The Hong Kong Polytechnic University

Department of Health Technology and Informatics

ASSESSMENT OF THE ANATOMY AND PHYSIOLOGY OF THE THYROID GLAND OF THE INDO-PACIFIC BOTTLENOSE DOLPHIN, *TURSIOPS ADUNCUS*, USING ULTRASONOGRAPHY

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A thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

April 2010

Certificate of Originality

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Signed _____

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(Candidate)

Abstract of thesis entitled
"Assessment of the Anatomy and Physiology of the Thyroid Gland of the Indo-Pacific Bottlenose Dolphin, *Tursiops aduncus*, using ultrasonography"
submitted by **Brian Chin Wing Kot**
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Abstract

This 3-year study was the first to systematically monitor 18 *Tursiops aduncus*, at Ocean Park, Hong Kong. At the end of the study, the mean age of the population was 16.0 years (range 3 - 37 years). There were 7 males and 11 females in the initial subject group. Of these, 2 animals died during the study period, 14 animals were sexually mature and 4 were immature. Ages were 3 – 37 years [mean 16 y]. Body weights were 106.1 – 185.1 kg [mean 132.3 kg]. Body lengths were 196 – 244 cm [mean 220.1 cm]. For all the subjects, sonographic examination of the thyroid gland was performed once a week. Blood samples were collected monthly whenever possible for thyroid hormone evaluation, using the corresponding enzyme immunoassay kits. A total of 1384 thyroid ultrasound examinations were performed and 241 blood samples for serum thyroid hormone levels were collected and analyzed.

The accuracy of four 2-D ultrasound methods in dolphin thyroid volume measurement was also investigated for the first time, with the standard of reference determined by 3-D ultrasound. The inter- and intra-operator variability of the four 2-D ultrasound and the 3-D ultrasound thyroid volume measurement methods were also evaluated. Results show both 2-D and 3-D ultrasound can be used to evaluate dolphin thyroid volume. Methods A and B are considered to be more accurate and reliable methods for 2-D ultrasound dolphin thyroid measurement, regardless of the dolphin thyroid configuration, due to the difficulty in judging the maximal dimension of the craniocaudal dimension of the thyroid lobe in Methods C and D.

Sonographic features of the normal dolphin thyroid gland and adjacent neck structures were also documented for the first time.

Possible association between thyroid morphology and serum thyroid hormone levels was evaluated for the first time in bottlenose dolphins. Various degrees of association were demonstrated between thyroid hormone levels and thyroid volume amongst sex, age group and sexual maturity. The most prominent association was identified when the analyses were stratified by age, possibly due to the energy requirements of somatic growth transiting to reproductive development with advancing age. Variations in dolphin thyroid morphology for somatic growth, reproductive development and different body sizes, as well as seasonality, were also evaluated for the first time. Results indicate that thyroid volume varies with age, sex, sexual maturity and body size, due to the differences in energy expenditure within these parameters. In terms of sonographic features, a significant difference was found in the echogenicity of the adult and juvenile thyroid gland.

This study was the first to evaluate the possible variations of thyroid morphology in female dolphins during different reproductive events and the estrous cycle. Reproductive event was considered to be a significant predictor for thyroid volume measurement, in which a significantly larger thyroid volume in lactating females was presented compared to estrous and anestrous females which may be due to the high energy requirements and milk production during lactation. Cyclic changes of thyroid volume during the estrous cycle were documented, with the minimum thyroid volume during the follicular phase and the maximum thyroid volume during the luteal phase, possibly related to the influence of female sex steroids.

The principal findings of this study have shown that sonography is a non-invasive, readily available, comparatively low cost and real-time imaging tool to assess the anatomy and function of the thyroid gland of bottlenose dolphins. In addition, for the first time, a baseline status of the thyroid gland in bottlenose dolphins was established, which provides a means of sonographic diagnosis in live animals with

thyroid abnormalities and guides corrective therapy. Techniques developed may be applied to other marine mammals and provide information about thyroid physiology of other species, allowing optimal captive management. Such techniques may also aid conservation of wild animals in the future.

Presentations and Publications Originating from the Present Study

Publications

1. **Kot BCW**, Ying MTC, Brook FM, Kinoshita RE. Evaluation of 2-D and 3-D ultrasound in the assessment of the thyroid volume of the Indo-Pacific Bottlenose dolphin, *Tursiops aduncus*. Journal of Zoo and Wildlife Medicine (In Press).

2. **Kot BCW**, Ying MTC, Brook FM, Kinoshita RE, Cheng SCH, Chan WK. Sonographic imaging of the thyroid gland and adjacent neck structures of the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. American Journal of Veterinary Research (In Press).

3. **Kot BCW**, Ying MTC, Brook FM, Kinoshita RE, Dave K, Chan WK. Sonographic evaluation of thyroid morphology during different reproductive events in female Indo-Pacific bottlenose dolphins, *Tursiops aduncus*. Marine Mammal Science (In Press).

4. **Kot BCW**, Ying MTC, Brook FM. A comparison of portable ultrasound unit and fully-equipped clinical ultrasound unit in the thyroid size measurement of the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*). PLoS ONE (In Press).

Conference Presentations

1. **Kot BCW**, Ying MTC, Brook FM, Kinoshita RE. Evaluation of 2-D and 3-D ultrasound in the assessment of the thyroid gland of the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. Proceedings of the International Association for Aquatic and Animal Medicine Conference 2007: 168-172 (abstract).

2. **Kot BCW**, Ying MTC, Brook FM, Fernando N, Kinoshita RE, Martelli P. Difference in age, sex, body weight, body length and sexual maturity in association with variations in thyroid size and morphology of Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. Proceedings of the International Association for Aquatic and Animal Medicine Conference 2008: 62-64 (abstract).

3. **Kot BCW**, Ying MTC, Brook FM, Fernando N, Kinoshita RE. Variation of thyroid volume during the normal estrous cycle in the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. Proceedings of the International Association for Aquatic and Animal Medicine Conference 2009: 151-152 (abstract).

4. **Kot BCW**, Ying MTC, Brook FM, Fernando N, Kinoshita RE. Sonographic assessment of the anatomy and physiology of thyroid gland in the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. Proceedings of the International Association for Aquatic and Animal Medicine Conference 2010 (abstract).

Publications in working progresss

1. **Kot BCW**, Ying MTC, Brook FM, Kinoshita RE. Sonographic evaluation of thyroid morphology during the normal estrous cycle in the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. Journal of Zoo and Wildlife Medicine (Submitted and currently under peer-review process).

2. **Kot BCW**, Ying MTC, Brook FM, Kinoshita RE. Sonographic evaluation of the thyroid gland in association with the serum thyroid hormone concentration of the captive Indo-Pacific bottlenose dolphin, *Tursiops aduncus*. PLoS ONE (At the final stage of preparation).

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Chapter One

Introduction

With increasing interest in the conservation of small cetaceans over the past two decades, substantial efforts have been made to develop new approaches to address threats that have contributed to the depletion of these animals. From the most immediate threats, such as by-catch in the fishery industry and habitat loss and degradation, these threats to the cetaceans have changed over time. The U.S. Marine Mammal Protection Act of 1972 directs that the primary objective of marine mammal management should be to maintain the health and stability of the marine ecosystem and, whenever consistent with this primary objective, to obtain and maintain "optimum sustainable populations" of marine mammals.

Removal of live cetaceans from the wild for captive display and research is no longer permitted in most places, making it mandatory that facilities maintain the present captive stocks. To achieve this objective, facilities must attain self-sustainability and manage the health of the available captive populations carefully in order to preserve genetic diversity and retain reproductive fitness (Montgomery et al., 1997). In the past decade, considerable knowledge about the reproductive physiology of small cetaceans has been acquired, through the use of sonography, endocrine studies and advanced reproductive technologies (Bryden and Harrison, 1986; Schroeder, 1989; Schroeder and Keller, 1990; Robeck et al., 1994; Schroeder, 1995; Brook, 1997, 2001; Boyd et al., 1999; Brook et al., 2000, 2004; Robeck et al., 2004, Brook and Kinoshita, 2005). These techniques have also provided important information about the general health status and endocrine system in captive cetaceans. Increased knowledge will contribute to more successful husbandry care and better survival of healthy, self-sustaining populations. The study of metabolic physiology is necessary to understand endocrine control and is fundamental in

ensuring the health and survival of any species (Wildt et al., 2003). St. Aubin (2001) stated that information on endocrine pathologies in marine mammals is scant when compared to that in terrestrial animals. Therefore, the activity of a specific endocrine organ, such as the thyroid gland, as reflected by the morphological and functional changes, may provide important information about the body status of the subject and may help guiding corrective therapy.

Thyroid diseases have been reported in many species of wildlife (Wallach, 1970; Kaptein et al., 1994; Colborn, 2002; Reese et al., 2005) but rarely in marine mammals. Environmental contaminants and local environmental influences have been implicated in thyroid hormone imbalances (Cowan and Tajima, 2006), and in the development of various thyroid abnormalities (Schumacher et al., 1993; Mikaelian et al., 2003; Das et al., 2006) leading to diseases (St. Aubin, 2001; Mikaelian et al., 2003; Cowan and Tajima, 2006; McAloose and Newton, 2009) and calf mortality (Garner et al., 2002; West et al., 2002; West et al., 2003; West and Ramer, 2005). Cowan and Tajima (2006) described the gross and histological features of thyroid glands in stranded Atlantic bottlenose dolphins, and they found that 31 out of 60 animals suffered thyroid pathologies. To the best of our knowledge, the formal literature is devoid of any reference to the diagnosis of thyroid diseases in living dolphins. In order to accurately diagnose and assess thyroid diseases in live animals, reliable methods for assessing the thyroid gland in live animals must be developed.

