gametes coexisted with closely-packed mature gametes which gave rise to convolution of the mesenteries. Moreover, gametes were only found in the region next to the body wall but were absent in the region proximal to the pharynx which was in turn occupied by mesenterial filaments. From longitudinal

section of gravid polyps (Fig. 2.3B), mesenterial filaments were present at the base of the polyps, supporting growing gametes above them.

2.3.4 Changes in geometric diameter of gametes over time

Oocyte and spermary developments between colonies were asynchronized over the sampling period from September 2008 to February 2010. At the beginning of the sampling period, the mean (± SD) size of the largest oocytes remained similar from 73.0 ± 15.0 μm (n=20) in September 2008 to 70.9 ± 13.2 μm (n=18) in November 2008 (Fig. 2.4). This was due mainly to the presence of large oocytes in September 2008. The oocytes disappeared in December 2008 and started to appear again in June 2009. They increased in size from 86.0 ± 4.6 μm (n=20) in June 2009 to 88.7 ± 5.9 μm (n=20) in October 2009. The oocytes remained absent starting from November 2009 until February 2010 (end of sampling).

Spermaries attained their maximum mean (± SD) size of 101.6 ± 11.1 μm (n=20) in September 2008 and gradually decreased in size to reach their minimum of 83.9 ± 8.1 μm (n=16) in November 2008. They disappeared in December 2008 and started to appear again in June 2009 at an average diameter of 85.2 ± 12.0 μm (n=20). They increased in size to 109.3 ± 11.0 μm (n=20) in October 2009 and disappeared in December 2009, without leaving any relics.

Judging from the gametogenic cycles observed, minor spawning events likely occurred continuously during summer and fall that ended up with a major mass spawning event in December 2008 and November 2009 when gametes in all the tagged

male and female colonies disappeared. Neither relic oocytes nor spermaries were found within the coelenterons after the mass spawning event. A new cycle of gametogenesis occurred in late spring or early summer in the subsequent year.

2.3.5 Developmental stages of gametogenesis

The development of oocytes and spermaries in *A.curvata* can be classified into four different stages, following Parker *et al.* (1997). These stages are described in more details below.

2.3.5.1 Oogenesis

Spherical oocytes can be identified by a prominent nucleus with nucleolus inside while the whole oocyte is surrounded by mesogleal elements. The level of oocyte maturity was marked by the amount of materials stored inside, which pushed the nucleus aside. The development of oocytes can be divided into four stages (Fig. 2.5):

Stage I:

The earliest oocytes have a proportionally larger nucleus which is located in the center of the oocyte. They are clustered and embedded in the mesoglea of the mesentery. The size of primordial oocytes ranged from 19 to 30 μm. The average diameter (± SD) of stage I oocytes was 26.6 ± 3.6 μm (n=117).

Stage II:

As oocytes developed, stage II oocytes were observed in polyp cavity and were connected to mesenteries by pedicles. Sizes of these oocytes ranged from 31 to 43 μm with an average diameter (± SD) of 36.9 ± 3.9 μm (n=424).

Stage III:

In stage III, oocytes undergo vitellogenesis during which yolk synthesis is active. The size range of stage III oocytes was between 43 to 73 μm. The average diameter (± SD) increased to 59.1 ± 10μm (n=813). These oocytes were detached from the pedicles. Nuclei were often found at the periphery of the oocytes as they were pushed aside by the yolk accumulating in the cytoplasm.

Stage IV:

Oocytes reached their maximum size at their mature stage, with the diameter ranging from 74 to 100 μm. The average diameter (± SD) of stage IV oocytes was 85.0 ± 7.1 μm (n=721). Usually a large number of mature oocytes were packed tightly together.

2.3.5.2 Spermatogenesis

Spermatogenic cycle can also be divided into four stages (Fig. 2.6).

Stage I:

Clusters of spermatogonia were observed in the mesoglea of mesenteries. Their sizes ranged from 20 to 30 μm with the average diameter (± SD) being 25.6 ± 3.3 μm (n=224).

Stage II:

Stage II spermaries were marked by the aggregation of newly-formed spermatocytes from spermatogonia. Spermatocytes were enclosed in the lumen of the spermaries that were present in the gastrodermis of the primary transverse mesentery. The sizes of stage II spermary ranged from 30 to 60 μm with the average diameter (± SD) of 46.7 ± 9.2 μm (n=315).

Stage III:

Stage III spermaries were characterized by the aggregation of spermatocytes and spermatozoa. Thick layer of lightly-stained spermatocytes formed the outer layer while darkly stained heads of spermatozoa were located in the lumen. Sizes of stage III spermary ranged from 60 to 100 μm, with an average (± SD) size of 79.3 ± 11.0 μm (n=595).

Stage IV:

Mature spermaries were featured by the presence of mature spermatozoa with tails projecting toward the center of the lumen. This is one of the features to indicate that the

spawning event is close. The completely mature spermaries increased to a mean (± SD) diameter of 106.5 ± 12.1 μm (n=631), with sizes that ranged from 87 to 123 μm.

2.3.6 Development of oocytes and spermaries over time

About 43.2% of oocytes in November 2008 were in stage IV. No oocytes were observed in all collected samples from December 2008 to May 2009. Oogenesis did not appear to be synchronized in *A. curvata* at the beginning of the reproductive cycle in June 09 (Fig. 2.7A). In this month, about 9.8% of all oocytes examined belonged to stage I while no stage I oocytes were found in October 2009. Stage IV oocytes increased from 24.8% in June 2009 to 70.2% in October 2009. No oocytes were found in all the black coral colonies sampled in November 2009 till February 2010. Thus, the pattern was consistent in the two consecutive years in which mass release of the oocytes likely occurred ,in late autumn (between November and December in 2008, and between October and November in 2009) and there was no sign of the start of oogenesis in winter in both 2009 and 2010.

The cycle of spermatogenesis (Fig. 2.7B) was generally more synchronized when compared with the oogenic cycle. The percentage of stage I spermaries in September 2008 was only 0.5% which was much lower than that of stage I oocytes in the very same period. About 46.8% of spermaries in November 2008 were in stage IV. No spermaries were observed in December 2008 to May 2009, which was consistent with that of oocytes in female samples collected. By June 2009, spermaries were again found in the monthly-collected samples. About 10.4% and 53.8% of the spermaries were in

stages I and II respectively with only 1.9% of them in stage IV. The percentage of spermaries in more mature stages increased from June to October 2009. In October 2009, 90.5% of the spermaries were in stage IV and none of them was in stage I. All stages of spermaries disappeared from the November samples in 2009. Thus, male colonies of *A. curvata* likely broadcasted their gametes enmass between October and November in 2009, a pattern consistent with that observed for the tagged female colonies. No relic spermaries were found in the gonads after December 2008 and November 2009.

Since both oogenesis and spermatogenesis started in June and ended in October 09 (or November in 2008), the complete development of gametes took less than half a year. Some earlier developments of the oocytes may have been missed, since stages III and IV oocytes were already present in some female colonies in June 2009, together with stage I oocytes. Nonetheless, gametogenic development, especially oogenesis, between colonies appeared to be asynchronous. This is shown more clearly when gametogenic development is examined in individual colonies in more details.

2.3.7 Correlation of black coral reproduction with seawater temperature

A stronger positive correlation was found between changes in seawater temperature and spermary size ($n = 7$; Spearman Rank Correlation Coefficient $r = 0.643$; $p = 0.119$) than between seawater temperature and oocyte size ($n = 7$; $r = 0.464$; $p = 0.294$). However, both correlations were not statistically significant. In spite of this, some relationship between the reproductive biology of *A. curvata* and seawater temperature could still be

detected. As indicated above, late gametogenic stages of both oocytes and spermaries in *A. curvata* were found from September to November 08, during which the seawater temperature hovered around 24 to 28°C (Fig. 2.4). This was followed by a rapid drop of 4°C in seawater temperature in December 08. No relic oocytes nor spermaries could be found in samples collected after this period. This rapid drop in seawater temperature might be the cue that triggered the mass spawning event that likely occurred in this period.

Gametes were absent in winter samples when seawater temperature was between 15°C to 23°C. The rise in seawater temperature in June 09, reaching 28°C, might also be one of the cues that triggered the onset of gametogenesis. Since some mature oocytes were already observed in June 09 samples, gametogenesis could have started earlier in May in some colonies, resulting in some colonies having different cohorts of oocytes developing at the same time. During gametogenic development from June to September 09, the seawater temperature hovered around 27 to 30°C. The pattern repeated in 2009 with the disappearance of all oocytes and spermaries being associated with a rapid drop in seawater temperature by 4°C from 27.7 to 23.3°C in November 09.

2.3.8 Gametogenesis in individual colonies

The average sizes of the gametes did not appear to vary significantly from June 2009, the start of gametogenesis, to October 2009, right before mass spawning was suspected to have occurred. One explanation for this would be that gametogenic development was asynchronous such that different sizes (or stages) of gametes were always present. A

more detailed examination of the frequencies of different developmental stages of gametes in individual tagged female and male colonies could reveal more information about this. However, it should be noted that the success of relocating the same tagged colonies every month was highly dependent on water clarity. Thus, certain colonies were missed in some months, resulted in data being discontinuous for some colonies. All in all, sufficient information were available only for 14 female and 8 male colonies in the study site to allow compilation of their individual gametogenic histories.

2.3.8.1 Female colonies

In May 09, 12 female colonies (i.e. nos. 2, 3, 4, 5, 6, 7, 8, 9, 10 11, 12 and 14) were sampled but none of them showed any signs of the development of young oocytes.

In June 09, a total of 12 female colonies (i.e. nos. 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13 and 14) were sampled but oocytes could only be found in colony no. 6. In this colony, stage I oocytes contributed the smallest proportion of all the four stages of oocytes, whereas 48% of oocytes sampled were in stage III while 25% were in stage IV (Fig. 2.8A). This explained the presence of large sized oocytes in June (Fig. 2.4), which masked the average size contributed by the smaller stage I oocytes.

In July 09, frequencies of different oocyte stages were highly inconsistent among the eight female colonies examined (Fig. 2.8B). Colony nos. 2 and 5 supported 100% and 71.4% respectively of stage I oocytes with little or no mature oocytes, while the proportion of stage I oocytes in colony nos. 1, 3, 4 ranged only from 30 to 45%. Two out of these five colonies had stage IV oocytes. Colony no. 6 consisted of about 36.7%

of stage IV oocytes, which was 11% higher than that in June 09. There was also an increase in the proportion of stage I oocytes by 25%, indicating that there were different cohorts of oocytes in this colony. Colony nos. 7 and 8 were different from the others in that they supported more mature oocytes than the immature ones. Over 78% of the oocytes in colony no. 7 were in stages III and IV while all oocytes found in colony no. 8 were in stage IV. This indicated a large discrepancy in oogenic development between the black coral colonies.

In August 09, the frequency of different stages of oocytes in seven tagged colonies examined was more consistent when compared with that of the previous two months (Fig. 2.8C). The proportion of stages III and IV oocytes in each colony ranged from 62.5% to 100%, indicating that the development of oocytes between colonies became more synchronized.

In September 09, of the total of eight female colonies examined (Fig. 2.8D), the proportions of stage III and IV oocytes were more similar when compared with those in August 09. In colony nos. 1 and 12 for examples, there was a large increase in the proportion of stage III oocytes by 63.4% and 66.7% respectively from August to September. Colony nos. 1, 2, 4, 6 and 8 supported about 68% to 90% of stages III and IV oocytes. In addition, young oocytes of stages I and II appeared in all female colonies examined, their proportions ranged from 4 to 28% and 10 to 43% respectively. These young oocytes were not observed in August, suggesting that a new cohort of oocytes continue to emerge in September and this phenomenon was quite synchronized among colonies. Among all three colonies examined in October 09 (Fig. 2.8E), at least 68% of

the oocytes were in stage IV, showing a consistent trend with the oocytes becoming more mature.

2.3.8.2 Male colonies

In May 09, five male colonies (i.e. nos. 1, 2, 3, 5 and 7) were sampled but none of them showed any signs of young spermaries.

In June 09, the same five male colonies were sampled and spermaries were found in only two of them, i.e. male colony nos. 1 and 2. Most of the spermaries examined in male colony nos. 1 and 2 were of stages II and III, while 4% and 12% respectively were of stage I (Fig. 2.9A).

In July 09, most of the spermaries in four colonies examined were of stages II and III, with a fraction of them (from 6% to 50% among three of the colonies) being mature (in stage IV) (Fig. 2.9B). Stage I spermaries originally present in male colony nos. 1 and 2 had developed into stage II, thus the proportion of stage II spermaries became higher than that in June 09 by 23% and 10% respectively in the two colonies.

In August 09, spermatogenic development was more synchronized among the three male colonies sampled as a majority (85%) of the spermaries were in stages III and IV (Fig. 2.9C). In September 09, male colony nos. 2 and 7 consisted of 2% and 20% of stage I spermaries respectively (Fig. 2.9D). This coincided with the appearance of stage I oocytes among female colonies within the same month. Spermaries continued to mature in male colonies examined in October 09 (Fig. 2.9E) with almost only stage IV

spermaries found. This supported the suggestion that mass spawning occurred thereafter between October and November 2009.

2.4 Discussion

Antipathes curvata from Lan Guo Shui in the present study is a gonochoric broadcaster. These reproductive characteristics are consistent with those found in other Antipatharian species, i.e. *Antipathes fiordensis* from New Zealand (Parker *et al.*, 1997) and *Cirrihapthes* cfr. *anguina* from Indonesia (Gaino and Scoccia, 2009). It is however, too premature to generalize the reproductive characteristics of all black corals based on only these few examples.

Compared with the study of Parker *et al.* (1997), both *A. curvata* and *A. fiordensis* shared many features in common. The reproductive period of both black coral species was seasonal. The gametogenesis cycle of *A. curvata* in Hong Kong lasted for about 6-7 months from June 2009 (late spring) to November 2009 (late autumn). Similarly, the gametogenic cycle of *A. fiordensis* in New Zealand lasted around 6 months from November 1995 (Austral spring) to April 1996 (Austral autumn). No relic gametes were found in both species after the annual mass spawning.

2.4.1 Asynchronization of gametogenic cycle

Despite these similarities, however, gametogenic development of *A. curvata* was more asynchronous within and between colonies. Different stages of gametes could be found

in the same colony or in different colonies within the same period. In contrast, gametogenic development in *A. fiordensis* was found to be synchronous (Parker *et al.*, 1997).

Within the study period, no signs of oocytes and spermaries were observed in samples collected from December 2008 to May 2009. Female colony no. 6 and male colony no. 1 were sampled in both May and June 2009 and no gametes were found in May 2009 in both colonies. However, in June 2009, some stages I and II oocytes with a larger proportion of stage III (48%) and IV (25%) oocytes were already present in female colony no. 6, whereas in male colony no. 1, 4% of the spermaries were in stage I and the remaining were in stage II (44%) and III (52%). Likewise, more colonies were detected to have oocytes or spermaries in different stages in July 09. It is possible that early gametogenesis developed very rapidly in the sampling interval of four weeks between May and June or between June and July, thus most stage I gametes were missed. On the other hand, as only one branch was normally sampled from each colony each time, it is also possible that gametogenic development in polyps from different branches within the same colony was highly asynchronous, such that polyps in different branches from the same colony did not start their gametogenesis at the same time. As a consequence, the few branches with polyps having developing gametes were missed in some of the samples in May or June. Subsequently in July and thereafter, gametogenesis would have been initiated in more and more of the polyps in different branches, thus increasing the chances of finding gametes in all of the samples collected.

From the 14 tagged female colonies monitored in this study, a few of them have oocytes that matured at an earlier period. Different cohorts of oocytes were formed

within the same polyp in certain colony. It is reasonable to deduce that the stage IV oocytes observed in June 09 would have been released before the main spawning event in November 09 with more, younger oocytes developing at the same time. Thus, some small scale spawning may be occurring continuously throughout the summer months. Overall, female colonies appeared less synchronized in their oocyte development at the beginning, i.e. in June, July 09. However, the process became more synchronized in later months, with the percentage of stage IV oocytes becoming more or less the same between colonies.