There are few reports of the anatomy of the dolphin thyroid gland (Arvy, 1970; Ridgway and Patton, 1971), and a vast majority of the literature is limited to the assessment of pathologies. This is not adequate for clinical diagnosis in living animals. Diagnosis of pathological disorders is invariably based on identifying variations from the normal conditions. Therefore, a clear understanding of the normal is crucial for an accurate diagnosis. In ultrasound examination of the dolphin thyroid gland, knowledge of the normal sonographic features is the basis for the diagnosis of pathology, and this information has not been reported in the literature.

Chapter 3 describes the sonographic morphology of normal thyroid gland and adjacent neck structures of a group of live bottlenose dolphins.

Ultrasound is a useful imaging tool in the assessment of thyroid morphology in humans (Hegedüs, 2001; AIUM, 2003; Khati et al., 2003) and companion animals (Cartee et al., 1993; Wisner et al., 2002; Reese et al., 2005; Brömel et al., 2006). Previous studies have tried to measure the size of bottlenose dolphin thyroid gland in-vivo using ultrasound, and efforts have been made to establish baselines values of hormonal parameters and related them to thyroid function (West et al., 2003; West and Ramer, 2005). However, West and Ramer (2005) claimed that ultrasound measurements were extremely variable because of skill differences between operators. Their study focused on determining anatomical landmarks and a few measurements rather than establishing a standardized scanning protocol for comprehensive dolphin thyroid ultrasound assessment. The results of a study are considered to be reliable only when the methodology is standardized, repeatable and reproducible. Thus, a standardized protocol for sonographic examination of the dolphin thyroid gland would be beneficial in reducing such variations. An investigation of the feasibility of using two-dimensional (2-D) and three-dimensional (3-D) ultrasound in the measurement of dolphin thyroid volume, and an assessment of the reliability of these ultrasound techniques in measuring dolphin thyroid volume are presented in Chapter 4.

Baseline values of serum thyroid hormones have been established in some common captive cetacean species (St. Aubin, 2001), but discrepancies exist due to the different methodologies and assays used. Although serum thyroid hormones analysis can give valuable insight to any alternation in thyroid function, the procedure involves needle puncture of the animal which may damage skin and blood vessels (Brook, 1997). As the examination procedure is invasive, repeated measurements for follow-up examinations may result in the loss of voluntary behaviour of the dolphin. Concentrations of free and total triiodothyronine (T3), free and total thyroxine (T4), as well as thyroid-stimulating hormone (TSH) should be

evaluated together as a panel (Cunningham, 2002). Different types of thyroid hormone test kits are available, but they have been species-specific developed mainly for humans and domestic mammals. Therefore, these thyroid hormone test kits may not be compatible for use in marine mammals due to the mismatch of corresponding antibodies. West and Ramer (2005) first reported the successful evaluation of serum TSH using radioimmunoassay (RIA) assay in bottlenose dolphins, but no detailed methodology of the test was provided. Therefore, serum thyroid hormones analysis cannot be used frequently to monitor thyroid physiology in live dolphins. Studies suggested that ultrasound may be a more sensitive index of thyroid disturbance than serum TSH concentration (Stewart et al., 1989). Knowledge of the possible association of the thyroid volume (measured by ultrasound) and function is essential to consider ultrasound as a primary means of investigation, before conducting more expensive and invasive procedures such as blood sampling for thyroid hormones analysis. This information is presented in Chapter 5.

Assessment of thyroid morphology and function is one of the diagnostic challenges in cetacean clinical endocrinology. Various factors affect the cetacean thyroid function and morphology, such as demographic parameters, physiological cycles and the health status of the animals (Docter et al., 1993; St. Aubin et al., 1996; Berghout and Wiersinga, 1998; St. Aubin, 2001; Cowan and Tajima, 2006; Myers et al., 2006; Mooney et al., 2008). It is important to recognize the potential influence of these factors on the thyroid morphology when assessing the thyroid gland. A number of studies have revealed that the thyroid volume in humans varies with different sex, race, age, weight, health conditions, dietary conditions and reproductive status of female subjects (Hegedüs et al., 1986; Loevner, 1996; Chan et al., 1998,1999; Ying et al., 1998; Barraclough and Barraclough, 2000; Hess and Zimmermann, 2000; Zimmermann et al., 2000; Hegedüs, 2001; Zimmermann et al., 2001; Khati et al., 2003; Senchenkov and Staren, 2004; Sheikh et al., 2004). In veterinary medicine, there are a few studies reporting the thyroid volume and sonographic features of the normal thyroid gland in dogs, cats and horses (Cartee et

al., 1993; Breuhaus, 2002; Reese et al., 2005; Brömel et al., 2006). However, the formal literature is devoid of any reference to possible determinants such as age, sex, sexual maturity, body size and season on thyroid morphology in bottlenose dolphins. The possible variations in thyroid morphology of a population of Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) in captivity with different demographic factors are addressed in Chapter 6.

Apart from the pathological changes, physiological alternations of thyroid morphology with changes of female hormonal environments and dietary iodine intake have been observed. Previous studies in humans have reported that the thyroid volume varied significantly during the normal menstrual cycle in females of reproductive age, and thyroid physiological characteristics altered during different reproductive phases of a women's life (Hegedüs et al., 1986; Hegedüs, 1990; Chan et al., 1998; Hegedüs, 2001; Krejza et al., 2004). In a companion animal study, serum thyroid hormone concentrations were found to be higher in diestrous females than in anestrous, proestrous and lactating females (Reimers et al., 1984). Thyroid function and morphology are likely to be affected by the cyclic change of the hormonal environment during the estrous cycle and in different reproductive events in females (Hegedüs et al., 1986; Miki et al., 1990; Chan et al., 1999; Hegedüs, 2001; Sekulić et al., 2007; Zagrodzki et al., 2007). Previous literature is devoid of information on thyroid morphology as assessed by ultrasound in normal female bottlenose dolphins during the estrous cycle and in different reproductive states. Recognizing changes in the thyroid gland during the estrous cycle and in different reproductive events in female dolphins is essential to help the diagnosis of pathology and monitoring of the thyroid gland during treatment. This information is presented in Chapters 7 and 8 respectively.

Chapter Two

Literature review

The thyroid anatomy, physiology and function of the bottlenose dolphin

2.1 Anatomy of the bottlenose dolphin thyroid gland

There are scarce literatures that have comprehensively described the gross anatomy of cetaceans, with particular attention to their neck region (Turner, 1862; Harrison, 1969; Arvy, 1970; Shimokawa et al., 2002). Others have reported briefly on the general appearance, size and the weight of the cetacean thyroid gland (presented in thyroid weight to body weight ratios) in general necropsy reports of a considerable number of stranded animals or post-mortem examination of a single captivity subject (Neuville, 1928; Jacobsen, 1941; Slijper, 1958; Pilleri and Gihr, 1969; Gihr and Pilleri, 1969; Harrison and Young, 1970; Ridgway and Patton, 1971; St. Aubin, 2001). These reports found that there is little variation in the histology of the thyroid gland among cetaceans. However, the gross morphology of the cetacean thyroid gland has been shown to vary between individuals of the same species as well as between different species.

2.1.1 Location

Due to the greatly foreshortened neck in dolphins, the location of the thyroid gland differs from other mammalian species. Reports regarding the location of the thyroid gland are scarce and mainly referred to a very limited number of subjects with unknown demographic data. Turner (1860) examined the thyroid gland of 3 common porpoises (*Phocoena communis*) by removing the large sterno-hyoid and

smaller sterno-thyroid muscles in the neck region of the specimens. A distinct and well-defined glandular mass was seen lying on the anterior and lateral surfaces of the trachea at its upper end, and extending slightly upwards on each side over the outer surface of the cricoid cartilage. The thyroid gland was described as a structure extending across the midline and attached to the front and the 2 sides of the trachea.

Neuville (1928) described the location of the thyroid gland of a young unknown species of dolphin. The thyroid gland was situated along the median cervical line and appeared as a mass of tissues bearing traces of a division into 2 lobes in its anterior region. In some specimens this division might have been completed in which the thyroid gland would form 2 roughly symmetrical latero-cervical glandular masses. In 1 of the Risso's dolphin specimens, its left lobe laid along the windpipe whereas in contrast, the right lobe of the gland contained an anterior region lying against the right edge of the windpipe and a posterior region that curved back towards the medio-cervical line, in front of the trachea, reaching nearly as far as the left lobe of the thyroid gland (Neuville, 1928). Ten common (Pacific) dolphin (*Delphinus delphis bairdi*) thyroid glands were examined post-mortem, and the investigators found that the thyroid glands consisted of 2 large lobes situated on either side of the trachea and joined by a narrow isthmus (Harrison and Young, 1970).

Shimokawa and his team (2002) conducted a morphological study of the thyroid gland in 7 Risso's dolphins (*Grampus griseus*) by examining the gross anatomy of the thyroid gland. The thyroid gland was located at the lateral-ventral side of the thyroid cartilage. Hayakawa et al. (2004) found the thyroid gland of 3 adult false killer whales, which was situated on the rostral part of the trachea as a dark brown mass. Cowan and Tajima (2006) described the location of the thyroid gland of 60 Atlantic bottlenose dolphins (*Tursiops truncatus*), which was situated in the upper anterior mediastinum, in close association with the trachea, just above the level of the aortic arch.

In baleen whales, Rosa et al. (2007) found that the thyroid gland of bowhead whales was located along the ventral trachea, cranial to where the trachea bifurcates into primary bronchi.

2.1.2 Gross anatomy

The gross morphology of cetacean thyroid gland has been examined and described (Galliano et al., 1966; Viamonte et al., 1968; Harrison, 1969; Arvy, 1970; Cowan and Smith, 1999; St. Aubin, 2001; Shimokawa et al., 2002; Cowan and Tajima, 2006). Early reports have only included brief observations about the shape, colour and location of the thyroid gland, with no record on the histology of the gland. The thyroid weight, as well as the thyroid weight to body weight ratio, were usually noted. However, the measurements were widely varied within a single species and among different species (Crile and Quiring, 1940; Cowan, 1966; Gihr and Pilleri, 1969; Harrison, 1969; Arvy, 1970; Harrison and Young, 1970; Cowan and Tajima, 2006). Moreover, no constant relationship could be established between the thyroid volume and the body size of the cetacean specimens.

Turner (1862) was the first to publish a macroscopic description of the thyroid gland in the cetacean. The thyroid gland of the adult common porpoise (*Phocoena communis*) was described as a single uniform mass of about 2 inches long and with a dark purple tint. The supero-inferior diameter of the middle portion of the thyroid gland was similar to that of the lateral portion. The supero-inferior diameter of the middle portion of thyroid gland was measured ¾ inch and ¼ inch in adult and fetus respectively.

Neuville (1928) further reported the gross anatomy of the thyroid gland of a few specimens belonging to an unknown dolphin species. The thyroid gland was observed as a mass of soft tissues showing a division into 2 lobes. However, Neuville (1928) also found that other specimens had a complete division of the thyroid gland forming 2 separate roughly symmetrical latero-cervical glandular

masses. The author also observed that the thyroid gland of rough-toothed porpoise (*Steno rostratus*), which was considerably larger among dolphin species. In 1 of the investigated specimens, its left thyroid lobe was flattened and irregular in shape, while the right thyroid lobe formed an 'L' shape. Only with scarce observations, Nenville (1928) concluded that the cetacean thyroid gland was a single soft tissue structure, and is in a median position in young animals, and bi-lobulated in old animals.

Jacobsen (1941) studied a large population of blue whales (*Balaenoptera musculus*), and reported that in blue whales the thyroid gland was red with a greyish tinge, and the thyroid gland was about 35 cm in length. Blue whales and fin whales were found to have a bi-lobular thyroid gland with a small connecting bridge and exhibiting distinct lobulation (Slijper, 1958). The thyroid gland of a Risso's dolphin (*Grampus griseus*) was investigated macroscopically and it was observed to have a coarse lobular appearance, with various lobules being separated from one another by connective and adipose tissue (Pilleri and Gihr, 1969). The thyroid glands of the Ganges River dolphins were also examined and they appeared as a large dark brown mass measuring 3.1 cm x 1.6 cm x 0.8 cm in transverse diameter, longitudinal diameter and thickness respectively and weighing 3g.

More recent findings have continued the debate regarding the gross anatomy of the dolphin thyroid gland. Hayakawa et al. (1998) studied the thyroid gland of 6 Risso's dolphins, and found that the thyroid gland consisted of 2 large lobes joined by a narrow isthmus, and the thyroid gland was described as a dark brown mass with numerous indentations on its surface. The size of the thyroid gland was measured, ranging from 10.1 to 13.7 cm for transverse diameter, 3.6 to 5.1 cm for longitudinal diameter and 1.7 to 2.5 cm for the thickness. In contrast, Shimokawa et al. (2002) found that the left and right lobes of the dolphin thyroid gland were not clearly discriminated and was devoid of the isthmus. Lobules of the thyroid gland were clearly identified due to the presence of interlobular connective tissues. The crown-tail length (long axis) of the thyroid lobe, was ranging from 5 to 15 cm, and the

thickness was ranging 1.5 to 2 cm.

The most comprehensive study to date on dolphin thyroid gland has thoroughly evaluated different gross configurations of 60 stranded Atlantic bottlenose dolphins (Cowan and Tajima, 2006). The authors categorized the thyroid gland into 4 different gross configurations: 1) two lobes joined by an isthmus (45% of animals) (Figure 2.1); 2) two separate lobes, usually of equal size, one on each side of the trachea, with no connecting isthmus (28% of animals); 3) a shield-like, single mass, roughly diamond-shaped, located anteriorly (ventrally) on the trachea (20% of animals) (Figure 2.2); 4) irregular, multi-lobular and grape cluster-like mass, with adjacent but separate lobules (Figure 2.3). The authors also stated that the cetacean thyroid gland was encapsulated by thin fibrous tissues.

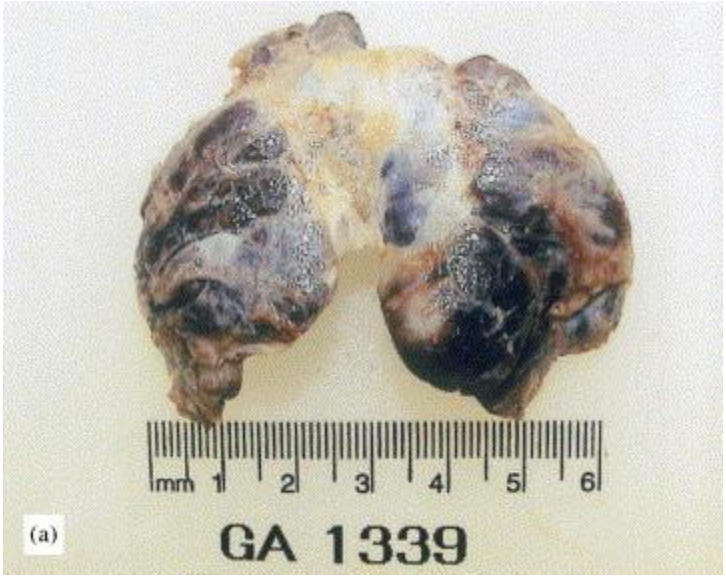

Figure 2.1: Picture shows the most common dolphin thyroid configuration - two lobes joined by an isthmus (adapted from Cowan and Tajima, 2006).

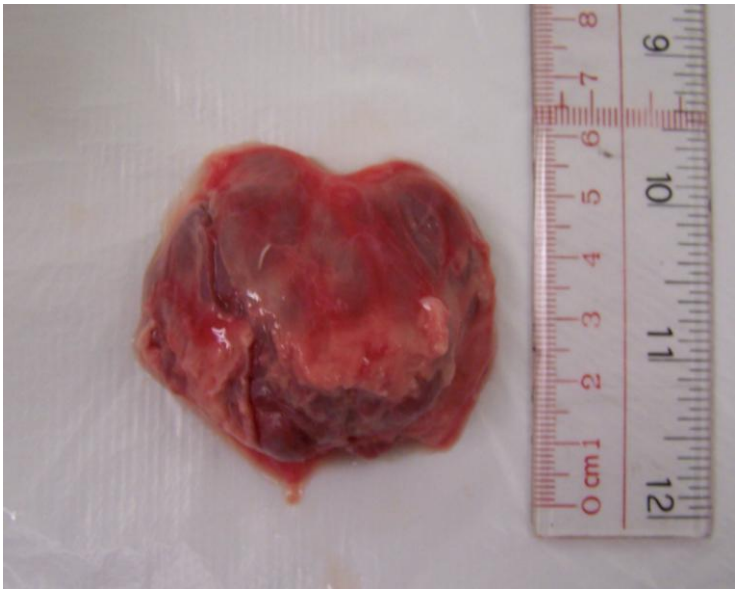

Figure 2.2: Picture shows another type of dolphin thyroid configuration - a shield-like, single mass, roughly diamond-shaped, located anteriorly (ventrally) on the trachea.

Figure 2.3: Picture shows an atrophic and fibrotic dolphin thyroid gland (adapted from Cowan and Tajima, 2006)**.**

In baleen whales, Rosa et al. (2007) described that the right and left lobes of the bowhead whale's thyroid gland were easily discriminated and no isthmus was observed between the 2 lobes.

Thyroid gland anomalies or normal variations may occur due to the disturbance of the descensus of the thyroid gland or the regression of the thyroglossal duct. The presence of an accessory thyroid gland tissue (the pyramidal lobe) was reported in both human and cetaceans. In humans, the presence of a pyramidal lobe has been reported in 15% to 75% of the population (Blumberg, 1981; Ahuja, 2000; Bruneton et al., 2002; Beazley, 2005). There is no sex difference in the presence of a pyramidal lobe (Bruneton et al., 2002; Beazley, 2005). The origination of the pyramidal lobe varied, and it could be found from the right side of the isthmus; the middle of the isthmus; left part of the isthmus; and from the left lobe of the thyroid gland. In cetaceans, Turner (1862) was the only study reported the presence of a pyramidal lobe in the thyroid gland in porpoise. The pyramidal lobe of the thyroid gland appeared as a dark purple tint, with a soft and succulent texture (Turner, 1862).

Few studies have investigated the parathyroid gland of marine mammals, including dolphins (Pilleri and Gihr, 1969; Kamiya et al., 1978; Hayakawa et al., 1998). Pilleri and Gihr (1969) claimed that 2 parathyroid glands, about 2 cm or less in diameter, were found macroscopically in Risso's dolphins. Hayakawa et al. (1998) examined 2 species of dolphins (*Tursiops truncatus* and *Grampus griseus*) and found that 2 to 4 parathyroid glands in varying sizes and locations were found either on the surface or within the thyroid gland. The parathyroid glands of the Risso's dolphins reported by Hayakawa et al. (1998) were much smaller than those reported by Pilleri and Gihr (1969). This might be possibly accounted by the presence of several cervical lymph nodes observed proximal to the thyroid gland, which were misidentified as parathyroid glands in Pilleri and Gihr's study (Hayakawa et al., 1998). The mean size of the parathyroid glands of Risso's dolphins and bottlenose dolphins was 0.27 x 0.15 cm and 0.2 x 0.13 cm respectively (Hayakawa et al., 1998). By considering the greater body size of these dolphins, Hayakawa et al. (1998) believed that the parathyroid glands of Risso's dolphins and bottlenose dolphins were comparatively smaller than those of other mammals. It may reflect a lower functional state, and suggest that the activity of the dolphin's parathyroid gland could be adapted to the

calcium homeostasis in cetaceans, enabling these dolphins to live in a weightless and aquatic environment (Hayakawa et al., 1998).