From the eight male colonies followed, spermatogenic development appeared to be much more synchronized within and between colonies than among the female colonies throughout the reproductive period. Right at early part of the reproductive cycle, the proportion of different developmental stages of spermaries did not vary much between colonies. As the process continued, all male colonies were having a higher and comparable proportion of mature spermaries between them.

Relatively smaller percentage of younger oocytes was still present in the female colonies in September and October 09. This was less apparent in the male colonies. In the absence of any relic male or female gametes of any stages found in November 09 (and also December 08) samples and in those collected in the subsequent winter months, these premature gametes may either have been discharged with the mature gametes during mass spawning, or may have been reabsorbed. One thing is at least certain, that no developing gametes stayed in a "dormant" stage over the winter in this black coral species.

Detailed analysis on gametogenesis in tagged individual colonies has rarely been done on other coral species. Results from the present study revealed a far more dynamic picture of gametogenic development in different colonies of black coral than would have been shown from random or haphazard sampling of these colonies alone., the latter being a more common approach in other coral reproductive studies. Different colonies, or branches within the same colony, appeared to initiate gametogenesis not at the same time. This is particularly so among the female colonies. However, most of them eventually caught up with one another that ended with a main spawning event towards late autumn.

2.4.2 Possible effect of seawater temperature on reproduction of *A. curvata*

Synchronized sexual reproductive cycle of corals has been shown to be regulated by environmental factors (Giese and Pearse, 1974; Olive, 1984). These factors can provide cues to synchronize gamete maturation and reproductive cycle and can act as ultimate causes that exert evolutionary selective pressure through enhanced reproductive success (Harrison and Wallace, 1990; Harrison, 2011). For instance, synchronized spawning among coral populations enhanced the success of fertilization (Oliver and Babcock, 1992; Willis *et al.*, 1997; Levitan *et al.*, 2004).

Seasonal change in seawater temperature is often cited as an important environmental cue in controlling gametogenic cycles or the timing of planulae release in scleractinian corals (Kojis, 1986; Mendes and Woodley, 2002; Penland *et al.*, 2004). Numerous correlative evidences showed that changes in seawater temperature provide

seasonal cue for reproduction of various broadcasting corals (Van Veghel, 1994; Fan and Dai, 1995; Mendes and Woodley, 2002).

Such apparent correlation between seawater temperature and oogenesis was also seen in *A. fiordensis* in New Zealand (Parker *et al.*, 1997). The monthly temperature from November 1994 to May 1995 was 2 to 3°C lower than that in previous year. Histological analysis showed that the development of gametes was almost completed in February 1995, but spawning did not occur until March 1995 which was one month later than in previous year.

The lack of a significant positive correlation between seawater temperature and gametogenesis in *A. curvata* in the present study may be due to the asynchronous development of gametes in this black coral species. Among the female colonies, some matured faster than the others such that relatively high proportion of stages III and IV oocytes were present along with younger stages I and II oocytes. These larger oocytes contributed to the large average size of oocytes even in June and July 2009, at the beginning of the oogenic cycle. Thus, the mean sizes of the oocyte throughout the reproductive period were not significantly different. Although development of spermaries in male colonies appeared more synchronized, there was still a mix of spermary of different stages and sizes in different months. This also contributed to the lack of a distinctive trend in the increase of spermary sizes throughout the whole reproductive period.

Nonetheless, it can still be shown that the onset of gametogenesis in *A. curvata* was coincident with the warming of seawater temperature by 5°C within two months from April to June 2009 whereas mass spawning in November 2009 was coincident with the

drop of seawater temperature by 4°C within one month from October to November 2009. Moreover, the yearly variation in seawater temperature in 2008 and 2009 could be the reason for the different timing of spawning in these years (December in 2008 vs. November in 2009). The seawater temperature from May to October 2009 (late spring to late autumn) was about 1 to 2°C higher than that of the same period in 2008. Relatively warmer summer in 2009 may directly increase the rate of gametogenic development or indirectly through increase in food availability (Sundback *et al.*, 1996), causing *A. curvata* to mature faster and to spawn one month earlier at a higher temperature (23.3°C) than in the previous year (20°C). But what is more notable is that a drop of 4°C in seawater temperature was recorded one month before the putative mass spawning event in both years. This clearly suggests that drop in a certain range of temperature is more critical in triggering spawning than reaching a defined threshold temperature. Changes in temperature thus appear to play an important role in the reproductive development of *A. curvata.*

It has been suggested that lower sea temperature resulted in late spawning of corals (Harrison *et al.*, 1984). The extension of breeding period was the response to the unfavorable environmental conditions posed on coral reproduction. This extension could help to maximize reproductive success (Tomascik and Sander, 1987). Such response was also apparent in the black coral *A. curvata.* The relationship between seawater temperature and the timing of different critical reproductive events in black coral is likely to be similar to those found in other hard and soft corals. This suggests that although information on black coral reproduction is very limited, this group of

marine organisms is unlikely to be spared from any effects that may be brought about by projected global seawater temperature change.

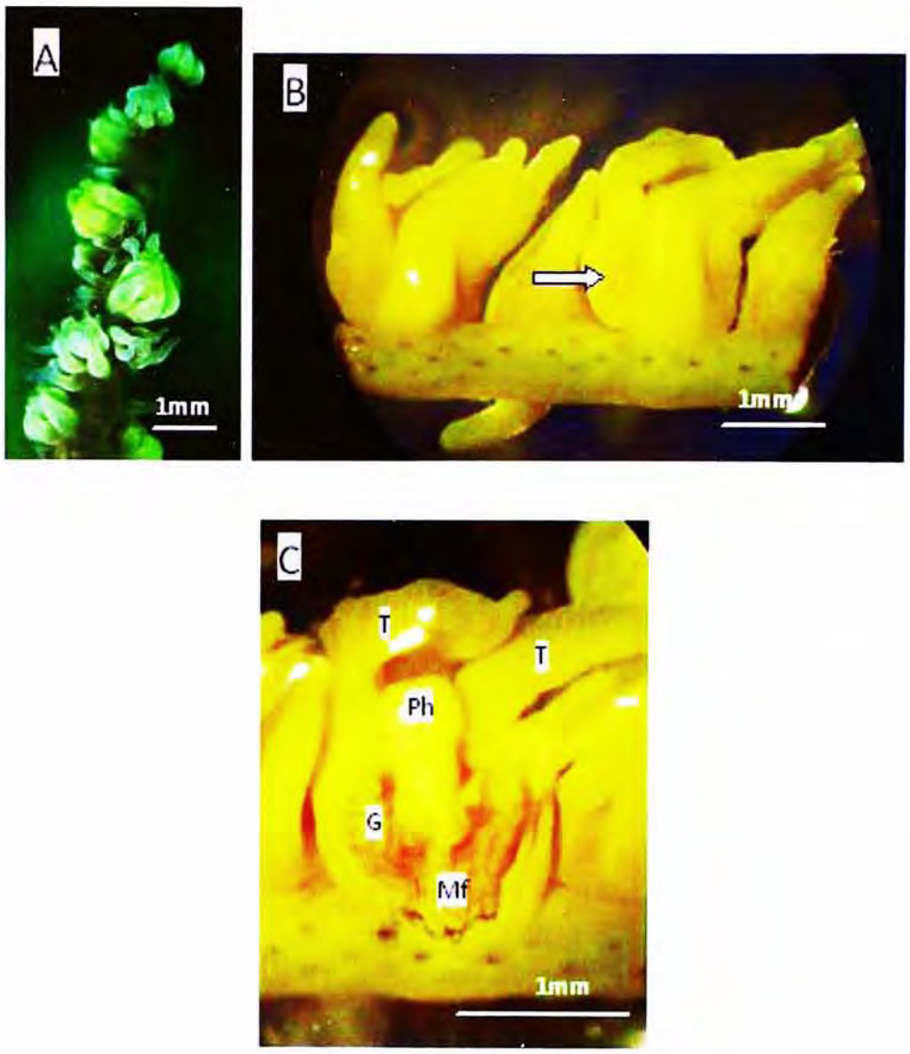

Fig. 2.1 **A.** Polyps of *A. curvata* taken underwater showing six contractable tentacles. **B.** Polyps of *A. curvata* around the skeleton showing the bulging base of the lateral tentacle (arrow) that is due to the presence of growing gametes inside. **C.** Longitudinal section showing the arrangement of tissues inside a polyp. Pharynx (Ph) is surrounded by tentacles (T). Growing gametes (G) are embedded in mesentery supported by mesenterial filaments (Mf).

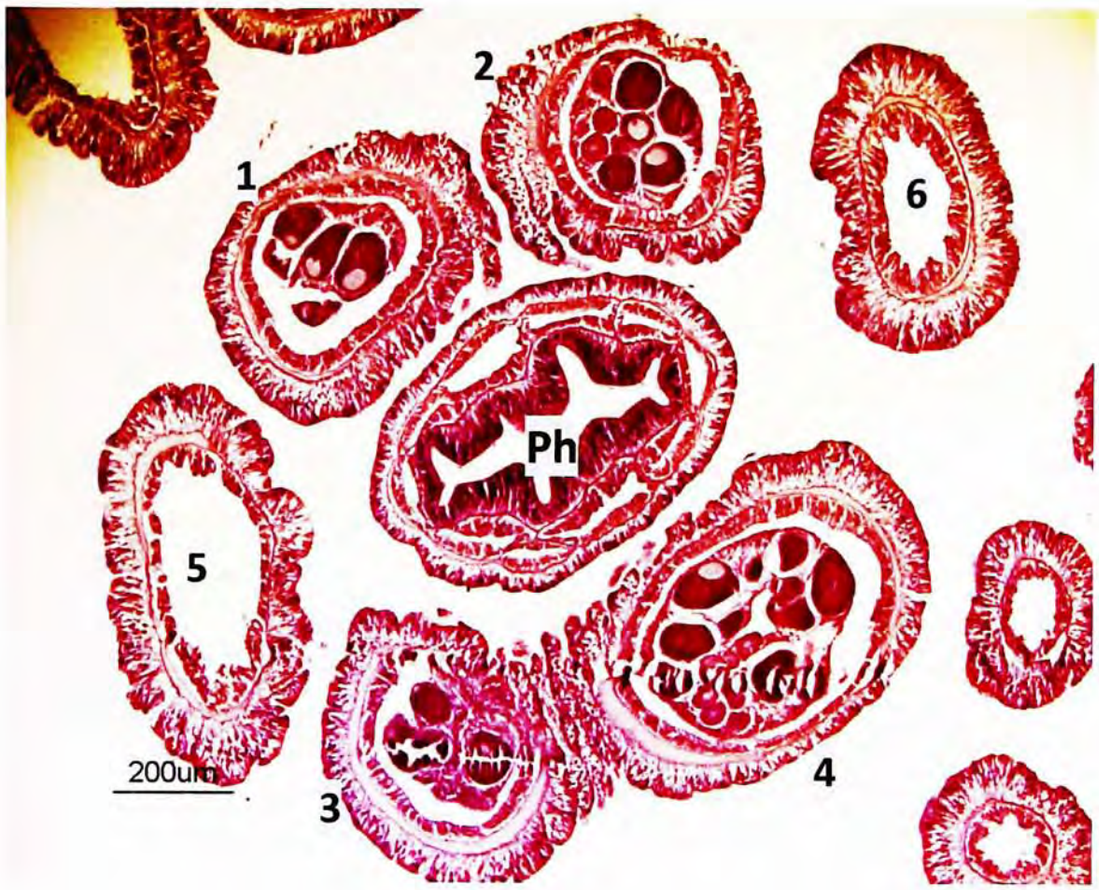

Fig. 2.2 A histological cross-section of the oral region of a gravid female polyp of *A. curvata*. The central pharynx (Ph) is surrounded by six tentacles, the lateral tentacles (1-4) and the sagittal tentacles (5, 6). Gametes are found only in the four lateral tentacles and not in the two sagittal tentacles.

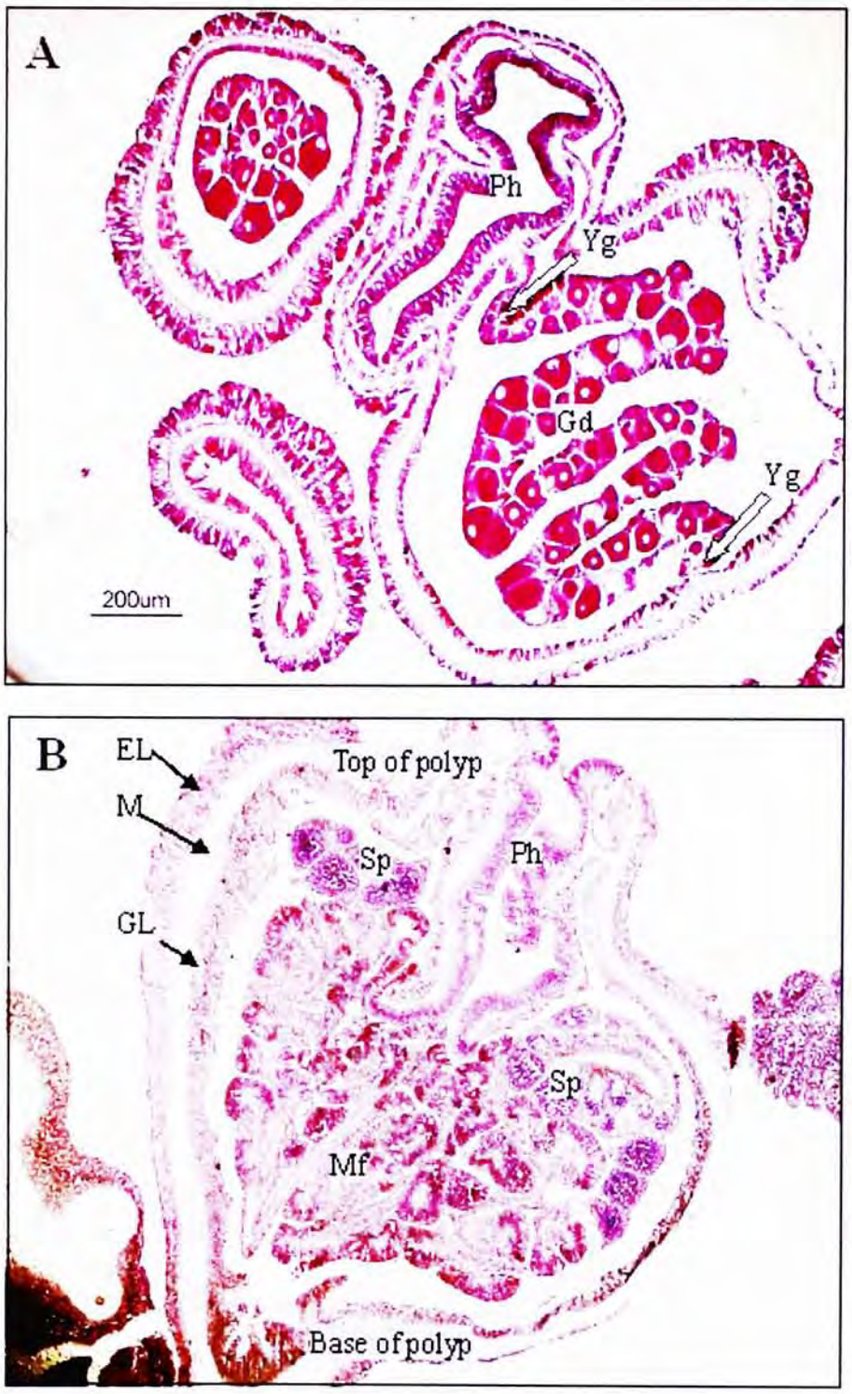

Fig. 2.3 **A.** Histological cross-section near the basal region of a gravid female polyp of *A. curvata*. Young gametes (Yg, arrows) tend to gather in mesoglea proximal to the region where mesentery originated from the gastroderm (Gd) lining the wall of the body and in region next to the pharynx (Ph). **B.** Histological longitudinal section of a male polyp showing growing spermaries (Sp) on top of the mesenterial filaments (Mf). Note EL, epidermal layer; M, mesoglea; GL, gastrodermal layer.