Hayakawa et al. (1998) reported the location of the dolphin parathyroid glands, which were found on the dorsal surface of the thyroid gland, buried within the parenchyma of the thyroid gland or appeared along the connective tissue surrounding the dorsal side of the thyroid gland in the specimens. Each parathyroid gland was divided into several lobules by connective tissue. Hayakawa et al. (2004) also examined the thyroid gland of 3 adult false killer whales and found that only 1 to 2 parathyroid glands were present in each animal.

2.1.3 Volume and weight

Previous studies have evaluated the weight of the thyroid gland in various cetacean species (Harrison, 1969; Arvy, 1970; Cowan and Tajima, 2006) (Table 2.1). There was substantial variability of the weight of the thyroid gland among individuals of the same species, as well as in subjects of different species. Owing to the legality of commercial whaling in the past, previous studies have acquired abundant information regarding with measurements of various internal organs including the thyroid weight.

Table 2.1: Summary of thyroid weight and the corresponding demographic parameters in various cetacean species.

Suborder	*Species*	Common Name	Study	Body Weight (kg)	Body Length (cm)	Thyroid Gland Weight (g)	Sex	State
Mysticeti	*Balaenoptera musculus*	Blue Whale	Crile and Quiring, 1940 (n=1) Jacobsen, 1941	58,059 -	-	3,450 6,000 - 8,000	-	-
	Balaenoptera physalus	Fin Whale	Quiring, 1943 (n=1)	59394	2100	3,970	F	-
	Megaptera novaeangliae	Humpback Whale	Quiring, 1943 (n=3)	37,195 - 40,823	1200	2,960 - 3,237	M	-
Odontoceti	*Phocoena phocoena*	Harbor Porpoise	Crile and Quiring, 1940 (n=1) Slipjer, 1958; Harrison, 1968 (n=24)	142 6.5 - 60	- 80 - 172	18.29 6 - 43	M,F M,F	Newborn, Sexually Immature, Immature, Mature, Sexually Mature
	Physeter catodon	Sperm Whale	Quiring, 1943 (n=1)	39,009	1400	800	M	-
	Delphinapterus leucas	Beluga Whale	Crile and Quiring, 1950 (n=6)	303 - 447	-	66 - 111	M,F	-
	Delphinus delphis	Short-beaked Common Dolphin	Gihr and Pilleri, 1969 (n=6)	37 - 54	-	4.8 - 25	M,F	-
	Delphinus delphis bairdi	Short-beaked Common (Pacific) Dolphin	Young and Harrison, 1969 (n=12)	4.8 - 84	75 - 198	1.3 - 16.3	M,F	Newborn, Immature, Mature
	Globicephala melaena	Long-finned Pilot Whale	Harrison, 1969 (n=2)	140	200	22	F	Immature
	Globicephala scammoni	North Pacific Pilot Whale	Harrison, 1969 (n=1)	159	215	32.6	F	Juvenile
	Grampus griseus	Risso's Dolphin	Pilleri and Gihr, 1969 (n=1)	234	-	90	-	-
	Lagenorhynchus obliquidens	Pacific White-sided Dolphin	Harrison, 1969 (n=2)	36.3 - 79	136 - 183	23 - 29.9	M,F	Immature, Juvenile
	Sotalia plumbea	Plumbeous Dolphin	Harrison, 1969 (n=1)	41	166	10	F	Immature
	Stenella attenuata	Pantropical Spotted Dolphin	Harrison, 1969 (n=1)	35.9	168	10	F	Immature
	Stenella coeruleoalba	Striped Dolphin	Gihr and Pilleri, 1969 (n=4)	45 - 71	-	6 - 12.2	M,F	-
	Stenella coeruleoalba	Striped Dolphin	Harrison, 1969 (n=1)	33.5	159	9.75	F	Immature
	Stenella graffmani	Eastern Pacific Coastal Spotted Porpoise	Harrison, 1969 (n=3)	22.7 - 80.5	141 - 244	3.1 - 11.6	M,F	Suckling, Mature
	Stenella longirostris	Spinner Dolphin	Harrison, 1969 (n=4)	30.5 - 54.5	160 - 182	4.5 - 8.9	M	Immature, Sexually Mature
	Tursiops truncatus	Common Bottlenose Dolphin	Harrison, 1969 (n=4)	16.5 - 180	110 - 254	3.3 - 31.5	M,F	Newborn,Mature, Recently Pregnant
	Pseudorca crassidens	False Killer Whale	Hayakawa et al., 2004 (n=3)	500	400	45	-	Mature
	Tursiops truncatus	Common Bottlenose Dolphin	Cowan and Tajima, 2006 (n=49)	16.4 - 258	109 - 249	11 - 58	M, F	Neonate, Immature, Mature, Pregnant, Lactating

Various comparisons have been made between cetacean thyroid mass and body mass. It has found that the thyroid weight to body weight ratio was particularly higher in the aquatic and terrestrial mammals that live in the Arctic (Crile and Quiring, 1940). Even for mammals of similar size, the thyroid weight to body weight ratio varied; it measured approximately 0.015% in the horse, a terrestrial mammal, while in the beluga whale it was 0.05 %. (Crileand Quiring, 1940). Slijper (1958) reported the absolute weight of the thyroid gland ranging from 14 to 28 g in a large population of harbour porpoises (*Phocoena phocoena*), with the mean thyroid weight of females larger than that of the males.

2.1.4 Histology

The functional components of the thyroid gland are the thyroid follicles, which consists of a cuboidal epithelium arranged as a single layer surrounding a lumen that contains a colloid material (Foktin et al., 2010). The epithelial secreting cells are in the irregular follicles separated by connective tissue where the blood as well as lymph vessels and nerves are carried. In the normal state, the follicles are filled with the homogeneous colloidal substance. The epithelium exhibits a peculiarity in that a basement membrane is lacking. This peculiarity is of significance during secretion and absorption of the substance secreted in the follicle. The epithelial cells vary in size and number, which is dependent on the activity of the thyroid gland. In a hyperactive thyroid, hyperplasia or abnormal increase of epithelial cells occur (Hartoft-Nielsen et al., 2005). The histology of the thyroid gland is quite uniform in vertebrates. Homogeneous colloidal material, which is found in many of the follicles, is the common feature of a stained thyroid gland section (Foktin et al., 2010).

Histological examination of the thyroid gland in marine mammals has revealed no significant difference from the typical mammalian arrangement (Harrison, 1969; Young and Harrison, 1969; Arvy, 1970; Ridgway and Patton, 1971; Cowan and Tajima, 2006). Similar to humans and other mammals, the volumetric fraction and

activity of different histological components in the thyroid gland (follicular cells, C-cells, colloid, and interstitial tissue) change considerably throughout the course of development (Conde et al., 1991). Both phocids and cetaceans have shown a marked variation in the activity of thyroid follicular cells at various times during development and life history (Harrison et al., 1962; Amoroso et al., 1965; St. Aubin and Geraci, 1989; Little, 1991; St. Aubin et al., 1996).

Cowan (1966) investigated the thyroid gland in 55 Newfoundland pilot whales (*Globicephala melaena*) and observed that the thyroid follicles are more regular in younger animals. In older animals, more variability of follicle size and colloid density was observed. Another study also reported that the thyroid gland of Long-finned pilot whales (*Globicephala melaena*) exhibited noticeable variation in size and appearance with advancing age, and active formation of follicles was observed at all stages of development in young Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) (Harrison, 1969). The average follicle diameter in young Pacific white-sided dolphins was 0.3 mm. The thyroid histology in young Pacific spotted dolphins (*Stenella graffmanim*) was similar to that in young Pacific white-sided dolphins, but the Pacific spotted dolphins have a more obvious follicular development with smaller follicles measuring 0.1 mm in diameter on average. The follicular epithelial cells appeared cuboidal and the colloid stained deeply. The thyroid gland of young North Pacific pilot whales (*Globicephala scammoni*) and common pilot whales (*Globicephala melaena*) also showed similar histological appearances. The average diameter of the follicles measured 0.25 mm. The epithelium was uniformly presented as a low cuboidal type with the colloid evenly and moderately stained. In the thyroid gland of the newborn bottlenose dolphin and common dolphin, the follicles are irregularly divided into groups by thick connective tissue septa. Some small follicles, measuring between 30 and 100 μm in diameter, were present in the peripheral part of the thyroid gland. The follicular epithelial cells were cuboidal, 6 to 10 μm in height and the colloid was lightly stained (Harrison, 1969).

In summary, observations showed that thyroid follicles appeared to be more regular

in young animals. The colloid was staining readily (slightly chromophile), and its appearance was more fluid and the epithelial cells were columnar and tall (Figure 2.4). In contrast, the colloid of older animals was less hydrated in appearance, the epithelial cells were cuboidal and a large number of lamellar basophile masses were present in the thyroid gland (Arvy, 1970) (Figure 2.5).

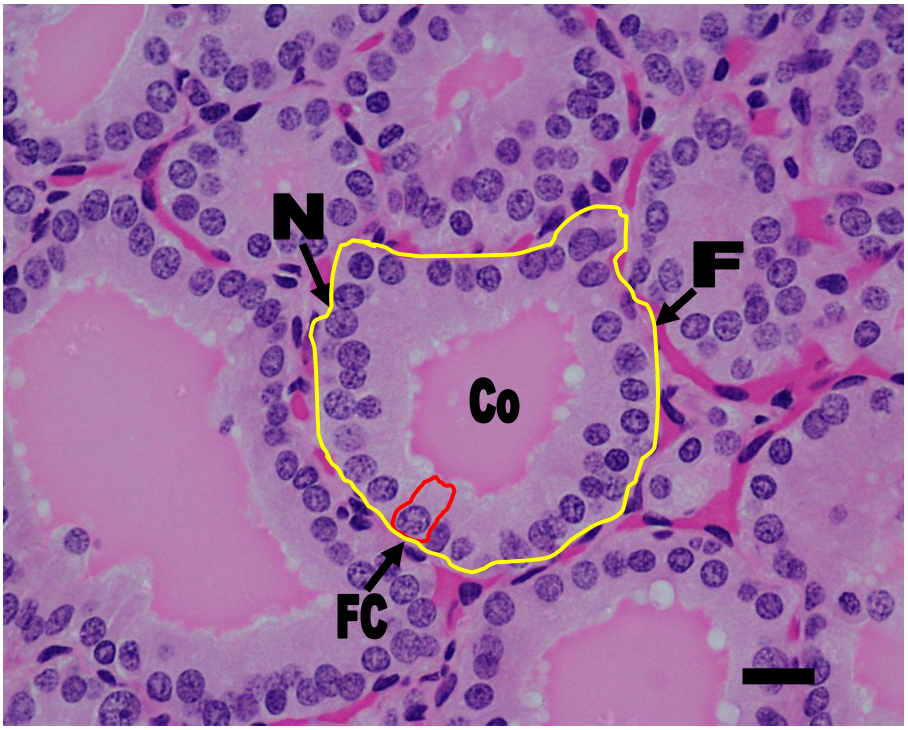

Figure 2.4: A normal young dolphin thyroid gland section with Haematoxylin and Eosin Stain. The thyroid follicle (F) (outlined in yellow solid line) were lined by a single layered columnar follicular cell (FC) (outlined in red solid line) with round nuclei (N). The lumen contained homogeneous colloid (Co) material. Bar, 20 µm.

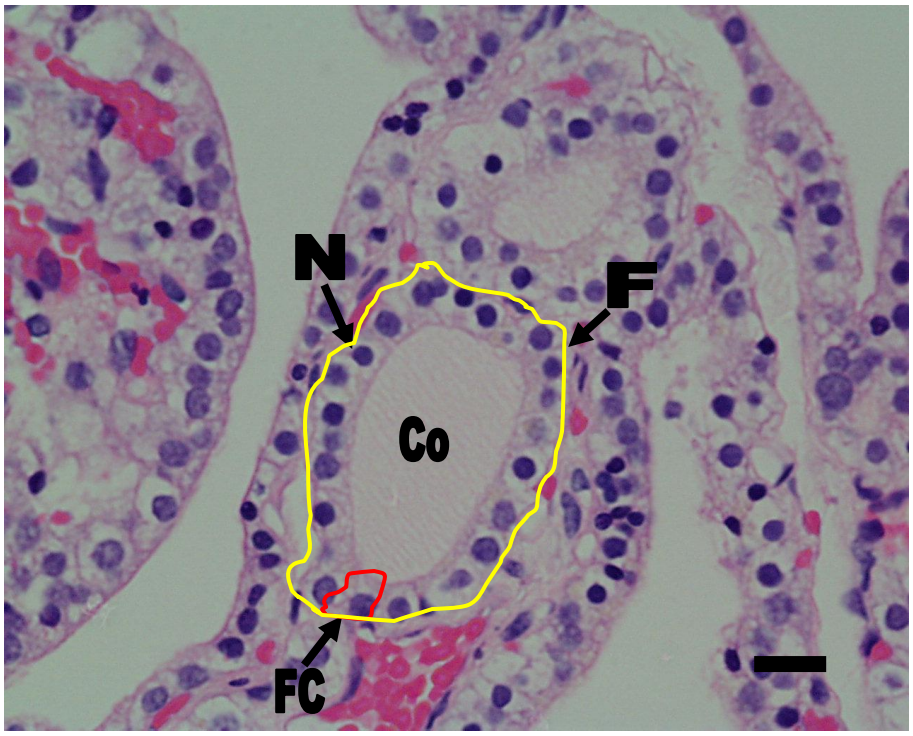

Figure 2.5: A normal elder dolphin thyroid gland section with Haematoxylin and Eosin Stain. The thyroid follicle (F) (outlined in yellow solid line) were lined by a single layered columnar follicular cell (FC) (outlined in red solid line) with round nuclei (N). The lumen contained homogeneous colloid (Co) material. Bar, 20 µm.

More recent studies on cetacean thyroid histological investigation have revealed similar findings. Shimokawa et al. (2002) described the thyroid histology of 7 male Risso's dolphins, in which irregular or oval follicular lumens were observed in the parenchyma of the thyroid gland, and surrounded by follicular epithelial cells. Stratified follicular epithelial cells were often invaginated into the follicular lumen. The size of follicular lumen was larger in the central regions than in the peripheral regions. The diameter of the follicular lumen ranged from 98.1 to 120.3 µm. Mikaelian et al. (2003) found a substantial degree of histological variability among beluga whales living in cold northern water. Hayakawa et al. (2004) reported the morphology of the thyroid gland of 3 adult false killer whales microscopically, in which the mean height of the follicular epithelium of the false killer whale was 12.1

µm. In baleen whales, Rosa et al. (2007) reported the thyroid histomorphology in 24 bowhead whales and described that the gland consisted of follicles of variable size lined by simple epithelium of variable height.

Among all the investigated cetaceans, marked seasonal differences in thyroid histology were only observed in bowhead whales and beluga whales. Thyroid activity was examined among 3 seasonal phases: spring, summer and fall (St. Aubin and Geraci, 1989). Thyroid epithethial cells in beluga whales were cuboidal during spring, columnar during summer, and reverted back to cuboidal during fall. These changed were consistent with increased synthesis and secretion of thyroid hormones. Intake of iodine from seasonal foraging and change in water temperature of where the whales seasonally occupied might be attributed to this observation (St. Aubin and Geraci, 1989; Rosa et al., 2007)

Harrison (1969) demonstrated that the thyroid glands of some stranded animals kept in captivity for varying periods showed dramatic changes. A female *Tursiops truncatus* that was kept in captivity for nearly 2 years possessed numerous interlacing septa of connective tissues, and exhibited a mucoid degeneration. The follicles were small, with an average diameter of 40 µm, and the follicular epithelial cells were columnar with the colloid condensed and densely stained. The majority of the follicles contained only remnants of a colloid, suggesting severe thyroid gland depletion. Suspected thyroid gland depletion was also found in a short-beaked common dolphin (*Delphinus delphis*) and a beluga whale (*Delphinapterus leucas*). One bottlenose dolphin (*Tursiops truncatus*) was reported to have acute thyroiditis and was devoid of follicles in its thyroid gland.

2.1.5 Blood supply

Although information is scarce on the description of the anatomy of the cervicothoracic vascular system of dolphins, most organs in marine mammals are similar to those of terrestrial mammals, and their central blood supplies are also

similar (Rommel and Lowenstine, 2001). Particular attention on the vascular adaptation and specialisations in cetaceans has been demonstrated in previous studies (Galliano et al., 1966; Viamonte et al., 1968), regarding the relationship between the blood supply of the brain and the associated mechanisms in which the brain may receive blood under a steady perfusion pressure during diving.

The arterial blood supply of the thyroid gland is from the superior thyroid artery arising from the brachiocephalic trunk, the inferior thyroid branch of the subclavian artery and a branch of the carotid artery (De Kock, 1959; Galliano et al., 1966; Viamonte et al., 1968). These arteries form numerous anastomoses so that obstruction of one or the other artery does not seriously hinder blood flow to the organ (De Kock, 1959; Galliano et al., 1966; Viamonte et al., 1968). Cervical arteries such as the brachiocephalic trunk, subclavian arteries, internal carotid arteries, external carotid arteries, omooccipital artery and superior thyroid arteries were identified in anatomical dissection as well as in invasive surgical procedures such as guided angiography on living bottlenose dolphins.

Venous drainage, which is plexiform in nature, pours into the jugular and innominate channels. Cervical veins such as the brachiocephalic vein, internal jugular veins and superior thyroid veins were also identified in anatomical dissection as well as in invasive surgical procedures on living bottlenose dolphins. The venous blood of the thyroid gland is drained into the superior thyroid vein which then drains into the internal jugular vein (Galliano et al., 1966; Viamonte et al., 1968).

2.1.6 Lymph nodes

Due to the foreshortened neck in bottlenose dolphins, direct comparisons of anatomical positions to terrestrial mammals are not possible. In bottlenose dolphins (*Tursiops truncatus*), there is an observable variability of definition of the individual nodes. In some animals, lymph nodes within a group may be clumped together, but

they remain distinct. However, in other animals the nodes fuse to form an irregular, lobular mass. Cowan and Smith (1999) investigated the morphology of the lymphoid organs of 50 stranded bottlenose dolphins, and found that the cervical nodes occurred consistently in the neck area, in front of and behind (ventral and dorsal to) the middle of the diagonal mastohumeral muscle. They were apparently found beneath the fascia, and the upper group might extend deeply between the muscles. The lymph nodes in the neck region were usually discrete. The dorsal group of lymph nodes was often larger, consisting of 6 to 8 ovoid nodes.

In addition, several nodes were always associated with the thymus and the thyroid gland, which were distinguishable from both of these organs by their gross appearance on sectioning. Cowan and Smith (1999) described that, as many as 10 nodes could be found in the aortic arch region, although 6 to 8 were more frequently counted. The thyroid gland, thymus, and nodes, together with the blood and lymphatic vessels, were invested within a delicate fascial plane in the mediastinum. These organs and their associated lymph nodes were found in the mediastinum close to the aortic arch.

2.2 Functions of the mammalian thyroid gland

The mammalian thyroid gland maintains basal metabolism in the tissues in a way that is optimal for normal function (Hegedüs, 2001). The thyroid gland is the site of hormone synthesis and storage for both thyroxine (T4) and tri-iodothyronine (T3). Both T4 and T3 have vital roles in the regulation of a variety of metabolic functions. Thyroid hormones stimulate the oxygen consumption of most of the body cells, help regulate lipid and carbohydrate metabolism, and are essential elements for normal growth and maturation (St. Aubin, 2001; Foktin et al., 2010). Although the thyroid gland is not essential for survival, absence of thyroid gland can cause mental and physical retardation, poor resistance to cold, and dwarfism (Foktin et al., 2010). Conversely, excess secretion of thyroid hormones leads to body wasting,

nervousness, tachycardia, tremor, and excess heat production (Foktin et al., 2010).