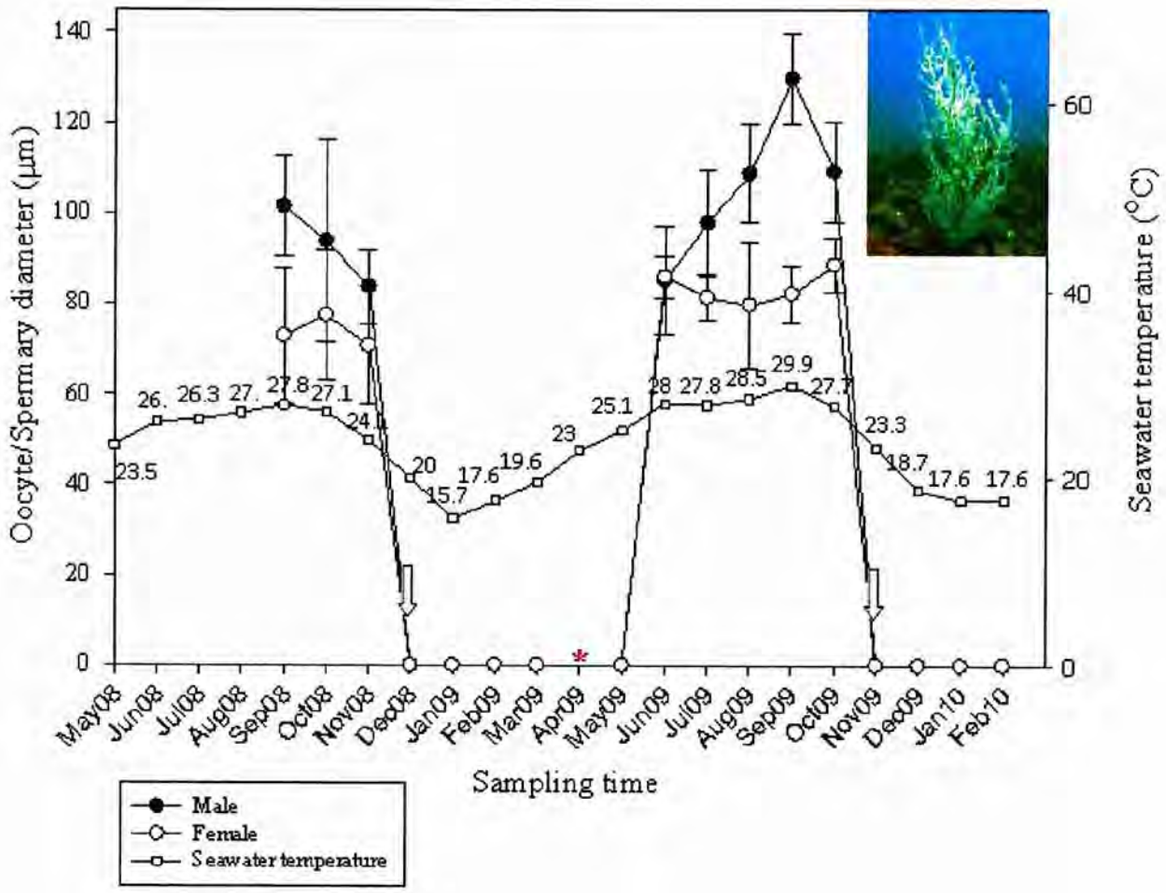

Fig. 2.4 Changes in the average sizes (± SD) of oocytes and spermaries of tagged *A. curvata* colonies from September 2008 to February 2010. Mass spawning likely took place in December 2008 and November 2009 (arrows). Numerical numbers above the solid triangle indicate the mean monthly seawater temperature. Samples were missed in April 2009 (*).

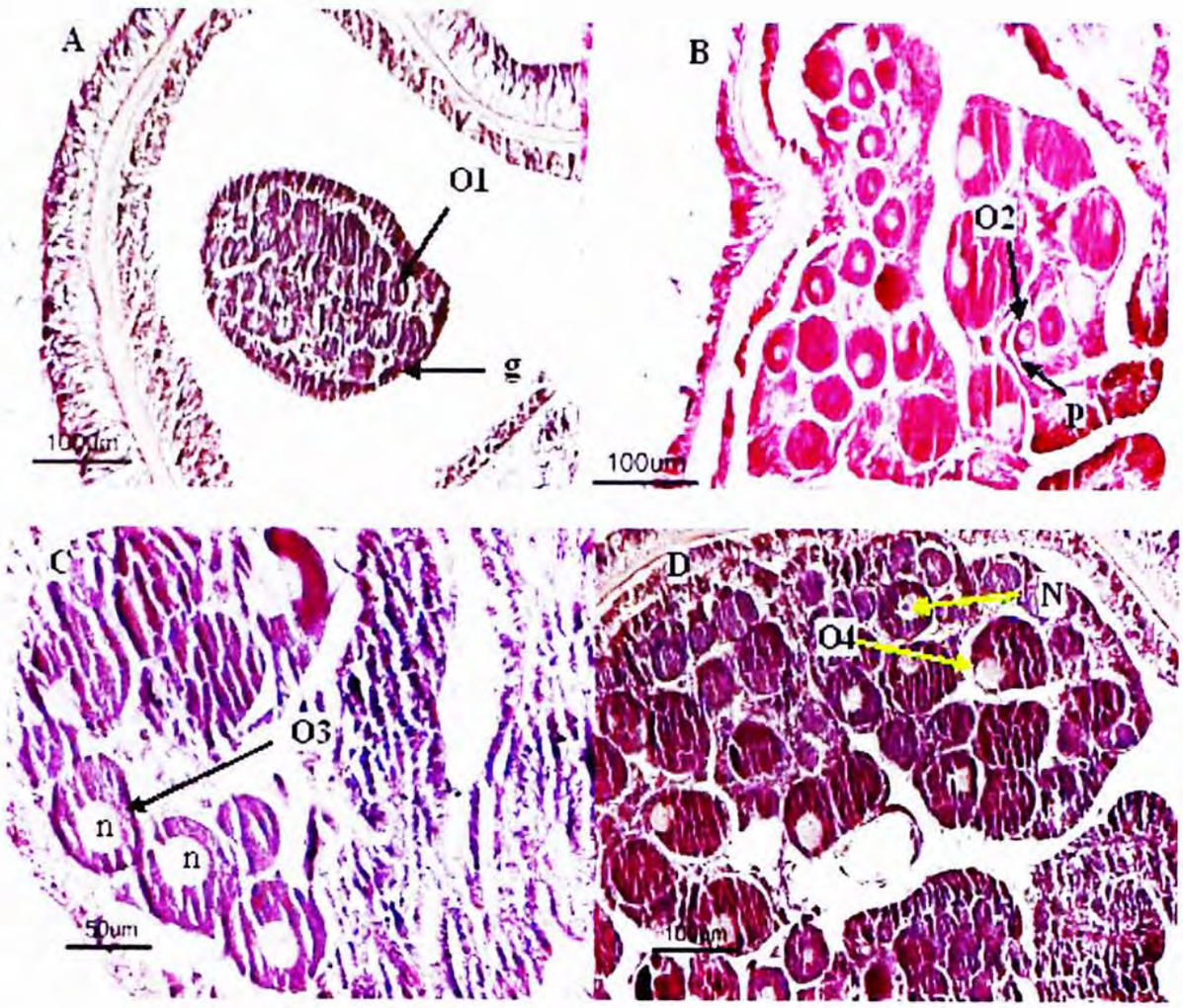

Fig. 2.5 Oogenic developmental stages of *A. curvata*. **A.** Stage I (O1): oocytes in their early stage are embedded in gastroderm in mesentery. They are characterized by a high nucleus to cytoplasm ratio. **B.** Stage II (O2): oocytes are found inside polyp cavity with a pedicle (P) connected to the mesentery. **C.** Stage III (O3): vitellogenesis begins in stage III oocytes and yolk started to accumulate in their cytoplasm. Ratio of nucleus to cytoplasm diminishes. A spherical nucleus is situated in the center of the oocytes. **D.** Stage IV (O4): oocytes in their mature stage have reached their maximum sizes. Nucleus moves to the periphery of the oocytes. Note: n, nucleus; N, nucleolus; g, gastroderm; p, pedicle.

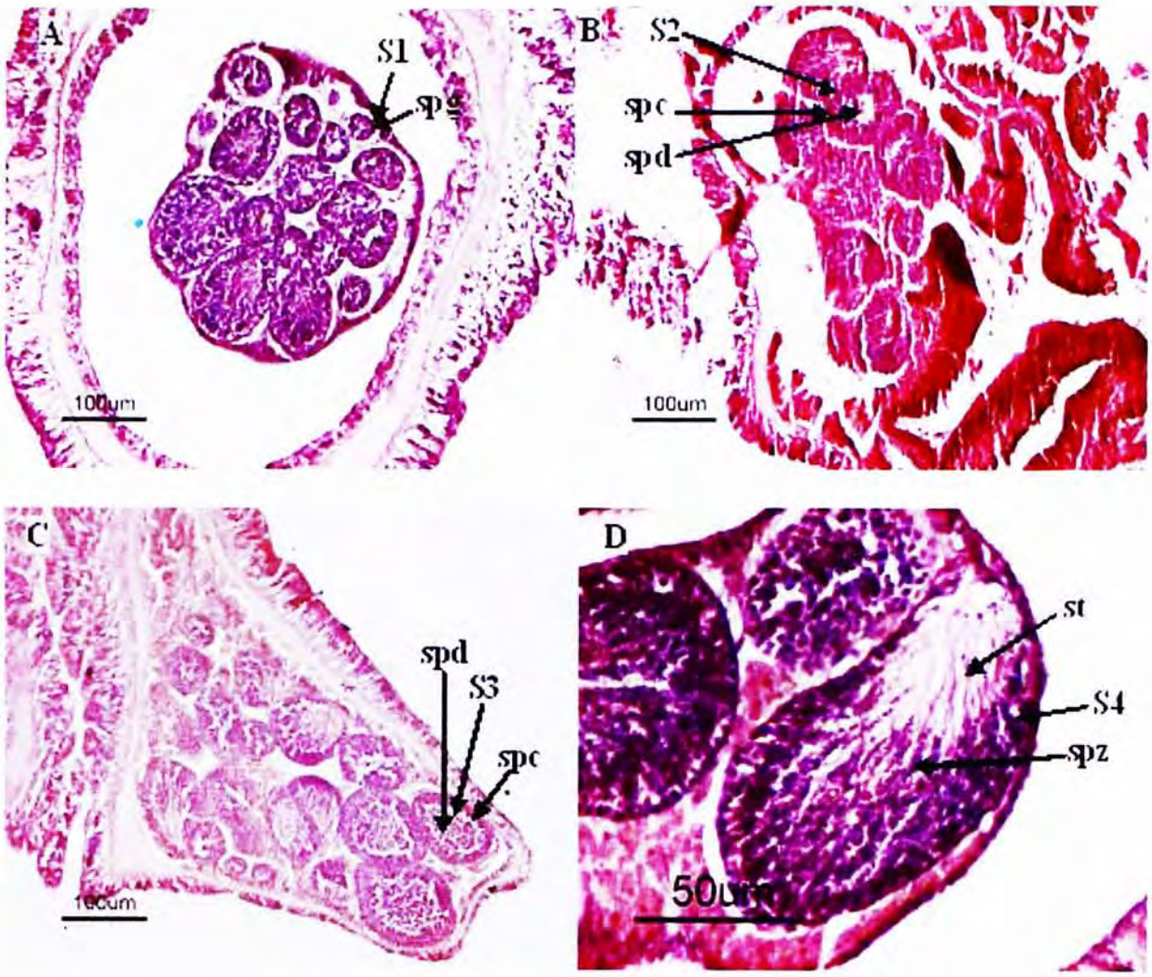

Fig. 2.6 Spermatogenic developmental stages of *A. curvata*. **A.** Stage I: spermaries with spermatogonia. **B.** Stage II: spermaries contain a group of spermatocytes which are undergoing meiosis. **C.** Stage III: thick layer of spermatids form the outer layer while spermatozoas are located in the lumen. **D.** Stage V: spermatozoa with tails all pointing towards one direction in the lumen. Note: spg, spermatogonia; spc, spermatocytes; spd, spermatids; spz, spermatozoa; st, spermatozoa tail.

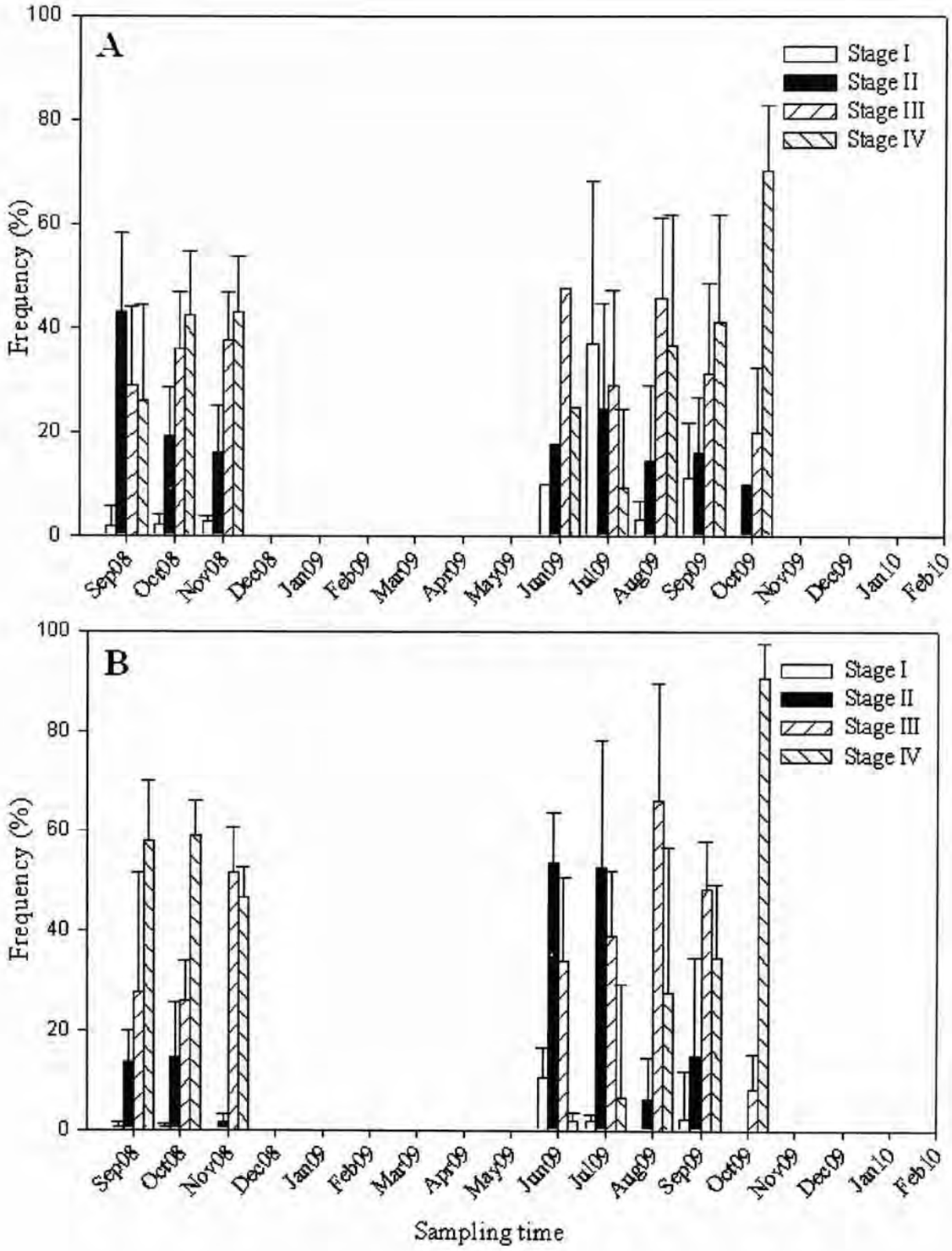

Fig. 2.7 Frequency (%) of different stages of **A.** Oocytes and **B.** Spermaries in *A. curvata* from September 2008 to February 2010. No oocytes and spermaries were found from December 2008 to May 2009 (samples in April 2009 were missed), and from November 2009 to February 2010.

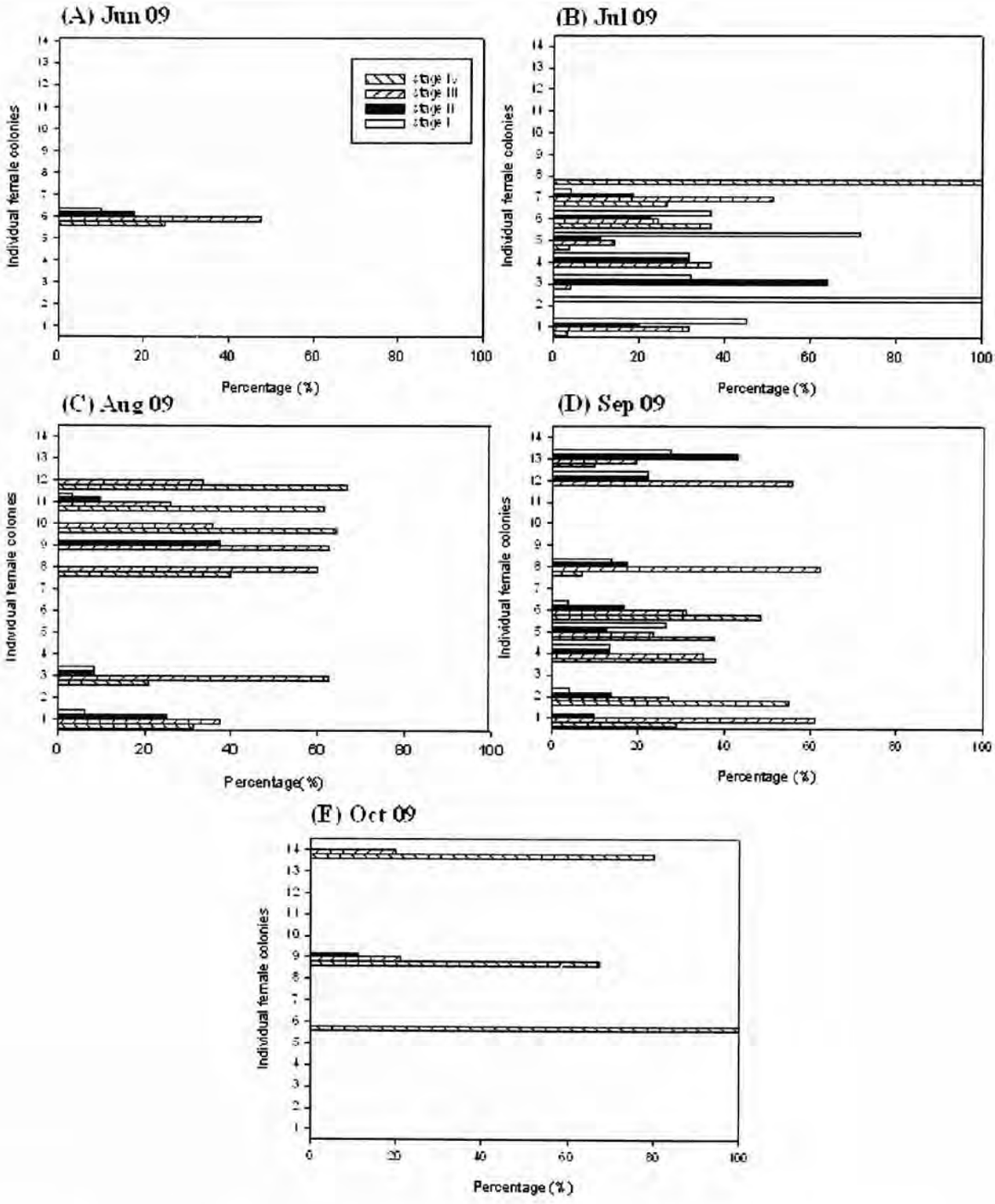

Fig. 2.8 Frequency (%) of different developmental stages of oocytes in 14 different tagged female colonies over time. No bars indicate that either the colonies were not sampled in that particular month or no oocytes could be found in them.

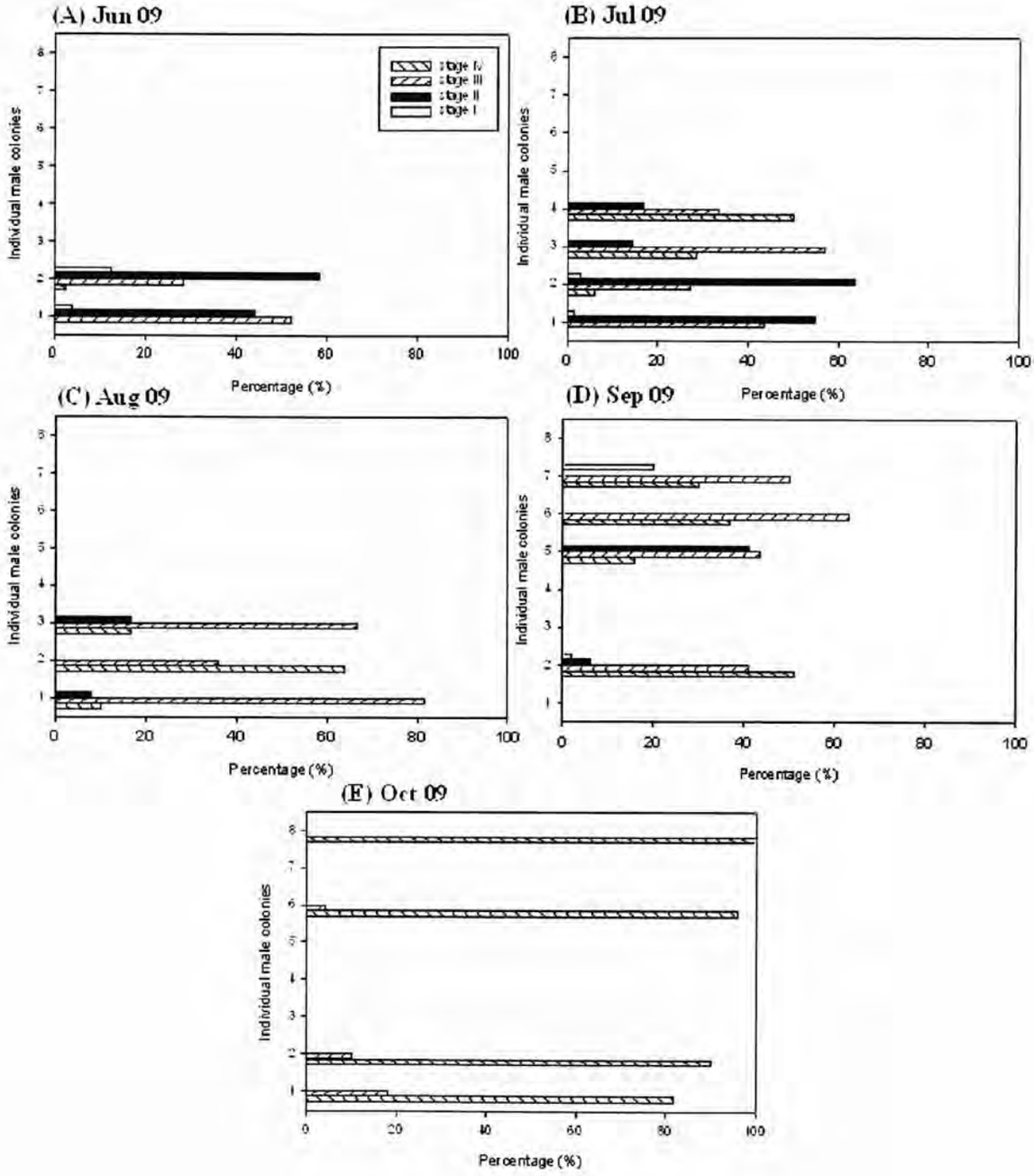

Fig. 2.9 Frequency (%) of different developmental stages of spermaries in eight different tagged male colonies over time. No bars indicate that either the colonies were not sampled in that particular month or no spermaries could be found in them.

Chapter 3 Detection of the Sex Steroid 17β-estradiol and its Possible Roles on Gametogenesis in Black Corals *Antipathes curvata* from Hong Kong

3.1. Introduction

In previous studies on the triggering factors of spawning in corals, environmental and endogenous physiological factors were always discussed separately. However, they are always of connected relationship. For any environmental cues that induce spawning, they must be translated into action by the organisms' endogenous control mechanisms which initiate some endocrine changes that trigger mass spawning finally. These organisms are seen as a "black box" which receives external inputs while gives out "response" which is spawning (Oliver, 1995). Recent studies found that photoperiod and temperature affect endocrine system which affects gametogenesis of an organism (Bentley and Pacey, 1992; Lawrence, 1996).

Vertebrate endocrine system is one of the good examples on the high complexity of metazoans which are the organisms with rich diversity of body plans, high degree of compartmentalization and a complicated communication network between organs and systems within the body (McLachlan, 2001). Bio-regulatory molecules and hormones secreted by specialized cells and organs are transported in circulatory system to targeted

cells and organs to regulate various kinds of physiological processes, including reproduction which is controlled by sex hormones.

Among invertebrates, many possess discrete endocrine organs that share similar functions with the corresponding ones in vertebrates. In this aspect, arthropods in particular insects are the most extensively studied group whereas Cnidaria is one of the least studied groups among invertebrates (Segal, 1993; Karlson, 1996; Klowden, 2003; Swevers and Iatrou, 2003). Corals which are in the phylum Cnidaria, have a nerve net, diploblastic tissue with a simple structure, which is of two cell layers, namely epidermis and gastrodermis. They are not organized into organs or systems, but only generally differentiated into tissues, and that circulation generally occurs by simple diffusion.

Due to the basal position in metazoans and simple organization in cnidarians, it was expected that their bio-regulation would be very different from that in vertebrates and that many vertebrate-features would be absent in corals. However, several classical vertebrate hormones, such as estradiol, estrone, testosterone, have been identified in coral tissues (Slattery *et al.*, 1999; Tarrant *et al.*, 1999; Pernet *et al.*, 2002; Twan *et al.*, 2003) though neither the homologs to steroid receptors in vertebrates have been detected in cnidarians nor the regulatory signaling pathways have been investigated. The elucidation of signaling pathways of sex steroids in corals help to build insight into the evolution of sex hormones and on the roles of these hormones in different levels of organisms. These studies could also help to provide baseline information on the investigation of physiological studies on cnidarians in the future. Moreover, a better understanding of endocrine-like bio-regulation in cnidarians could help in maximizing the effectiveness of conservation programs on coral reefs.

3.1.1 Roles of sex hormone, 17β-estradiol (E2), in the reproduction of vertebrates

Reproductive cycle in vertebrates is controlled by hormonal levels in the body which in turn is regulated by the CNS hypothalamic-pituitary-gonadal axis (Terakado, 2001; Dubois *et al.*, 2002). Hypothalamus secretes a small peptide, gonadotropin-releasing hormone (GnRH), namely luteinizing-releasing hormone. In turn, luteinizing-releasing hormone regulates the release of gonadotropins, such as luteinizing hormone (LH) and follicule-stimulating hormone (FSH) from the anterior pituitary gland. LH and FSH then stimulate the synthesis of sex steroids, such as estrogens (e.g. estradiol), androgens (e.g. testosterone) and progestins (e.g. progesterone), from targeted organs. Generally, steroids act by binding to intracellular receptor proteins in the nuclear receptor superfamily which bind to response element DNA and in turn regulate the transcription of targeted genes (Evans, 1988).

In vertebrates, 17β-estradiol (E2) is a predominant sex hormone present in females. It could also be produced as an active metabolic product from testosterone in males. E2 is a kind of steroid, derived from cholesterol. During reproductive years of females, most E2 is produced by aromatization of androstenedione to estrone in granulosa cells of ovaries and would be subsequently converted into E2. A smaller amount of E2 is synthesized in adrenal cortex or in testes in men.

On the other hand, E2 was synthesized not only in gonads, but could also be converted from testosterone from theca cells and in turn undergoes conversion by aromatase into E2 in both sexes. It could also be synthesized in arterial walls and in brain.

In females, E2 promotes the growth of reproductive organs, supporting the vaginal lining, the fallopian tubes. It also enhances the growth of myometrium, as well as maintaining oocytes in the ovary. Moreover, E2 would induce ovulation as it triggers a surge in luteinizing hormone in hypothalamic-pituitary event via positive-feedback mechanism. It prepares the endometrium for implantation in luteal phase. Furthermore, E2 helps to maintain pregnancy and its level increases as placenta develops.

3.1.2 Roles of vertebrate-type sex steroids in Cnidaria

GnRHs are well-conserved and form a family in vertebrates and protochordates (Powell *et al.,* 1996; Terakado, 2001) which stimulate the release of gonadotropins and act as neurotransmitters throughout the body. As cnidarians lack endocrine system and circulatory system, hypothalamic-pituitary-gonadal axis is also absent. However, studies found that synthetic GnRHs can stimulate gametogenesis and spawning of mollusks (Pazos *et al.,* 1999; Gorbman *et al.,* 2003). GnRHs immunoreactivity has been identified in two species of anthozoa, including a sea pansy, *Renilla koellikeri* and a sea anemone, *Nematostella vectensis*. Both cnidarian GnRH-like compounds and vertebrate GnRH inhibit peristaltic contraction in *R. koellikeri* while it can be blocked by GnRH analog (Anctil, 2000). It is still uncertain about the physiological roles of GnRH-like compounds in cnidarians whether they stimulate the production of other peptides, which may be homologs to vertebrate gonadotropins. Nevertheless, a receptor which highly resembles vertebrate glycoprotein hormone receptor family, including somatotropins

and gonadotropins, has been identified in a sea anemone (Nothacker and Grimmelikhuizen, 1993).

It was found that an immunoreactive (ir)GnRH could be detected in tissues of scleractinian coral, *Euphyllia ancora* by using reversed phase HPLC (Twan *et al.*, 2006). Even if coral irGnRH has different retention time than GnRHs in other animals, such as lamprey (l)GnRH-I, chicken (c)GnRH-II, salmon (s)GnRH, seabream (sb)GnRH and mammalian (m) GnRH in HPLC profiles, both coral irGnRH (10^{-5}-10^{-9}M) and (m)GnRH (10^{-6}-10^{-10}M) agonist could dose-dependently stimulate the release of luteinizing hormone in pituitary cell culture of black porgy (Twan *et al.*, 2006). Moreover, mGnRH receptor antagonist dose-dependently inhibited the stimulation on the release of luteinizing hormone which was caused by the presence of coral irGnRH. It was also found that aromatase activity, irGnRH concentrations, free estradiol and estradiol glucuronide concentrations increased in a parallel manner at the time of coral spawning. irGnRH and aromatase concentration during coral spawning was 9 times and 10 times higher than that in non-spawning period, respectively. When applying mGnRH agonist to cultivated corals kept in seawater aquarium, aromatase activity, free estradiol and testosterone concentrations and their conjugates concentrations increased (Twan *et al.*, 2006), suggesting that sex steroids have conserved roles in reproduction across vertebrates and invertebrates even before the appearance of endocrine system and pituitary gland as a relay.

E2 was first detected in coral eggs during a mass coral spawning in 1992 (Atkinson and Atkinson, 1992). In scleractinian corals, *Montipora capitata*, the concentrations of estradiol and estrone varied throughout the year, with estradiol peaked in February and

March while estrone peaked in April, prior to spawning in June and July (Tarrant *et al.*, 1999). For *Euphyllia ancora,* peak concentrations of free estradiol, glucuronided estradiol and glucuronided testosterone were found in the coral tissue during months of spawning (Twan *et al.*, 2003). Octocoral *Renilla koellikeri* showed elevated level of estradiol prior to spawning while the level in female colonies were greater than that in males (Pernet *et al.*, 2002). Vertebrate-type sex steroids, estradiol, progesterone and testosterone were detected in three species of alcyonacean soft corals. For *Sinularia polydactyla*, there was a sharp drop of testosterone level following spawning month in both female and male colonies (Slattery *et al.*, 1999). Among females, there was a sudden drop of progesterone right after the spawning.