2.2.1 Hypothalamic-pituitary-thyroid axis

Thyroid hormones are released from the thyroid gland in response to a feedback mechanism initiated by the pituitary-hypothalamic axis. The secretion of thyroid hormones is mediated by thyrotropin-stimulating hormone (TSH) secreted by the anterior pituitary gland. The secretion of TSH, in turn, is mediated by thyrotropin-releasing hormone (TRH) secreted by the hypothalamus. T4 and T3 induce a negative feedback loop that inhibits TSH and TRH secretion, thereby providing regulation to thyroid hormone secretion and serum thyroid hormone concentrations. The synthesis of T4 and T3 is dependent on the nutritional availability of iodine, as T4 and T3 are formed from the iodination of tyrosyl residues within the glycoprotein thyroglobulin (Tg). Tg is a large glycoprotein dimer that acts as the storage form for thyroid hormones and iodide. Synthesis of Tg takes place at the rough endoplasmic reticulum and becomes packaged into membrane-bound granules in the Golgi apparatus. Non-iodinated tyrosine residues are initially added into Tg before being bound to active iodide. The coupling of 2 iodinated thyrosines yields an iodinated thyronine. Thyrocytes are able to digest Tg in lysosomes for T4 and T3 secretion. In the evaluation of thyroid status, it is important to consider that thyroid hormones regulate cellular processes in their free, unbound form, while Tg as a binding protein regulates the delivery, availability and activity of thyroid hormones (Feldman and Nelson, 2004; Dvorakova et al., 2008; Shivaraj et al., 2009; Foktin et al., 2010).

2.2.2 Thyroid hormone action

Thyroid hormones influence many major processes in mammals, such as metabolism, growth and differentiation, and reproduction (Feldman and Nelson,

2004; Foktin et al., 2010). Thyroid hormones also accelerate glucose oxidation or oxidative phosphorylation uncoupling. Moreover, thyroid hormones augment the actions of growth hormone on its target tissues, regulating secretion from the pituitary as well as cellular uptake. Differentiation processes such as hair replacement are also affected by thyroid hormones. Furthermore, proper thyroid function is essential for normal gonadal development and function. Hypothyroidism has been associated with delayed sexual maturation, reduced androgen synthesis in males and reduced ovarian weight and irregular ovarian cycling in females (Feldman and Nelson, 2004; Foktin et al., 2010).

2.2.3 Domestic mammals

Serum free T4, total T4, and total T3 concentrations have been assessed in healthy dogs, dogs with hypothyroidism, and euthyroid dogs with concurrent dermatopathy or illness (Nelson et al., 1991). The mean serum total T4 and total T3 concentrations in a population of 62 healthy dogs were 21 ng/ml (compared to 6 ng/ml in hypothyroid dogs) and 0.7 ng/ml (compared to 0.5 ng/ml in hypothyroid dogs) respectively. The mean serum free T4 and total T4 concentration in healthy dogs (0.51 ng/dl and 21 ng/ml respectively) were significantly higher than that in dogs with hypothyroidism (0.10 ng/dl and 6 ng/ml respectively). However, there was no significant difference in mean serum total T3 concentrations between healthy dogs, dogs with hypothyroidism, or euthyroid dogs with concurrent illness. Beale et al. (1992) evaluated the baseline serum total T4, free T4 and total T3 concentrations in 58 dogs with generalized dermatologic disease, and compared these values with the serum hormone concentrations of 200 healthy dogs. No significant difference was observed in serum total T4 and free T4 concentrations between dogs with generalized dermatologic disease and healthy dogs; both euthyroid and hypothyroid dogs with generalized dermatologic disease had baseline serum total T4 or free T4 above the low range (≤ 0.68 μg/ml and ≤ 0.145 ng/dl respectively). The serum total T3 concentration was found to be inaccurate in differentiating dogs with euthyroid and hypothyroid, as both of them had serum total T3 concentrations within the

normal range (≥ 90.86 ng/dl). Miller et al. (1992) found that mean serum total T4 and total T3 concentrations did not vary significantly within each group alone (euthyroidal, dogs with hypothyroid and euthyroid dogs with atopic dermatitis). However, the mean serum total T4 concentration was found to be significantly higher in both healthy euthyroid dogs and euthyroid dogs with atopic dermatitis when compared to dogs with hypothyroidism. Various assaying kits for measuring serum T4 concentrations suggested the clinically normal range to be between 1.0 - 5.0 μg/dl (Kemppainen and Birchfield , 2006).

For basal TSH in euthyroid dogs, a normal reference range of 0 to 0.41 ng/ml was determined (Ramsey et al., 1997). In Bruner et al. (1998), mean TSH concentration of healthy dogs (0.11 ng/ml) appeared to be lower than that of dogs with naturally developing hypothyroidism (0.55 ng/ml).

In healthy cats, the mean baseline concentration of serum T4 and T3 have been reported (33.7 nmol/L and 0.72 nmol/L respectively) (Mooney et al., 1996). Various assaying kits were used to measure serum T4 concentrations, and results suggested that the clinical normal range of serum T4 was 0.7 - 5.2 μg/dl (Kemppainen and Birchfield, 2006).

2.2.4 Marine mammals

In numerous species of marine mammals, serum concentrations of total and free thyroid hormones have been reported (St. Aubin, 2001). Baseline values for serum thyroid hormones have been established in the bottlenose dolphin (*Tursiops truncatus*) (Greenwood and Barlow, 1979). In a population of 29 bottlenose dolphins, serum T4 measurements ranged from 158 to 261 nmol/L. In the evaluation of sex difference, the range of serum T4 level in female bottlenose dolphins was between 143 and 275 nmol/L, whereas the serum T4 levels in male bottlenose dolphins ranged from 175 to 247 nmol/L. In addition, the serum T3 level ranged from 0.93 to 4.14 nmol/L in the general population. In female bottlenose dolphins,

the range of serum T3 levels ranged was from 0.76 to 4.10 nmol/L, while the serum T3 level in males ranged between 1.51 and 4.04 nmol/L. There was no significant sex difference in any thyroid hormone measurements (Greenwood and Barlow, 1979). Further study indicated that wild female bottlenose dolphins possessed significantly higher levels of total T4, free T4, and free T3 than the males (St. Aubin et al., 1996). This result may have been attributed to reproduction or lactation exclusive to female subjects, suggesting the need to closely observe thyroid activity during these 2 important life events. West et al. (2001) analyzed 60 serum samples in a population of bottlenose dolphins (n = 11). Serum concentrations of the total T4 and total T3 were found to be slightly lower in pregnant animals. For T4, the average serum concentration measured 113.1 ng/mL for pregnant individuals and 125.5 ng/mL for non-pregnant individuals. Conversely, the average serum T3 concentration in pregnant individuals (1.08 ng/mL) was higher than that in non-pregnant animals (0.95 ng/mL). Despite the slight difference in the thyroid hormone levels between pregnant and non-pregnant dolphins, the difference was not statistically significant (West et al., 2001). Additional research in this population also found no significant difference in thyroid hormone measurements with respect to water temperature, seasonality, or location (West et al., 2002). However, this study asserted that adult females likely had greater variability in thyroid hormone levels due to an initial rise in early pregnancy, followed by a decline during gestation. West et al. (2002) also suggested that serum total T3 concentration might be the most suitable thyroid hormone for diagnostic purposes as it had the lowest degree of measurement variability. However, St. Aubin et al. (1996) found that reverse T3 concentrations were significantly higher in wild male dolphins during the summer than those during the winter, and this was the only thyroid hormone in dolphins found to have seasonal variations.

In beluga whales (*Delphinapterus leucas*), St. Aubin and Geraci (1988) measured thyroid hormone changes in response to stress from captivity. Serum T3 levels notably decreased in the first 24 hours after capture, followed by a similar reduction in serum T4 levels in the next 2 to 4 days. It was concluded that the subjects had an

acute sensitivity of thyroid hormone balance in response to stress. The authors noted that belugas are the only cetaceans to have a comparable cycle in thyroid hormones (St. Aubin and Geraci, 1989). St. Aubin and Geraci (1992) further investigated the effect of captivity on thyroid hormone changes, and found that in the 10 newly captured beluga whales the serum T4 and T3 levels declined steadily while reverse T3 increased transiently during the first 24 to 36 hours of captivity.

While studying a population of captive rough-toothed dolphins (*Steno bredanensis*), Gaspar et al. (2002) found that the thyroid hormone changes reflected the treatment outcome of the animals. The thyroid hormone concentrations increased for those animals that were successfully rehabilitated, but decreased for those that had poor treatment response.

For bowhead whales (*Balaena mysticetus*), Rosa et al. (2007) found a low seasonal-related variability in the concentration of serum thyroid hormones. A low variability in the serum thyroid hormone concentration was also found between subjects of different ages, sexes and reproductive states. The existence of strong homeostatic mechanisms for the maintenance of serum thyroid hormone levels was asserted.

In the common seal (*Phoca vitulina*), the very high fat content in their milk may be related to a high rate of thyroid function (Harrison et al., 1962; Amoroso et al., 1965). In addition, there might also be a relationship between the annual molt of common seals and the blood plasma titers of T4 and cortisol (Riviere et al., 1977). The cessation of hair growth was marked by a decrease in T4 and an increase in cortisol. When the molt was complete and hair growth resumed, the level of T4 increased and cortisol returned to its baseline value. However, Renouf and Brotea (1991) believed that there might be no involvement of thyroid hormones during molting. A weekly measurement of free T4, total T4, and T3 hormone levels in 5 common seals over 12 months concluded with no statistically significant relationship between molting and serum thyroid hormone levels. Furthermore, it was observed that the serum thyroid hormone levels in common seals were

substantially lower than those found in other mammalian species (Renouf and Brotea, 1991).