Antipatharians are widely distributed from tropical to temperate seas and that they have been harvested for a long period of time. These black coral beds provide commercial resources for humans. As a result, maintaining the harvesting of marine resources such as black coral beds in a sustainable way is especially crucial for the benefits of our offsprings. To achieve this, understanding the endogenous regulation on reproduction of *Antipathes curvata* can serve to fill the knowledge gap of biology of black corals. Moreover, it can provide insight into the evolution of endocrine-like bioregulation from simple organisms to higher animals.

Considerable progress has been made in identifying and quantifying different endogenous sex steroids in hard corals and soft corals as mentioned above (Atkinson and Atkinson, 1992; Slattery *et al.,* 1997, 1999; Tarrant *et al.*, 1999; Pernet *et al.,* 2002; Twan *et al.,* 2006). This is, however, not the case with the antipatharians (black corals). Even though antipatharians were listed in CITES Appendix II as they were being

intensively harvested for jewelry industries, no such studies have ever been carried out partly due probably to their being found mostly in deep-water habitat. This makes systematic studies in identifying and quantifying their endogenous sex steroids logistically challenging. Nonetheless, identifying and understanding the roles of endogenous sex steroids in black coral reproduction are essentially important for their conservation. In Hong Kong , the black coral *Antipathes curvata* can be abundantly found in waters as shallow as 7-8m below Chart Datum (C.D.) (Ang *et al.*, 2010). This provides an excellent opportunity to study black coral reproduction as well as the presence of sex steroids and their roles in coral reproduction. The objectives of this research were therefore: 1). to quantify the endogenous E2 levels in *A. curvata* coral tissues; and 2). to correlate the levels of E2 with the patterns of gametogenesis in both female and male colonies of this species. In the present study, we tested the hypothesis that sex steroid E2 is important in gametogenesis and mass spawning of *A. curvata* and should be reflected in its higher level prior to the onset of gametogenesis and mass spawning events.

3.2 Materials and methods

3.2.1 Study site

Hong Kong is located in the southern coast of China at the mouth of Pearl River (See Chapter 1). Lan Guo Shui, on the southern side of Tung Ping Chau Marine Park (TPCMP), a relatively isolated island in northeast Hong Kong, supports a high density of *A. curvata*. Sample collection and field observations were carried out in this site using SCUBA.

3.2.2 17β-estradiol (E2) extraction

Branchlets (~12cm) were collected from tagged black coral colonies on a monthly basis from October 2008 to February 2010, immediately frozen in liquid nitrogen on board the research vessel and stored at -80°C in the laboratory for long-term storage. Gloves were worn when handling the coral samples to minimize steroid contamination.

As coral colonies were previously sexed, coral fragments collected were therefore classified into three groups: male, female and unsexable (i.e. no gonads found throughout the whole research period). In each group, coral tissues were separated from the skeleton by sonication and known amount of coral tissues (g) was homogenized.

Each group was further divided into unspiked and spiked portions in which the latter was spiked by a known amount of E2 standard (Cat. No. 80-0114. Assay Designs, Ann Arbor, MI, USA). The extraction of E2 from the coral tissues followed the protocol described by E2 ELISA kit manual (Cat. No. 900-008. Assay Designs, Ann Arbor, MI, USA.) and by Liu et al. (2010). Briefly, coral homogenate was extracted 3 times by 5-fold (v/v) of dichloromethane, i.e. the lower solvent fraction which contains steroids was isolated after the first extraction and the upper aqueous fraction was further extracted by dichloromethane for two more times. Steroids in the organic solvent was then concentrated to dryness using a gentle stream of pure nitrogen gas. Dried extract was re-suspended in 0.5ml methanol and 5ml buffered 0.07M Na_2HPO_4 (pH 7.4).

E2 in coral extracts after liquid-liquid extraction was purified and concentrated using Supelclean LC-18 Solid Phase Extraction (SPE) Tubes (6ml, 0.5g, Cat. No. 57054, Supelclean, Bellefonte, PA, USA). SPE tubes were first conditioned with 5ml methanol, followed by 10ml Milli-Q water. Methanol wets the surface of the sorbent and penetrates bonded alkyl phases that allowed water to wet the silica surface more efficiently. The re-suspended samples were pre-filtered, then adsorbed onto the pre-conditioned LC-18 Sep-Pak cartridges. SPE columns were washed with 10ml distilled water and 5ml 10% methanol. Steroids were finally rinsed with 10ml of eluting solvent (hexane: dichloromethane, 3:7) at a flow rate of 2ml/min (Liu *et al.*, 2010). The eluates were collected and dried under pure nitrogen gas. Dried extract was re-suspended in 10% methanol and in ELISA buffer for enzyme immunoassay in later stage.

3.2.3 17β-estradiol (E2) assay

The E2 level in 10% methanol and ELISA assay buffer was determined using commercial E2 ELISA kit (Cat. No. 900-008. Assay Designs, Ann Arbor, MI, USA.). This kit employs a competitive enzyme immunoassay wherein E2 in the standard or sample and the alkaline phosphatase molecule which has an E2 covalently attached to it would bind onto polyclonal antibodies in a competitive manner. After incubating at room temperature, excess reagents were washed away and the substrate was added for enzymatic reaction to take place. The phosphatase bound to the antibodies catalyzed the transformation of *p*-nitrophenyl phosphate into a yellow product, the absorbance of which was read on a microplate reader at 405nm. The color intensity was inversely proportional to the E2 concentrations in the samples and in the standards.

3.2.4 Calculation and assay validation

Standard curve with E2 standards was plotted by Sigma Plot 10.0 and E2 levels in the unknowns were calculated from optical density values. The assays were validated by demonstration of the parallel displacement curves with serially diluted black coral extracts for 3 times (1:2, v/v). The results suggested that E2 in black coral extracts bound to antibodies in a similar manner as E2 used in the standard curve (Fig. 3.1).

Extraction efficiency was determined by spiking a series of sample homogenates separately with a known amount of E2 standard for recovery analysis. Same experimental procedures including liquid-liquid (L-L) extraction and solid-phase extraction (SPE) were applied to both spiked and unspiked portions of the black coral extracts. The levels of E2 were then measured at the same time to calculate the recovery rates. After L-L extraction and SPE purification steps, recovery ranged from 88% to 92%.

3.2.5 Gametogenesis of *A. curvata*

The gametogenetic cycle of tagged, previously sexed *A. curvata* colonies were followed monthly from September 2008 to February 2010. In each month, terminal branchlets, one from each colony were cut and preserved in 10% buffered formalin in seawater (v/v) for 7 days before being transferred into 75% ethanol for long-term preservation. Each sample was fixed and embedded in paraffin. Serial histological transverse sections were then cut at 7μm thickness from oral to aboral side, mounted on slides, stained with Harris haematoxylin and eosin (Winsor, 1984), permanently mounted using Permount® and examined under the light microscope. The geometric diameter (Wallace, 1985) of 20 largest oocytes and spermaries from each sample was measured. Proportions of different oocyte and spermary developmental stages in each female and male colony respectively were calculated (Lau and Ang 2011). Classification of gametogenic development into four stages (Stages I to IV) followed that of Parker *et al.* (1997).

3.2.6 Seawater temperature and statistical analysis

Seawater temperature was measured at a depth of about 5 to 6m below Chart Datum in A Ma Wan, about 800 m away from Lan Guo Shui collection site, using a Minilog data loggers (VEMCO, AMIRIX Systems Inc., Canada).

All monthly data on E2 concentrations, gamete sizes and proportion (%) of different developmental stages were expressed as means ± SD. The difference in the profile of steroid levels between female and male colonies was analyzed by Mann-Whitney U test and the difference in the E2 levels over time was analyzed by parametric One-way ANOVA with Tukey's post hoc test to detect difference between months. Kruskal Wallis One way ANOVA was used to compare monthly differences in the sizes of oocyte and spermaries. Spearman Rank Correlation was used to evaluate the correlative relationship between changes in the seawater temperature, E2 levels, and sizes of oocytes and spermaries over time.

3.3 Results

3.3.1 Seasonal profile of E2

There was sex-specific difference in E2 concentrations in which those in female colonies were significantly higher (Mann-Whitney U Test, $P < 0.001$) than those in males throughout the sampling period when E2 was detected (Fig 3). Mean (± SD) E2

concentrations in female coral colonies ranged from 4.34 ± 1.50 to 65.13 ± 9.57 ng/g tissues. In 2009, the E2 level was first detected in March, and the highest level (65.13 ± 9.57 ng/g tissues) recorded in April. This dropped back to the basal level in May (4.91 ± 1.09 ng/g tissues), followed by a gradual rise until another peak was reached in July (17.92 ± 4.02 ng/g tissues). The level then stayed more or less the same till October (ANOVA with Tukey's HSD test, $P > 0.05$) until it dropped again in November (9.39 ± 2.84 ng/g tissues) and became undetectable in Dec 09. On the other hand, mean (± SD) E2 concentrations in male coral colonies ranged from 2.04 ± 0.62 to 7.41 ± 1.31 ng/g tissues. It was first detected in Mar 09, with a small but significant peak recorded in May (7.41 ± 1.31 ng/g tissues) (ANOVA with Tukey's post hoc test: $P < 0.001$). This was then followed by a drop in June (3.93 ± 0.87 ng/g tissues) with the level gradually decreasing until October (2.35 ± 0.59 ng/g tissues) (Fig. 3.2). No E2 was detected in both female and male coral colonies after the likely mass spawning events in 2008 and 2009 (Fig. 3.2). No E2 was also detected in any of the unsexable (sterile) colonies throughout the sampling period.

3.3.2 Gametogenesis

Antipathes curvata is a gonochoric broadcasting black coral in which there is no major morphological difference between male and female colonies. Gametogenesis in both colonies generally began in June (Fig. 3.3) (Lau and Ang, 2011). Oogenic cycles appear asynchronous, especially at the beginning in late spring and summer with different

developmental stages of oocytes found at the same time in the same colony and between colonies (Fig. 3.3A). This being the case, the average size of the oocytes between June 09 to August 09 was not significantly different (One way ANOVA, n = 57, $p = 0.063$). The oogenic cycle became more synchronized between different colonies towards autumn when more and more of the oocytes became mature (70.2% in Stage IV). In contrast, spermatogenesis appeared more synchronized between male colonies throughout the whole reproductive cycle (Fig. 3.3B). In June and July, a large proportion of spermaries was immature (10.4% and 53.8% were in stages I and II respectively), whereas a majority of spermaries contained mature spermatozoans in August (27.5% in Stage I) to October (90.5% in Stage IV). Some spawning likely occurred throughout the reproductive season with a major one occurring in December 2008 and November 2009 (Lau and Ang, 2011).

3.3.3 Correlation with seawater temperature

A significantly positive correlation was found between seawater temperature and E2 concentration in both female and male coral colonies over time (Fig. 3.2), with the correlation being stronger in female (n = 17, Spearman rank correlation coefficient r = 0.813, $p < 0.001$) than in male coral colonies (n = 17, r = 0.495, $p = 0.043$). Positive correlation between oocyte size and E2 concentrations in female colonies was also significant (n = 17, Spearman rank correlation coefficient r = 0.658, $p = 0.004$) but not between spermary size and E2 levels in male colonies (n = 17, r = 0.233, $p = 0.368$).

When only months with oocytes and spermaries were analyzed, a stronger positive correlation was found between seawater temperature and spermary size (n = 7; Spearman rank correlation coefficient r = 0.643; p = 0.119) than between seawater temperature and oocyte size (n = 7; r = 0.464; p = 0.294). However, these correlations were not significant. Presence of more mature, larger oocytes even at the early period of oogenesis contributed to similar mean sizes of oocytes from different monthly samples, thus masking the possible relationship between temperature change and change in oocyte mean sizes (Lau and Ang, 2011). Nonetheless, start of gametogenesis appeared to occur with a 5°C increase in mean seawater temperature from April to June 2009 and mass spawning likely occurred with a rapid drop of 4°C in mean seawater temperature between November to December 2008 and October to November 2009.

3.4 Discussion

It took about six months for both oocytes and spermaries of *A. curvata* colonies to attain maturity. This is comparable to that for *A. fiordensis* reported in New Zealand (Parker *et al.*, 1997). The development of oocytes in *A. curvata* was, however, less synchronized especially at the beginning of the oogenic cycle in June and July. Several cohorts of oocytes in different developmental stages can be found at the same time. This is in contrast to that in *A. fiordensis* in which only a single cohort of oocytes was observed. Indeed, seasonal maturation of gametes has been reported in scleractinian corals (e.g. *Pocillopora verrucosa* (Fadlallah, 1985), *Acropora clathrata* (Fadlallah, 1996), *Pavona*

varians (Glynn *et al.*, 2000), *Diploastrea heliopora* (Guest *et al.*, 2004), *Fungia repanda* (Loya *et al.*, 2009), *Acropora* spp. (Hanafy *et al.*, 2010)), soft corals (e.g. *Parerythropodium fulvum* (Benayahu and Loya, 1983), *Clavularia hamru* (Benayahu, 1989), *Dendronephthya gigantea* (Hwang *et al.*, 2007), *Sarcophyton elegans* (Hellstrom *et al.*, 2010)), and gorgonians (e.g. *Plexaura flexuosa* (Pakes *et al.*, 2008), *Pseudoplexaura porosa* (De Putron *et al.*, 2009)) as well.

Even though the mechanisms of how sex steroids regulate reproduction in invertebrates are still poorly understood, strong correlation between the levels of endogenous estrogens, progesterone, testosterone and gametogenesis as well as with spawning events have been reported in the starfish *Asterias vulgaris* (Hines *et al.*, 1992) and *Sclerasterias mollis* (Xu and Barker, 1990), sea urchins *Lytechinus variegates* (Wasson *et al.*, 2000), sea pansy *R. koellikeri* (Pernet *et al.*, 2002), hard corals *M. verrucosa* (Tarrant *et al.*, 1999), *E. ancora* (Twan *et al.*, 2003) and soft corals *S. polydactyla* (Slattery *et al.*, 1999). In our study, annual variation of E2 levels detected from both female and male *A. curvata* colonies suggested that there was some hormonal control over gametogenesis. E2 was first detected in female *A. curvata* colonies in March 09, and reached a high peak in April 09. Similarly, E2 was first detected in male colonies in March 09, and reached a peak in May 09, albeit at a much smaller level than that in female colonies. As gametes were not observed until June 09, such peaks before June suggested that E2 may be involved in triggering the onset of gametogenesis. Some oocytes observed in June were already of stages II or III, suggesting that the onset of oogenesis could have started earlier in May or April and may simply have been missed in April and May samples (Lau and Ang, 2011). After dropping back to basal level in

May 09, E2 concentration in female colonies gradually increased again in previtellogenic period from June 09 to August 09. This is a good indication demonstrating that this sex hormone can sustain oocyte development. Indeed, very similar pattern of E2 fluctuation was observed in the bivalves *Mya arenaria* (Gauthoer-Clerc *et al.*, 2006), with the highest E2 concentration being measured during pre-vitellogenic period and a second peak at spawning. Such variation in E2 levels may reflect their roles as endogenous modulator of reproduction.

Moreover, E2 concentrations among female coral colonies were significantly higher than those in males throughout the sampling period (October 2008 to November 2009) when E2 was detected. A significant positive correlation was recorded between E2 concentration and mean oocyte diameter, as was reported in scleractinian corals *M. verrucosa* (Tarrant *et al.*, 1999) and in sea pansy *R. koellikeri* (Pernet *et al.*, 2002). Similar sex-specific differences in the pattern of E1 and progesterone concentrations were recorded in the starfish *A. rubens* (Voogt and Dieleman, 1984), as well as for progesterone and testosterone in the soft coral *S. polydactyla* (Slattery *et al.*, 1999). On the other hand, no significant correlation was observed between spermatogenesis and E2 levels in male colonies. Gametogenesis in male colonies might be regulated by other sex steroids, such as testosterone, as in the starfish *A. vulgaris* (Hines *et al.*, 1992) and soft coral *S. polydactyla* (Slattery *et al.*, 1999). Finally, unlike scleractinian corals *M. verrucosa* (Tarrant *et al.*, 1999) and *E. ancora* (Twan *et al.*, 2003), no obvious peaks in E2 concentration were observed before mass spawning in female and male colonies of *A. curvata* and some levels of E2 continued to be detected in female colonies in December 08 and November 09 when spawning could have already occurred (Lau and

Ang, 2011). The absence of a significant E2 peak prior to spawning in *A. curvata* and the presence of more or less equal levels of E2 in females from July to October might be a result of asynchronous development of the oocytes. The appearance of mature stage IV oocytes from June onwards suggested the presence of different cohorts of oocytes within and between colonies. This is further confirmed from detailed analysis of oogenic development at the individual colony level (Lau and Ang, 2011). Spawnings from individual colonies could be taking place all throughout the summer months and some local peaks of E2 level from individual colony could have occurred to trigger such localized spawning event. But these local peaks cannot be detected in the present sampling scheme when monthly tissue samples from different colonies were combined for E2 extraction to minimize the amount of tissue needed to be removed from each colony each time. Nonetheless, the consistently high level of E2 throughout the summer months when oogenesis was active and its absence during the winter months and also in unsexable (sterile) colonies strongly suggests its active role in oogenesis.

Environmental factors, such as lunar periodicity, seawater temperature, irradiance, tidal surge and physical disturbances can affect physiological responses in marine invertebrates which in turn mediate the timing of their gametogenesis and mass spawning via the intrinsic hormonal signaling pathway (Vize, 2009). Even though our study did not attempt to prove the relationship between seawater temperature and the timing of various critical reproductive events, it was observed that the warming of seawater temperature might be one of the primary influences over the rising of endogenous E2 in female coral colonies and the onset of gametogenesis in *A. curvata*. This is supported by the past study on starfish that seasonal fluctuations in seawater

temperature regulate their gametogenesis by influencing the activity and kinetics of metabolic enzymes necessary to initiate germ call proliferation (Watts *et al.*, 1990).

Our work is the first record of the presence of vertebrate-type sex steroid E2 in a black coral. The similarities of sex steroids in vertebrates and corals indicate an evolutionary conservation of the reproductive system and the signaling pathway involved. This provides additional evidence to show that sex steroids are ancient and are conserved among the eukaryotic phyla. However, the physiological roles of sex steroids in black corals or in other cnidarian, as well as their interaction with extrinsic and intrinsic factors to affect gametogenesis, remain a challenging area for more extensive investigation in the near future.

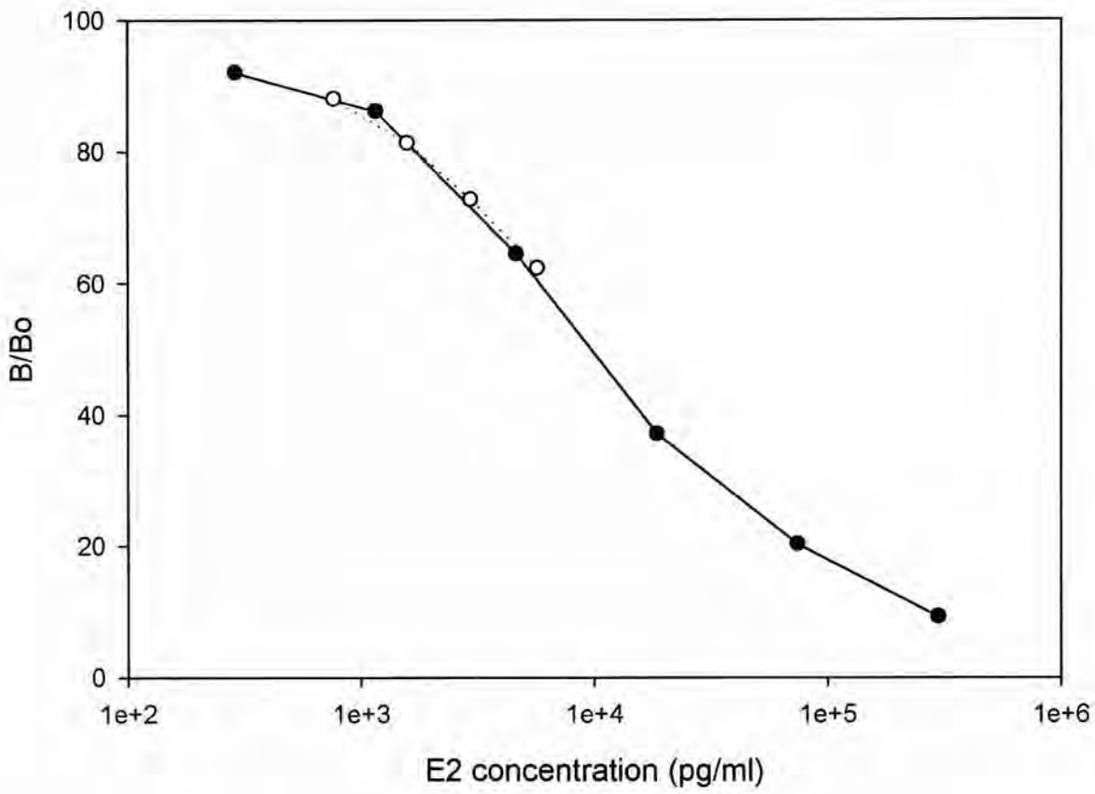

Fig. 3.1 The parallel relationship in EIA between the 17β-estradiol standard curve and different concentrations of the coral fractions from the SPE column. Serial dilutions of black coral extracts (1:2, v/v) are indicated by open circles.

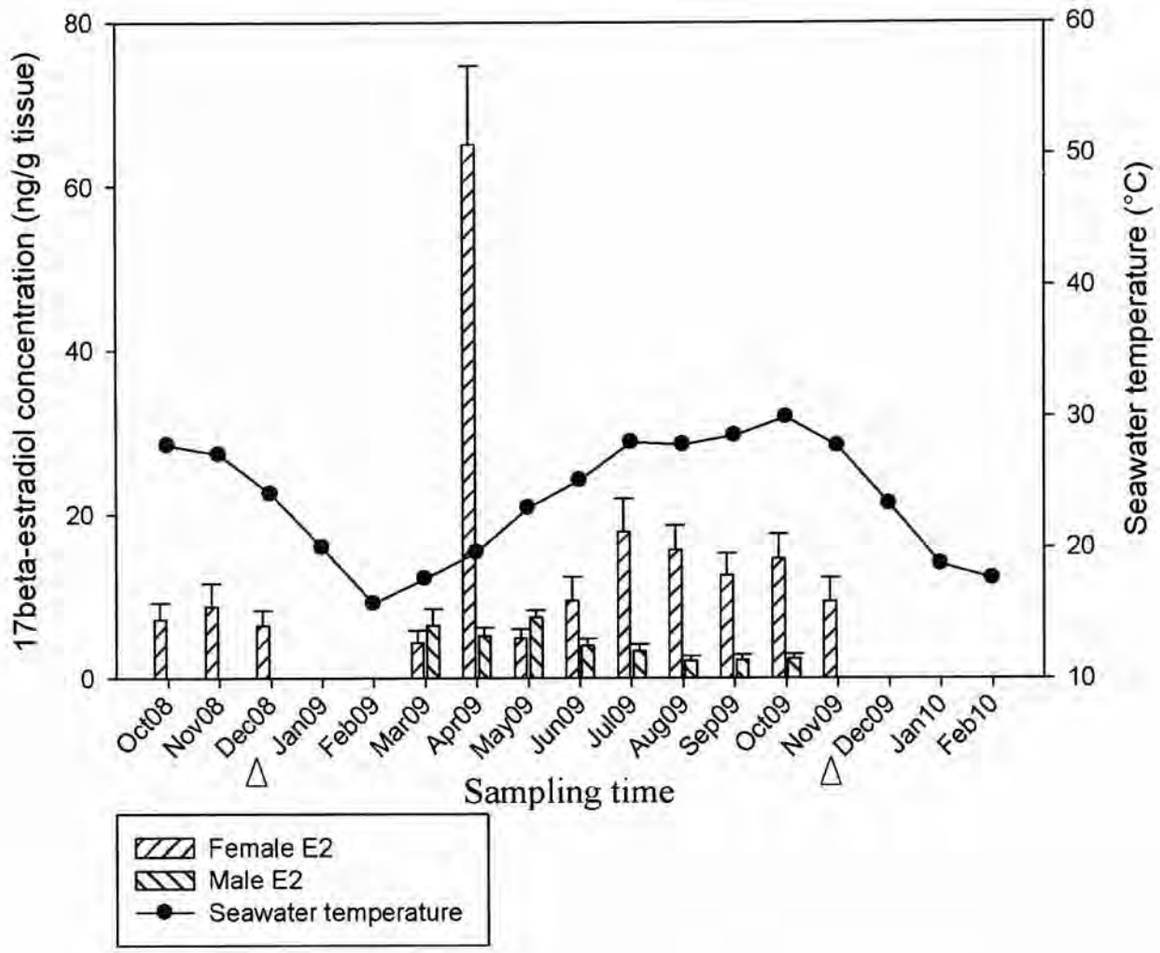

Fig. 3.2 Mean (± SD) monthly variations of E2 levels in female and male colonies of the black coral *A. curvata*. Mass spawning months in December 2008 and November 2009 were indicated by red triangles. E2 concentrations among female and male colonies showed significant difference. Female colonies: ANOVA with Tukey's HSD test: MS=0.152 , F =76.856, $P < 0.001$; Male colonies: Kruskal-Wallis Test: $P < 0.001$. Between March to October 09, E2 levels among male colonies showed significant difference (ANOVA with Tukey's post hoc test: MS = 12.535, F = 11.640, $P < 0.001$). For other months with empty bars, samples were collected but E2 concentrations were not detected. The mean monthly variation of seawater temperature of the study site is indicated by the solid line.

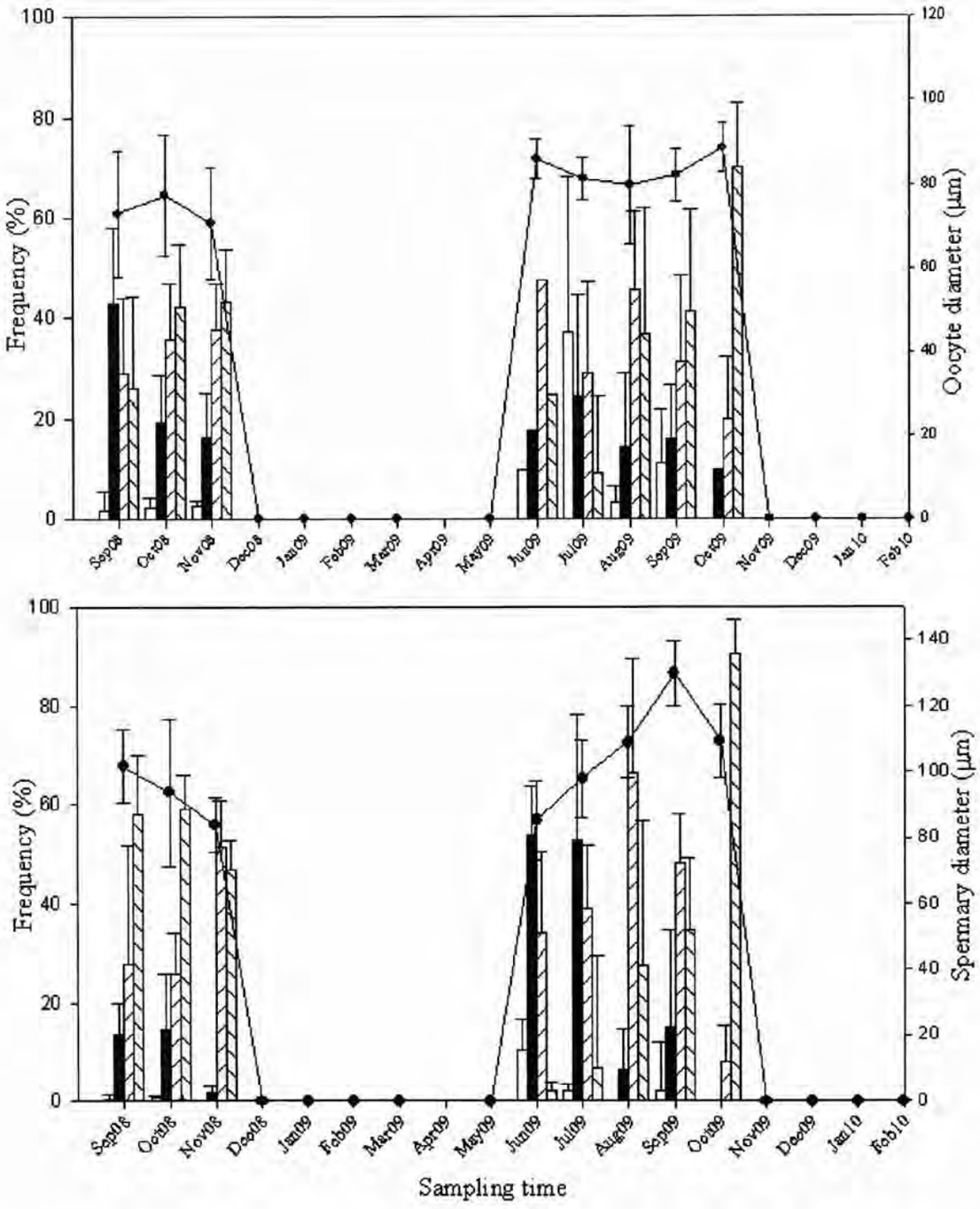

Fig. 3.3 Frequency (%) of different stages of **A.** Oocytes and **B.** Spermaries in *A. curvata* with variation of their mean geometric diameter (± SD) (line) from September 2008 to February 2010. No oocytes and spermaries were found from December 2008 to May 2009 (samples in April 2009 were missed), and from November 2009 to February 2010.

Chapter 4 Summary and Perspectives

The present study is a pioneering work on sexual reproduction of the black coral, *Antipathes curvata*, correlating its gametogenic cycle with the levels of sex steroid, 17β-estradiol, found in its colonies. Black coral communities in Hong Kong are mainly distributed from northeastern waters like Tung Ping Chau, to southern waters like Po Toi. They could be found at relatively shallow depth (≈ 6m C.D.), in contrast to many other black coral communities outside Hong Kong which are found at greater depths. This allows the opportunity to monitor the basic reproductive pattern of this black coral species, thus filling the knowledge gap about black coral reproductive biology. Knowing the reproductive mode, sex ratio and seasonality of spawning of *A. curvata* is crucial to the establishment of better policies for its management and conservation.

Sex steroids are well-studied among the vertebrates. They are essential for the regulation of sexual reproduction. Biologically active vertebrate-type sex steroids, such as estrogens, testosterone and progesterone, have also been found in many invertebrates, including cnidarians. It is believed that environmental cues, such as seawater temperature, tidal level and food availability are detected by the nerve net of corals which in turn triggers the secretion of sex steroids from their gonad tissues, resulting in the development of gonads (Tarrant *et al.*, 1999; Twan *et al.*, 2003). Such reproductive pattern and the presence of corresponding sex steroids in black corals have never been studied before. This thesis research therefore monitored the reproductive cycle of *A. curvata* colonies found in Lan Guo Shui (LGS), Tung Ping Chau Marine Park. At the

same time, the levels of female sex hormone 17β-estradiol (E2) in male, female and unsexable colonies were quantified over time and correlated with the critical events in reproduction, such as the onset of gametogenesis and spawning. This helps to investigate the presence of endogenous sex hormones in black corals and their roles in black coral reproduction.

The reproductive biology of *A. curvata* was examined monthly from September 2008 to March 2010 in LGS. Oocytes and spermaries were not found to coexist in the same polyp or colony nor were brooding larvae observed within the polyps. Therefore, *A. curvata* is believed to be a gonochoric broadcaster. Sex ratio among 38 tagged colonies was approximately 1:1. Minimum mature size among the tagged colonies was 32cm tall while the largest immature size was 50cm. The average density of oocytes was 256 ± 68 oocytes/mm^2 of polyp transverse area while that of spermaries was 165 ± 46 spermaries/mm^2 of polyp transverse area. The reproductive cycle of this black coral species covered a period of about six months with oocytes and spermaries appearing in histological sections in June 2009 and disappearing in November 2009.