A number of variables were found to influence thyroid hormone levels in male common seals (Renouf and Noseworthy, 1991). Free T4 levels positively correlated with food intake and water temperature. Free T4 levels also negatively correlated with body mass and fat changes, while the reverse held true for the T3 to free T4 ratio. Over winter, free T4 levels were significantly lower with augmented mass and blubber despite a lower food intake, and when food intake was directly related to mass accumulation, the T3 to total T4 ratio and free T4 levels were reduced. These effects were only observed in the males but not in the females.

Haulena et al. (1998) highlighted the dynamic nature of thyroid hormones and their involvement in the regulation of metabolism during the postnatal period of the common seal. Between mothers and pups, it was found that the pups had significantly higher total T4, free T4, total T3, free T3, reverse T3, percentage of free T4, and ratio of reverse T3 to T3. During the lactation period, the total T4, free T4, percentage of T4, free T3, total T3, reverse T3 and ratio of reverse T3 to T3 decreased significantly in the pups, while the maternal free T4 and total T4 increased significantly.

Oki and Atkinson (2004) investigated seasonal changes of thyroid hormones in the common seal, and found that the total T4, total T3, and free T3 levels of the common seal in the winter were significantly higher than those in the summer. The findings indicate a possible adaptive mechanism for coping with the low temperature during winter.

The thyroid gland of the southern elephant seal (*Mirounga leonina*) exhibited markedly increased secretory activity during the first 24 hours after birth (Little, 1991). From birth, plasma T4 increased 3-fold, peaking at 6 hours postpartum and then steadily declined. Plasma T3 increased 8-fold between birth and 24 hours

postpartum, remained high up until the 5th to 7th day, after which it decreased until the 20th day. The author concluded that the thyroid gland played a vital role in maintaining the body temperature of newborn seals when they entered the sub-Antartic environment.

Hall et al. (1998) measured the thyroid hormone levels in grey seals, and found no apparent effect of sex or stage of lactation on free T4, total T4 and T3 level, but found significant age-related variability. The pups had significantly higher total T4 concentration than the adults, and all serum thyroid hormone levels declined with the progression of age. A similar study conducted in grey seals showed that the total T4 concentration was the highest in the first 2 days after birth, after which it dropped to a lower, stable level (Woldstad and Jenssen, 1999). Total T3 concentrations were the lowest in neonatal pups, but it increased as a function of age, possibly due to increased T4 deiodination activity. In addition, there was no variation of free T4 concentration as a function of age.

Thyroid hormone concentrations were reported in captive and feral polar bears (*Ursus maritimus*) (Leatherland and Ronald, 1981). The T4 and T3 of 1 captive adult male were at the peak level during the winter despite being maintained in a constant environment with regards to temperature, photoperiod and diet.

Free-ranging West Indian manatees (*Trichechus manatus*) exhibited significantly greater total T4 and free T4 concentrations than captive adults, regardless of diet (Ortiz and Worthy, 2000). The findings might be associated with the increased lipolytic activity.

Various methods were used to quantify the serum thyroid hormone concentrations, rendering direct comparison of different species difficult. Nevertheless, serum total T4 concentrations in cetaceans tend to be higher than in most other species, although the concentrations in dolphins are comparable to humans.

2.3 Thyroid abnormalities found in marine mammals

2.3.1 Cetaceans

Cowan (1966) performed a systematic autopsy examination on 55 Pilot whales (*Globicephala melaena*) and found that the thyroid gland exhibited substantial variability in size and histology with the progression of age. Thyroid glands that were of similar weight had large microscopic differences. Some of the glands were noted to have nodular hyperplastic epithelium, with many macroscopic follicles containing solidified colloid as well as granulomata.

In 11 perinatal Atlantic bottlenose dolphins (*Tursiops truncatus*), Garner et al. (2002) used histopathy to diagnose diffused hyperplastic goiter. The authors observed reduced follicular luminal diameter, reduced or absent luminal colloid, follicular epithelium hypertrophy, and follicular dysplasia. According to the study, the goiter might have been attributed to an imbalance of maternal dietary iodine levels and an inherited dyshormonogenetic inability to synthesize or secrete adequate amounts of thyroid hormones leading up to the time of birth. The thyroid glands of the affected calves were also noticeably pale, with slightly nodular irregularities and less bilateral symmetry. Further study of bottlenose dolphins has been conducted and numerous pathologies, including adenoma, discrete hyperplastic nodules, macroscopically identifiable colloid-filled cysts, squamous cysts, patchy or diffuse interstitial fibrosis, amyloidosis, thyroiditis, and vasculitis were identified (Cowan and Tajima, 2006).

An evaluation of thyroid gland lesions was conducted on the beluga whale population (n = 30) at St. Lawrence estuary and Hudson Bay (Mikaelian et al., 2003). The study identified follicular cysts and nodules of adenomatous hyperplasia, which positively correlated with age. The cause of the thyroid disorders was

suggested to be endocrine disruption following polychlorinated biphenyl (PCB) exposure. Decreased plasma concentration of thyroid hormones was followed by a compensatory increase of TSH through the hypophysis, leading to follicular hyperplasia and follicular neoplastic transformation. Rolland (2000) found that neoplastic lesions, secondary bacterial infections and other pathologic lesions were prevalent in beluga whales. Post-mortem examinations of beluga whales revealed a number of thyroid abscesses and adenoma.

Das et al. (2006) investigated the relationship between PCBs, polybrominated diphenyl ethers (PBDE), dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT) compounds and inter-follicular fibrosis in the thyroid gland of the harbour porpoise (*Phocoena phocoena*). Connective tissue strands of various dimensions were found between follicles, and thyroid follicles were eventually replaced by the connective tissues. This observation was thought to be related to severely impair thyroid function. Schnitzler et al. (2008) performed histological and immunologic investigations on the thyroid glands of 36 harbour porpoises, and significant association between trace elements (cadmium, selenium, and copper) and thyroid fibrous was found.

2.3.2 Pinnipeds

Morphological changes in the thyroid gland have been associated with elevated tissue burden of persistent organochlorines (Rolland, 2000). Colloid depletion and inter-follicular fibrosis were observed among common seals (*Phoca vitulina*). In immature northern elephant seals (*Mirounga angustirostris)*, a skin disease known as generalized ulcerative dermatitis, was possibly inducedby a combination of PCB exposure and depressed levels of thyroid hormones and retinoid (Rolland, 2000). The affected animals had lower total T3 and total T4 when compared with the unaffected control group. PCB exposure was suggested to potentially underlie thyroid or retinol alternations and induce diseases. However, it remains unclear whether depressed retinol or thyroid hormone levels contribute directly towards the

etiology of the disease.

Organochlorine compounds have been shown to affect the thyroid hormone level (Sørmo et al., 2005). Plasma concentrations of T3 and T4 were examined in association with the concentration of various organochlorine pollutants in the blubber of the free-ranging, newly weaned gray seal pups. The total T3 and free T3 concentrations were significantly lower in polluted seals, and correlated negatively with blubber concentrations of analyzed PCB congeners and DDT compounds.

2.4 Methods of investigating thyroid physiology

2.4.1 Laboratory investigations

In the differential diagnosis of thyroid diseases, various examinations have been used; however each has its limitations (Hall et al., 1989; Foktin et. al, 2010). Thyroid function tests like serum thyroid hormone analyses are commonly used in the assessment of thyroid disorders. Serum thyroid hormone analysis is an indirect method to assess thyroid function by investigating the end-products of thyroid metabolism. A number of tests are needed in order to provide an overview of the thyroid function. Concentrations of free T3 and total T3, free T4 and total T4, as well as TSH should be evaluated for a comprehensive assessment. Although different types of thyroid hormone test kits are available, they have been developed exclusively for humans and may not be compatible for usage in other species due to a possible mismatch of corresponding antibodies. Development of a new assay specific to a particular species is costly and time consuming. Another important consideration in the evaluation of thyroid function is the degree to which thyroid hormones are bounded by circulating carrier proteins (Cunningham, 2002). However, efforts to demonstrate the binding protein concentration in belugas and bottlenose dolphins have proved unsuccessful using methodologies established for

other mammals (St. Aubin, 2001). Blood sampling itself is also an invasive procedure which involves needle puncture of the animal and may damage skin and blood vessels (Brook, 1997). Repeated needle puncture may also negatively affect the voluntary behaviour of the animals. Therefore this method cannot be used frequently to monitor thyroid physiology in live dolphins.

Thyroid function tests undoubtedly give valuable information about thyroid activity. Thyroid stimulation tests have been performed in the beluga whale and bottlenose dolphins, with marked differences in response to TSH, but the technique has yet to be refined (St. Aubin, 1988; St. Aubin, 2001; West and Ramer, 2005). Moreover, the morphology of the thyroid gland cannot be assessed with these tests.

2.4.2 Physical examination (palpation)

For years in human and animal medicine, palpation was the only method available for estimating the thyroid volume. In the evaluation of human thyroid volume, the common criterion is "when a thyroid gland whose lateral lobes have volume greater than the terminal phalanges of the thumbs of the person examined, it will be considered as goitrous". However, when this criterion is met, the thyroid volume is at least 4 to 5 times greater than normal. Clinical examination, by means of palpation and inspection, is a relatively insensitive and observer-dependent method for detecting any enlargement or nodularity of the thyroid gland (Sheikh et al., 2004). The accuracy of palpation in the assessment of thyroid disorders was only 60% (Nygaard et al., 2002). In companion animals, palpation was validated and considered as an important and reliable aid for diagnosing feline hyperthyoridism in an early stage to prevent development of deleterious complications (Paepe et al., 2008). In marine mammal medicine, the presence of blubber renders the palpation of the thyroid gland (Reidarson, 2003), which made the investigation of their thyroid gland impossible.