At the beginning of gametogensis, the smallest oocyte and spermary were 25.2 μm and 28.4 μm in geometric diameter respectively. Both oocytes and spermaries became mature after 6-7 months with mean (± SD) size of oocytes and spermaries in October 2009 reaching 88.7 ± 5.9 μm (n=20) and 109.3 ± 11.0 μm (n=20) respectively. Oogenesis was asynchronous especially at the earlier part of the reproductive cycle with both mature and immature oocytes found within or among different female colonies. However, oogenic development appeared to become more synchronized at the later dates with at least half of the oocytes becoming mature before they disappeared in

November 2009 (or December 2008 in the previous season). The development of spermaries was more synchronous since most spermaries found at the onset of spermatogenesis were of the immature stages, i.e. stages I and II. The spermaries continued to develop until they also disappeared in November 2009 (or December 2008 in the previous season). Neither immature nor mature gametes could be found in the mesenteries of the coral polyps after November 2009 (December 2008), suggesting that *A. curvata* carried out an annual spawning event around this period in early winter.

Past studies have shown that sex steroid also regulates gametogenesis or acts as a pheromone in mass spawning in simple organisms like cnidarians. In the present study, the monthly level of sex steroid 17β-estradiol (E2) in *A. curvata* was investigated from October 2008 to February 2010. The mean (± SD) monthly levels in female colonies from October to December 2008 were 7.22 ± 2.01, 8.74 ± 2.84, 6.38 ± 1.92 ng/g tissues respectively; and from March to November 2009 were 4.34 ± 1.5, 65.13 ± 9.57, 4.91 ± 1.09, 9.43 ± 2.93, 17.92 ± 4.02, 15.63 ± 2.98, 12.56 ± 2.71, 14.52 ± 3.08 and 9.39 ± 2.84 ng/g tissues respectively. The levels were undetectable from January to February 2009 and from December 2009 to February 2010. On the other hand, mean (± SD) monthly E2 levels in male colonies from October 2008 to February 2010 were undetectable. From March to October 2009, the monthly levels were 6.45 ± 2.02, 5.18 ± 0.97, 7.41 ± 0.9, 3.93 ± 0.87, 3.28 ± 0.84, 2.04 ± 0.62, 2.18 ± 0.7 and 2.35 ± 0.59 ng/g tissues respectively. It then became undetectable again from November 2009 to February 2010.

There was sex-specific difference in E2 concentration in which the highest level of E2 (65.13 ± 9.57 ng/ g tissue) in females (April 2009) was 8 folds higher than the E2

peak (7.41 ± 0.9 ng/g tissue) in males (May 2009), suggesting that E2 has conserved roles in being a female sex hormone. Those peaks occurred one to two months before oocytes and spermaries were observed in June 2009, implying that E2 may be involved in triggering the onset of gametogenesis.

The fact that E2 concentrations in females started to increase from basal level during previtellogenic period (June to August 2009) suggests the role of E2 in sustaining oocyte development. On the other hand, E2 concentrations did not show another peak prior to the major spawning event in both females and males (December 2008 and November 2009). However, E2 levels in females remained constantly about 3 to 4 folds higher than the basal level during the whole oogenic period from June to November 2009 whereas such fluctuation of E2 was not observed among male colonies. This is due likely to oogenesis being asynchronous, with mature oocytes often seen in some female colonies in June to August, so E2 was continuously being produced in female colonies to trigger minor spawnings in the summertime before mass spawning took place. Therefore, no sharp peak of E2 level was observed among female colonies. From June to November 2009, E2 levels in male colonies remained at basal level, suggesting that E2 might not be the sex hormone regulating maturation and mass spawning of spermaries. These reproductive events are likely regulated by other sex hormones in male.

A significantly positive correlation was found between seawater temperature and E2 concentration in both female and male *A. curvata* coral colonies over time, with correlation being stronger in females. Meanwhile, significantly positive correlation was observed between oocyte size and E2 concentrations in female colonies but not in male

colonies over time. Like other corals and marine invertebrates, environmental changes are the cues triggering different critical reproductive events in black corals. Changes in seawater temperature are likely perceived by nerve nets in these corals. This is believed to be translated into action by the organisms' endogenous control mechanisms which initiate the endogenous changes (i.e. rise or drop of E2 levels) that finally trigger certain critical reproductive events (i.e. the onset of gametogenesis, continuous minor or major spawning) (Watson *et al.*, 2000) in *A. curvata*. In such "transduction", the organism is seen as a "black box", receiving external inputs and producing a measurable response (reproductive events). Much still remains to be known about this black box and the detailed mechanisms involved in triggering the chain of reproductive events in black corals. With annual profile of the endogenous sex hormone (E2) level examined in *A. curvata* in the present study, further ecophysiological studies are needed to understand how sex hormone E2 functions in the absence of an endocrine system in corals and how bioregulatory functions of E2 are conserved across vertebrates and invertebrates.

Being one of the precious corals, the conventional black coral fishery strategy operates by means of "boom-and-bust" principle. It is a destructive method which quickly depletes a newly discovered stock and moves on to the next one. The conflict between sustainable management and short-term economical benefits led to the overexploitation of many black coral stocks. However, since black corals are deep water corals which are difficult to access, there has been a scarcity in their reproductive studies, making them one of the most under-studied corals. This present pioneering study provides baseline information on the reproductive cycle of the most common black coral species, *A. curvata*, in Hong Kong. These data could help to fill the existing

knowledge gap of deep water black corals. For example, a minimum height and a limit on stem diameter of black coral colonies could be established in selective harvesting to ensure that no juvenile black corals are collected.

On a more practical side, many studies have shown the increasing threat from exogenous estrogenic compounds from human wastes on the marine environment. Many of these compounds would accumulate in marine organisms, including corals, and disrupt their reproduction patterns. Thus, manipulative experiments are needed to investigate the effects of exogenous estrogens on coral, including black coral, reproductions. Information from this study will be useful as a baseline in comparing with studies looking into these effects. It is in such studies where the present findings may have the greatest value in contributing towards a better understanding of the biology and thus, the conservation of these corals.

References

Anctil M (2000) Evidence for gonadotropin-releasing hormone-like peptides in a cnidarian nervous system. General and Comparative Endocrinology 119:317-328

Ang PO, Choi MM, Tam TW, Tam MC, Choi LS (2006). Biological monitoring of coral communities in Tung Ping Chau Marine Park, Hong Kong SAR, China: changes before and after marine park designation. In: Proceedings of 10th International Coral Reef Symposium, pp. 1110-1117

Ang PO, Lee MW, Fung HL, Yeung CW (2010) Research Collection and Study on Octocorals and Black Corals in Hong Kong Waters. Final Report, March 2010. Submitted to Marine Parks Division, Agriculture, Fisheries and Conservation Department, Hong Kong SAR Government

Atkinson S, Atkinson MJ (1992) Detection of estradiol-17β during a mass coral spawn. Coral Reefs 11:33-35

Ayre DJ (1995) Localised adaptation of sea anemone clones: evidence from transplantation over two spatial scales. Journal of Animal Ecology 64:186-196

Babcock RC, Bull GD, Harrison PL, Heyward AJ, Oliver JK, Wallace CC, Willis BL (1986) Synchronous spawnings of 105 scleractinian coral species on the Great Barrier Reef. Marine Biology 90: 379-394

Baird AH, Birrel CL, Hughes TP, McDonald A, Nojima S, Page CA, Prachett MS, Yamasaki H (2009) Latitudinal variation in reproductive synchrony in *Acropora* assemblages: Japan vs Australia. Galaxea 11:101-108

Benayahu Y (1989) Reproductive cycle and developmental processes during embryogenesis of *Clavularia hamra* (Cnidaria, Octocorallia). Acta Zoologica 70:29-36

Benayahu Y, Loya Y (1983) Surface brooding in the red sea soft coral *Parerythropodium fulvum*. Biological Bulletin 165:353-369

Bengtsson BO, Ceplitis A (2000) The balance between sexual and asexual reproduction in plants living environments. Journal of Evolutionary Biology 13:415-422

Bentley MG, Pacey AA (1992) Physiological and environmental control of reproduction in polychaetes. Oceanography and Marine Biology: Annual Review 30:443-481

Bisby FA, Roskov YR, Orrell TM, Nicolson D, Paglinawan LE, Bailly N, Kirk PM, Bourgoin T, van Hertum J (eds) (2008) Species 2000 & ITIS Catalogue of Life: 2008 Annual Checklist. Digital resource at www.catalogueoflife.org/annual-checklist/2008/. Species 2000: Reading, U.K

Bo M, Bavestrello G, Canese S, Giusti M, Salvati E, Angiolillo M, Greco S (2009) Characteristics of a black coral meadow in the twilight zone of the central Mediterranean Sea. Marine Ecology Progress Series 397:53-61

Bo M, Di Camillo CG, Addamo AM, Valisano L, Bavestrello G (2009) Growth strategies of whip black corals (Cnidaria: Antipatharia) in the Bunaken Marine Park (Celebes Sea, Indonesia). Marine Biodiversity Records 2: e54

Bruckner A, De Angelis P, Montgomery T (2008) Case study for black coral for Hawaii. In International Expert Workshop on CITES Non-detriment Findings, Cancun, November 17th-22nd 2008

Brugler MR, France SC (2007) The complete mitochondrial genome of the black coral *Chrysopathes formosa* (Cnidaria: Anthozoa: Antipatharia) supports classificaiont of antipatharians within the subclass Hexacorallia. Molecular Phylogenetics and Evolution 42:776-788

Chadwick-Furman NE, Spiegel M, Nir L (2000) Sexual reproduction in the tropical corallimorpharian *Rhodactis rhodostoma*. Invertebrate Biology 119:361-369

Davidoff MV (1908) Russische Zoologische Station in Villefranche (Riviera). Internationale Revue der gesamten Hydrobiologie 1: 295–297

De Putron SJ, Ryland JS (2009) Effects of seawater temperature on reproductive seasonality and fecundity of *Pseudoplexaura porosa* (Cnidaria: Octocorallia): latitudinal variation in Caribbean gorgonian reproduction. Invertebrate Biology 128:213-222

Dubois EA, Zandbergen MA, Peute J, Good HJT (2002) Evolutionary development of three gonadotropin-releasing hormone (GnRH) systems in vertebrates. Brain Research Bulletin 57:413-418

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889-895

Fadlallah YH (1983) Sexual reproduction, development and larval biology in scleractinian corals. Coral Reefs 2:129-150

Fadlallah YH (1985) Reproduction in the coral *Pocillopora verrucosa* on the reefs adjacent to the industrial city of Yanbu (Red Sea, Saudi Arabia). In: Proceedings of 5th international coral reef congress, volume 4, Tahiti, 1985, pp. 313-318

Fadlallah YH (1996) Synchronous spawning of *Acropora clathrata* coral colonies from the western Arabian Gulf (Saudi Arabia). Bulletin of Marine Science 59:209-216

Fan TY, Dai CF (1995) Reproductive ecology of the scleractinian coral *Echinopora lamellose* in northern and southern Taiwan. Marine Biology 123:565-572

Gaino E, Boyer M, Scoccia F (2008) Sperm morphology in the black coral *Cirrhipathes* sp. (Anthozoa, Antipatharia). Invertebrate Biology 127:249-258

Gaino E, Scoccia F (2008) Female gametes of the black coral *Cirrhipathes* cfr. *anguina* (Anthozia, Antipatharia) from the Indonesia Marine Park of Bunaken. Invertebrate Reproduction and Development 51:119-126

Gaino E, Scoccia F (2009) Release of sperm clusters in sphere by the black coral *Cupressopathes pumila* (Anthozoa, Antipatharia). Coral Reefs 28:851-857

Gauthier-Clerc S, Pellerin J, Amiard JC (2006) Estradiol-17β and testosterone concentrations in male and female *Mya arenaria* (Mollusca bivalvia) during the reproductive cycle. General and Comparative Endocrinology 145:133-139

Giese AC, Pearse JS (1974) Introduction: general principles. In: Giese AC, Pearse JS (eds) Reproduction of marine invertebrates I. Academic Press, New York, pp. 1-49

Glynn PW, Colley SB, Ting JH, Mate JL, Guzman HM (2000) Reef coral reproduction in the eastern Pacific: Costa Rica, Panama and Galapagos Islands (Ecuador). IV. Agariciidae, recruitment and recovery of *Pavona varians* and *Pavona* sp. Marine Biology 136:785-805

Gorbman A, Sower SA (2003) Evolution of the role of GnRH in animal (Metazoan) biology. General and Comparative Endocrinology 134:207-213

Grange KR (1985) Distribution, standing crop, population structure and growth rates of an unexploited resource of black coral in the southern fjords of New Zealand. In: Proceedings of 5th International Coral Reef Congress, volume 6, pp. 217-221

Grange KR (1986) The underwater world of Fiordland. Forest Bird 17:10-13

Grange KR (1988) Redescription of *Antipathes aperta*, Totton (Coelenterata: Antipatharia) an ecological dominant in the southern fiords of New Zealand. New Zealand Journal of Zoology 15:55-61

Grange KR (1990) *Antipathes fiordensis*, a new species of black coral (Coelenterata: Antipatharia) from New Zealand. New Zealand Journal of Zoology 17:279-282

Grange KR (1997) Demography of black coral populations in Doubtful Sound, New Zealand: results from a 7-year experiment. In: Proceedings of the 6th International Conference on Coelenterate Biology. J.C. den Hartog (ed.). Leiden, The Netherlands: National Museum of Natural History. pp. 185–193

Grange KR, Singleton RJ (1988) Population structure of black coral, *Antipathes aperta*, in the southern fiords of New Zealand. New Zealand Journal of Zoology 15:481-489

Grigg RW (1965) Ecological studies of black coral in Hawaii. Pacific Science 19:244-260

Grigg RW (1993) Precious coral fisheries of Hawaii and the U.S. Pacific Islands. Marine Fisheries Review 55:50-60

Grigg RW (2001) Black corals: History of a sustainable fishery in Hawaii. Pacific Science 55:291-299

Grigg RW, Opresko D (1977) Order Antipatharia black corals. In Reef and shore fauna of Hawaii. Bishop Museum Special Publications 64:242-261

Guest JR (2004) Reproduction of *Diploastrea helipora* (Scleractinia: Faviidae) in Singapore. In: Proceedings of 10th International Coral Reef Symposium. Abstracts, Okinawa, 2004, p. 367

Guest JR, Baird AH, Goh BPL, Chou LM (2005) Seasonal reproduction in equatorial coral reefs. Invertebrate Reproduction and Development 48:207-218

Guest JR, Chou LM, Baird AH, Goh BPL (2002) Multispecific, synchronous coral spawning in Singapore. Coral Reefs 21:422-423

Hanafy MH, Aamer MA, Habib M, Rouphael AB, Baird AH (2010) Synchronous reproduction of corals in the Red Sea. Coral Reefs 29:119-124

Harrison PL (1985) Sexual characteristics of scleractinian corals: systematic and evolutionary implications. In: Proceedings of 5th International Coral Reef Congress, volume 4, pp. 337-342

Harrison PL (2011) Sexual reproduction of scleractinian corals. Coral Reefs: An Ecosystem in Transition 3:59-85

Harrison PL, Babcock RC, Bull GD, Oliver JK, Wallace CC, Willis BL (1984) Mass spawning in tropical reef corals. Science 223:1186-1189

Harrison PL, Wallace CC (1990) Reproduction, dispersal and recruitment of scleractinian corals. In: Dubinsky Z (ed) Ecosystems of the World: Coral Reefs. Chapter 7. Elsevier, New York, pp. 133-202

Hellstrom M, Kavanagh KD, Benzie JAH (2010) Multiple spawning events and sexual reproduction in the octocoral *Sarcophyton elegans* (Cnidaria: Alcyonacea) on Lizard Island, Great Barrier Reef. Marine Biology 157:383-392

Hines GA, Watts SA, Sower SA, Walker CW (1992) Sex steroid levels in the testes, ovaries, and pyloric caeca during gametogenesis in the sea star *Asterias vulgaris*. General and Comparative Endocrinology 87:451-460

Hwang SJ, Song JI (2007) Reproductive biology and larval development of the temperate soft coral *Dendronephthya gigantean* (Alcyonacea: Nephtheidae). Marine Biology 152:273-284

Jeng SS, Wan WCM, Chang CF (1978) Existence of an estrogen-like compound in the ovary of the shrimp *Parapenaeus fissures*. General and Comparative Endocrinology 36:211-214

Jokiel PL, Hunter CL, Taguchi S, Watari L (1993) Ecological impact of a fresh water "reef-kill" in Kanehoe Bay, Oahu, Hawaii. Coral Reefs 12:177-184

Karlson P (1996) On the hormonal control of insect metamorphosis. A historical review. International Journal of Developmental Biology 40:93-96

Kenyon JC (1995) Latitudinal differences between Palau and Yap in coral reproductive synchrony. Pacific Science 49:156-164

Klowden MJ (2003) Contributions of insect research toward our understanding of neurosecretion. Archives of Insect Biochemistry and Physiology 53:101-114

Kojis BL (1986) Sexual reproduction in *Acropora* (Isopora) (Coelenterata: Scleractinia). II. Latitudinal variation in *A.palifera* from the Great Barrier Reef and Papua New Guinea. Marine Biology 91:311-318

Kojis BL, Quinn NJ (1982) Reproductive ecology of two faviid corals (Coelenterata: Scleractinia). Marine Ecology Progress Series 8:251-255

Korringa P (1947) Relations between the moon and periodicity in the breeding of reef animals. Ecological Monographs 17:347-381

Kramarsky-Winter E, Loya Y (1998) Reproductive strategies of two fungiid corals from the northern Red Sea: environmental constraints? Marine Ecology Progress Series 174:175-182

Lapian HFN, Barucca M, Bavestrello G, Biscotti MA, Bo M, Canapa A, Tazioli S, Olmo E (2007) A systematic study of some Black Corals species (Antipatharia, Hexacorallia) based on rDNA internal transcribed spacers sequences. Marine Biology 151:785-792

Lau PL, Ang PO (2011) Asynchronous gametogenesis of Antipatharian black coral, *Antipathes curvata*, from Lan Guo Shui, Hong Kong, based on analysis of individual colonies. Coral Reefs (submitted)

Levitan DR, Fukami H, Jara J, Kline D, McGovern TM, McGhee KE, Swanson CA, Knowlton N (2004) Mechanisms of reproductive isolation among sympatric broadcast-spawning corals of the *Montastraea annularis* species complex. Evolution 58:308-323

Lawrence AJ (1996) Environmental and endocrine control of reproduction in two species of polychaete: potential bio-indicators for global climate change. Journal of the Marine Biological Association of the United Kingdom 76:247-250

Lewis DH (1973) The relevance of symbiosis to taxonomy and ecology, with particular reference to mutualistic symbioses and the exploitation of marginal habitats. Pp.

151-172 in Heywood (ed.) Taxonomy and Ecology. Systematics Association, London, Special Volume 5. Academic Press, London

Lin TP (2003) Reproduction Patterns of Scleractinian Corals from Tung Ping Chau, Hong Kong and the Effect of Physical Factors on These Patterns. MPhil Thesis. The Chinese University of Hong Kong.

Liu SH, Ang PO (2002) Fecundity and gametogenesis of the dominant coral *Platygyra sinensis* in Tung Ping Chau, Hong Kong. Asian Conference on Marine Science and Technology, Kuala Lumpur, Malaysia, 2002

Liu Y, Guan YT, Tam FY, Mizuno T, Tsuno H, Zhu WP (2010) Influence of rainfall and basic water quality parameters on the distribution of endocrine-disrupting chemicals in coastal area. Water Air and Soil Pollution 209:333-343

Loya Y, Sakai K, Heyward A (2009) Reproductive patterns of fungiid corals in Okinawa, Japan. Galaxea 11:119-129

Lumsden SE, Hourigan TF, Bruckner AW, Dorr G (2007) The state of deep coral ecosystems of the United States. NOAA Technical Memorandum CRCP-3. Silver Spring, MD: NOAA, National Marine Fisheries Service

McLachlan JA (2001). Environmental signaling: What embryos and evolution teach us about endocrine disrupting chemicals. Endocrine Reviews 22:319-341

Mendes JM, Woodley JD (2002) Timing of reproduction in *Montastraea annularis*: relationship to environmental variables. Marine Ecology Progress Series 227:241-251

Molodtsova TN (2006) New species of *Hexapathes* Kinoshita,1910 (Anthozoa, Antipatharia, Cladopathidae) from the South-West Pacific. Zoosystema 28:597-606

Montgomery AD (2002) The feasibility of transplanting black coral (order Antipatharia). Hydrobiologia 471:157-164

Miller K (1996) Piecing together the reproductive habits of New Zealand's endemic black corals. Water and Atmosphere 4:18-19

Nothacker HP, Grimmelikhuijzen CJP (1993) Molecular cloning of a novel, putative G protein-coupled receptor from sea anemones structurally related to members of the FSH, TSH, LH/CG receptor family from mammals. Biochemical and Biophysical Reseach Communications 197:1062-1069

Olive PJW (1984) Environmental control of reproduction in Polychaete. Fortschritte der Zoologie 29:17-38

Olive PJW (1995) Annual breeding cycles in marine invertebrates and environmental temperature: probing the proximate and ultimate causes of reproductive synchrony. Journal of Thermal Biology 20:79-90

Oliver JK, Babcock RC (1992) Aspects of the fertilization ecology of broadcast spawning corals: sperm dilution effects and in situ measurements of fertilization. Biological Bulletin 183:409-417

Oliver JK, Babcock RC, Harrison PL, Willis BL (1988) Geographic extent of mass coral spawning: clues to ultimate causal factors. In: Proceedings of 6th International Coral Reef Symposium, volume 2, pp. 803-810

Olsen DA, Wood RS (1980) Investigations on black coral in Salt River Submarine Canyon St. Croix, U.S. Virgin Islands. St. Croix, U.S. Virgin Islands: Division of Fish and Wildlife

Opresko DM (1972) Redescriptions and reevalutions of the antipatharians described by L.D. de Pourtales. Bulletin of Marine Science 22:950-1017

Opresko DM (1976) Redescription of *Antipathes panamensis* Verrill (Coelenterata, Antipatharia). Pacific Science 30:235-240

Opresko DM (2001) Revision of the Antipatharia (Cnidaria: Anthozoa). Part I. Establishment of a new family, Myriopathidae. Zoologische Mededelingen 75:347-374

Opresko DM (2002) Revision of the Antipatharia (Cnidaria: Anthozoa). Part II. Schizopathidae. Zoologische Mededelingen 76:411-442

Opresko DM (2003) Revision of the Antipatharia (Cnidaria: Anthozoa). Part III. Cladopathidae. Zoologische Mededelingen 77:495-536

Opresko DM (2004) Revision of the Antipatharia (Cnidaria: Anthozoa). Part IV. Establishment of a new family, Aphanipathidae. Zoologische Mededelingen 78:209-240

Pakes MJ, Woollacott RM (2008) Reproduction of the gorgonian *Plexaura flexuosa* in Bermuda. Journal of Experimental Marine Biology and Ecology 357:121-127

Parker NR, Mladenov PV, Grange KR (1997) Reproductive biology of the antipatharian black coral *Antipathes fiordensis* in Doubtful Sound, Fiordland, New Zealand. Marine Biology 130:11-22

Pazos AJ, Mathieu M (1999) Effects of five natural gonadotropin-releasing hormones on cell suspensions of marine bivalve gonad: Stimulation of gonial DNA synthesis. General and Comparative Endocrinology 113:112-120

Penland L, Kloulechad J, Idip D, van Woesik R (2004) Coral spawning in the western Pacific Ocean is related to solar radiation: evidence of multiple spawning events in Palau. Coral Reefs 23:133-140

Pernet V, Anctil M (2002) Annual variations and sex-related differences of estradiol-17β in the anthozoan *Renilla koellikeri*. General and Comparative Endocrinology 129:63-68

Poh K (1971) Economics and market potential of the precious coral industry in Hawaii. University of Hawaii Sea Grant Publications 71:1-22

Powell JF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM (1996) Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proceedings of the National Academy of Sciences USA 93:10461-10464

Reis-Hendriques MA, Le Guellec D, Remy-Martin JP, Adessi GL (1990) Studies of endogenous sterods from the marine mollusk *Mytitus edulis* L. by gas chromatography and mass spectrometry. Comparative Biochemistry and Physiology, Part B 95:303-309

Richmond RH (1997) Reproduction and recruitment in corals: critical links in the persistence of reefs. In: Birkeland C (ed) Life and Death of Coral Reefs. Chapman & Hall, New York, pp. 175-197

Richmond RH, Hunter CL (1990) Reproduction and recruitment of corals: comparisons among the Caribbean, the Tropical Pacific, and the Red Sea. Marine Ecology Progress Series 60:185-203

Roark EB, Guilderson TP, Dunbar RB, Fallon SJ, Mucciarone DA (2009) Extreme longevity in proteinaceous deep-sea corals. In: Proceedings of the National Academy of Sciences USA, volume 106, pp. 5204-5208

Roark EB, Guilderson TP, Dunbar RB, Ingram BL (2006) Radiocarbon-based ages and growth rates of Hawaiian deep-sea corals. Marine Ecology Progress Series 327: 1-14

Sanchez A, Zea S, Diaz JM (1998) Patterns of octocoral and black coral distribution in the oceanic barrier reef-complex of Providencia Island, Southwestern Caribbean. Caribbean Journal of Science 34:250-264

Segal D (1993) Prospects of using *Drosophila* for insect neuroendocrine research. Archives of Insect Biochemistry and Physiology 22:199-231

Sherman CDH, Ayre DJ, Miller KJ (2006) Asexual reproduction does not produce clonal populations of the brooding coral *Pocillopora damicornis* on the Great Barrier Reef, Australia. Coral Reefs 25:7-18

Sherwood OA, Edinger EA (2009) Ages and growth rates of some deep-sea gorgonian and antipatharian corals of Newfoundland and Labrador. Canadian Journal of Fisheries and Aquatic Science 66:142-152

Shick JM (1991) A Functional Biology of Sea Anemones, Chapman and Hall, London

Simpson CJ (1985) Mass spawning of scleractinian corals in the Dampier archipelago and the implications for management of coral reefs in Western Australia. Bulletin of Department of Conservation and Environment Perth 244:1-35

Slattery M, Hines GA, Watts SA (1997) Steroid metabolism in Antarctic soft corals. Polar Biology 18:76-82

Slattery M, Hines GA, Starmer J, Paul VJ (1999) Chemical signals in gametogenesis, spawning and larval settlement and defense of the soft coral *Sinularia polydactyla*. Coral Reefs 18:75-84

Stoddart JA (1983) Asexual production of planulae in the coral *Pocillopora damicornis*. Marine Biology 76:279-284

Sundback K, Nilsson P, Nilsson C, Jonsson B (1996) Balance between autotrophic and heterotrophc componenets and processes in microbenthic communities of sandy sediments: A field study. Estuarine Coastal and Shelf Science 43:689-706

Swevers L, Iatrou K (2003) The ecdysone regulatory cascade and ovarian development in lepidopteran insects: Insights from the silkmoth paradigm. Insect Biochemistry and Molecular Biology 33:1285-1297

Tarrant AM, Atkinson S, Atkinson MJ (1999) Estrone and estradiol-17β concentration in tissue of the scleractinian coral, *Montipora verrucosa*. Comparative Biochemistry and Physiology, Part A 122: 85-92

Terakado K (2001) Induction of gamete release by gonadotropin-releasing hormone in a protochordate, *Ciona intestinalis*. General and Comparative Endocrinology 124:277-284

Tomascik T, Sander F (1987) Effects of eutrophication on reef-building corals III. Reproduction of the reef-building coral *Porites porites*. Marine Biology 94:77-94

Tsounis G, Rossi S, Grigg R, Santangelo G, Bramanti L, Gili JM (2010) The exploitation and conservation of precious corals. Oceanography and Marine Biology 48:161-212

Twan WH, Hwang JS, Chang CF (2003) Sex steroids in scleractinian coral, *Euphyllia ancora*: Implication in mass spawning. Biology of Reproduction 68:2255-2260

Twan WH, Wu HF, Hwang JS, Lee YH, Chang CF (2005) Corals have already evolved the vertebrate-type hormone system in the sexual reproduction. Fish Physiology and Biochemistry 31:111-115

Twan WH, Hwang JS, Lee YL, Jeng SR, Yueh WS, Tung YH, Wu HF, Dufour S, Chang CF (2006) The presence and ancestral role of GnRH in the reproduction of scleractinian coral, *Euphyllis ancora*. Endocrinology 147:397-406

Van Veghel MLJ (1994) Reproductive characteristics of the polymorphic Caribbean reef building coral *Montastrea annularis*. I. Gametogenesis and spawning behavior. Marine Ecology Progress Series 109:209-219

Van Veghel MLJ, Kahmann MEH (1994) Reproductive characteristics of the polymorphic Caribbean reef building coral *Montastrea annularis*. II. Fecundity and colony structure. Marine Ecology Progress Series 109:221-227

Veron JEN (1995) Corals in space and time. The Biogeography and Evolution of the Scleractinia. UNSW Press, Sydney

Vize PD (2009) Transcriptome analysis of the circadian regulatory network in the coral, *Acropora millepora.* Biological Bulletin 216:131-137

Vogt PA, Dielemen SJ (1984) Progesterone and oestrone levels in the gonads and pyloric caeca of the male sea star *Asterias rubens*: a comparison with the corresponding levels in the female sea star. Comparative Biochemistry and Physiology, Part A 79:635-639

Wallace CC (1985) Reproduction, recruitment and fragmentation in nine sympatric species of the coral genus *Acropora*. Marine Biology 88: 217-233

Warner GF (1981) Species descriptions and ecological observations of black corals (Antipatharia) from Trinidad. Bulletin of Marine Science 31:147–163

Wasson KM, Gower BA, Hines GA, Watts SA (2000) Levels of progesterone, testosterone and estradiol and androstenedione metabolism in the gonads of *Lytechinus variegates* (Echinogermata: Echinoidea). Comparative Biochemistry and Physiology, Part C: Pharmacology, Toxicology and Endocrinology 126:153-165

Watson GJ, Williams ME, Bentley MG (2000) Can synchronous spawning be predicted from environmental parameters? A case study of the lugworm *Arenicola marina*. Marine Biology 136:1003-1017

Watts SA, Hines G, Lee K, Jaffurs D, Roy J, Smith FF, Walker CW (1990) Seasonal patterns of orithine decarboxylase activity and levels of polyamines in relation to the cytology of germinal cells during spermatogenesis in the sea star, *Asterias vulgaris.* Tissue and Cell 22:435-447

Willis BL, Babcock RC, Harrison PL, Oliver JK, Wallace CC (1985) Patterns in the mass spawning of corals on the Great Barrier Reef from 1981 to 1984. In: Proceedings of 5th International Coral Reef Congress, Tahiti, volume 4, pp. 343-348

Willis BL, Babcock RC, Harrison RL, Wallace CC (1997) Experimental hybridization and breeding incompatibilities within the mating systems of mass spawning reef corals. Coral Reefs 16:53-65

Winsor L (1984) Manual of Basic Zoological Microtechniques for Light Microscopy. Townsville: James Cook University of North Queensland

Wolanski E, Sarsenski J (1997) Larvae dispersion in coral reefs and mangroves. American Scientist 85:236-241

Xu RA, Barker MF (1990) Annual changes in the steroid levels in the ovaries and the pyloric caeca of *Sclerasterias mollis* (Echinodermata: Asteroidea) during the reproductive cycle. Comparative Biochemistry and Physiology, Part A 95:127-133

Yeung CW, Ang PO (2010) Sexual Reproduction in the Soft Coral Lobophytum sp. in Tung Ping Chau Marine Park, Hong Kong SAR, China. In: Proceedings of the 11th International Coral Reef Symposium, Florida, USA 2008. Session 11:371-374

Zou JM, Zhou RL (1984) Antipatharians from Hong Kong waters with a description of a new species. Asian Marine Biology 1:101-105