2.4.3 Diagnostic imaging

Extensive studies have been conducted in human medicine regarding the assessment of thyroid morphology using different imaging modalities, including nuclear medicine, computed tomography (CT), magnetic resonance imaging (MRI) and sonography (Ahuja, 2000; Bruneton et al., 2002; Hegedüs and Bennedbæk, 2005). However, there are only few reports on imaging assessment of thyroid gland in veterinary medicine, and they are focused on domestic mammals (Cartee et al., 1993; Breuhaus, 2002; Reese et al., 2005; Brömel et al., 2006).

In human medicine, nuclear medicine provides excellent functional information of the thyroid gland; CT and MRI provide important anatomical information in select clinical scenarios, especially in assessing advanced thyroid carcinomas, as well as in the evaluation of recurrent thyroid malignancy following thyroidectomy. Sonography remains the primary imaging modality for the assessment of thyroid diseases because high resolution ultrasound provides high quality images for the detection and characterization of diffuse and focal thyroid abnormalities (Ahuja, 2000; Bruneton et al., 2002; Hegedüs and Bennedbæk, 2005).

2.5 Diagnostic imaging assessment of the mammalian thyroid gland

2.5.1 Radionuclide imaging

Radioiodine is the radio-pharmaceutical commonly used for radionuclide imaging (RNI) assessment of the thyroid gland. The radioiodine uptake test provides a quantitative evaluation of the function of the thyroid gland. However, the examination is time consuming and involves ionising radiation, which provides a possible hazard to subjects. Moreover, subjects are required to keep very still during

the examination in order to avoid motion artifacts in the images. Therefore, RNI is not suitable for use in marine mammals as the animals cannot voluntarily remain still throughout the examination. The removal of subjects from the aquatic environment may also lead to risks such as respiratory compromise, psychosocial stress, and disturbances of thermoregulation and significant pre-existing diseases (Suzuki et al., 2008).

2.5.2 Computed tomography

Computed tomography (CT) provides cross-sectional images of the thyroid gland, which allows accurate localization of the thyroid gland and detection of thyroid masses. CT also helps in the assessment of tumour invasion on adjacent tissues, and aids treatment planning (Loevner et al., 2008). In addition, the accuracy of thyroid volume measurements based on CT is high (90-95%) (Hegedüs and Bennedbæk, 2005). Nevertheless, CT involves ionizing radiation, which poses an exposure hazard to the operators and subjects. Moreover, iodinated contrast medium is commonly used in CT examinations, which may lead to an allergic reaction on the subjects. Similar to RNI, CT examination may not be feasible for marine mammals as there are risks involved with taking an animal from its aquatic environment. The quality of CT images could be degraded by motion artifacts, as it may be difficult to keep the animals still during the CT examination.

2.5.3 Magnetic resonance imaging

Similar to CT, magnetic resonance imaging (MRI) generates cross-sectional images of the thyroid gland, aiding lesion localization and size measurement. However, MRI does not involve ionizing radiation and the contrast medium used is not iodinated, which reduces the risk of allergic reaction in the subjects. MRI also allows accurate estimation of thyroid volume with an overall accuracy of 90-95% (Hegedüs and Bennedbæk, 2005). However, MRI is not widely accessible as it is

expensive when compared to other imaging modalities such as CT and sonography (Andermann et al., 2007; Malago et al., 2008). Also, the long image-acquisition time of MRI examination does not favor its use in marine mammals as it is difficult to restrain the animal's movements for a long period of time during the examination. Moreover, MRI is costly and, once again, it is potentially harmful to remove the marine mammal from its aquatic environment.

2.5.4 Sonography

Sonography is commonly used in human medicine for the assessment of the thyroid gland. Grey scale ultrasound allows accurate estimation of thyroid volume, and provides comprehensive assessment of thyroid morphology. It also aids in the detection and characterization of diffuse and focal thyroid disorders (Shapiro, 2003; Hegedüs and Bennedbæk, 2005; Loevner et al., 2008). Furthermore, ultrasound is non-invasive, readily available, comparatively low cost and does not involve ionizing radiation (Andermann et al., 2007; Malago et al., 2008). Ultrasound can also help in guiding the fine needle aspiration procedure of sampling tissues from abnormal thyroid tissues for further cytological investigation.

Thyroid volume is 1 of the parameters used in the assessment of the thyroid gland. Enlargement of the thyroid gland may indicate thyroid pathologies. With the advancement of technology, sonography has been considered to be the most reliable method for thyroid volume estimation (Brunn et al., 1981; Hegedüs et al., 1983; Ueda, 1990). However, a clear understanding of the volume of normal thyroid glands is essential for an accurate diagnosis. Previous studies have revealed normative values for thyroid volume in humans of different sex, race, age, weight, health conditions, dietary conditions and the reproductive status of female subjects (Hegedüs et al., 1986; Loevner, 1996; Chan et al., 1998, 1999; Ying et al., 1998; Barraclough and Barraclough, 2000; Hess and Zimmermann, 2000; Zimmermann et al., 2000; Hegedüs, 2001; Zimmermann et al., 2001; Khati et al., 2003; Senchenkov and Staren, 2004; Sheikh et al., 2004). In veterinary medicine, there are only a few

studies reporting the normal thyroid volume and the sonographic characteristics of the normal thyroid gland in dogs, cats and horses (Cartee et al., 1993; Breuhaus, 2002; Reese et al., 2005; Brömel et al., 2006). To the best of our knowledge, the formal literature has scarce reference to the application of ultrasound in the assessment of the normal thyroid gland of any marine mammal species.

In human medicine, the volume of the thyroid gland is a useful clinical measure, particularly in the diagnosis of thyroid diseases and determining the appropriate dosage in radioiodine therapy. Serial ultrasound measurements of the diameters of thyroid gland have been proven to be useful in identifying thyroid diseases and monitoring treatment response. Assessment of thyroid gland diameters is usually performed by measuring the three dimensions of the thyroid gland (i.e. the craniocaudal, lateromedial and anteroposterior dimensions). However, it has been reported that thyroid volume is more accurate than thyroid gland diameters in the assessment of thyroid size. Ultrasound has been used to measure thyroid volume (Loevner, 1996; Ahuja and Metreweli, 2000; Hegedüs, 2001; AIUM, 2003; Senchenkov and Staren, 2004) and there was a good correlation observed between the thyroid volume measured with ultrasound and the actual thyroid volume, as well as the weight of the thyroid gland measured after autopsy (Needleman, 1990; Senchenkov and Staren, 2004).

With the availability of three-dimensional (3-D) ultrasound, thyroid volume can be measured in a 3-D matrix, improving accuracy. It has been reported that the thyroid volume measured with 3-D ultrasound has a high correlation with the volume of the surgical thyroid specimen (Andermann et al., 2007). Compared to two-dimensional (2-D) ultrasound, 3-D ultrasound has less measurement error and higher accuracy in thyroid volume measurement. However, 3-D ultrasound is not available in all facilities, and thus 2-D ultrasound is still commonly used in thyroid volume measurement (Rossi et al., 2002; Andermann et al., 2007; Ruggieri et al., 2008).

2.6 Sonography of the mammalian thyroid gland

2.6.1 Two-dimensional (2-D) ultrasound thyroid volume measurement

Two-dimensional ultrasound is commonly used to estimate the thyroid volume in humans. The volume estimation is based on the assumption that the thyroid lobes are ellipsoid in shape. With the use of 2-D ultrasound, the craniocaudal (CC), lateromedial (LM) and anteroposterior (AP) dimensions of each thyroid lobe are measured, and the thyroid volume is then calculated using the ellipsoid equation (Needleman, 1990):

Volume of a thyroid lobe = $\pi/6$ x (CC x LM x AP)

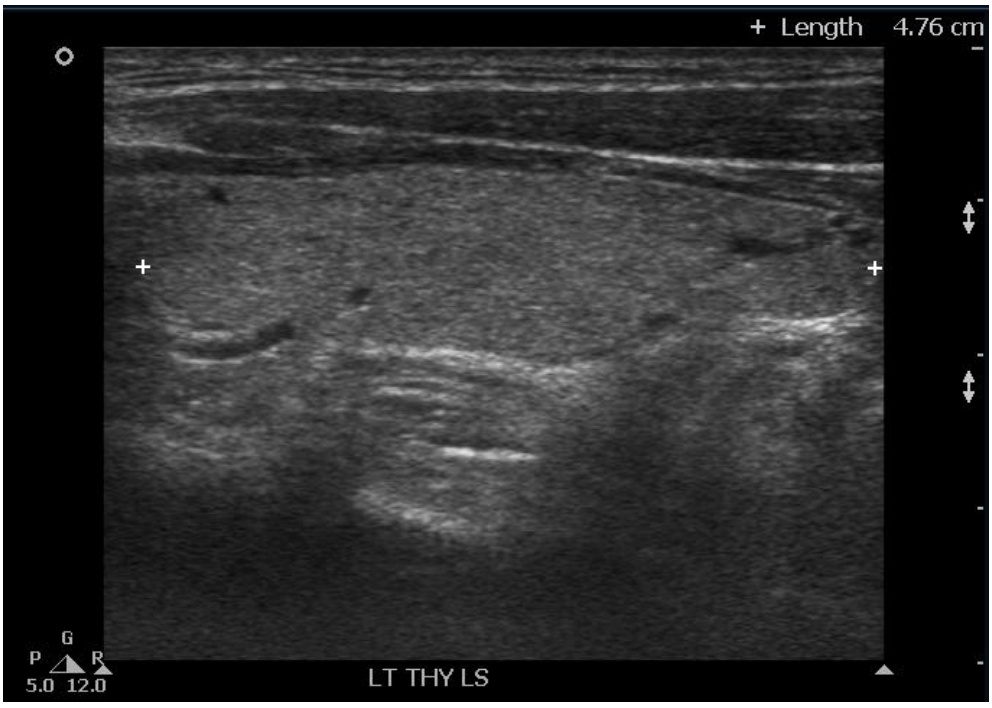

Figure 2.6: Longitudinal gray scale sonogram shows the measurement of the craniocaudal dimension (calipers) of the left lobe of a human thyroid gland.

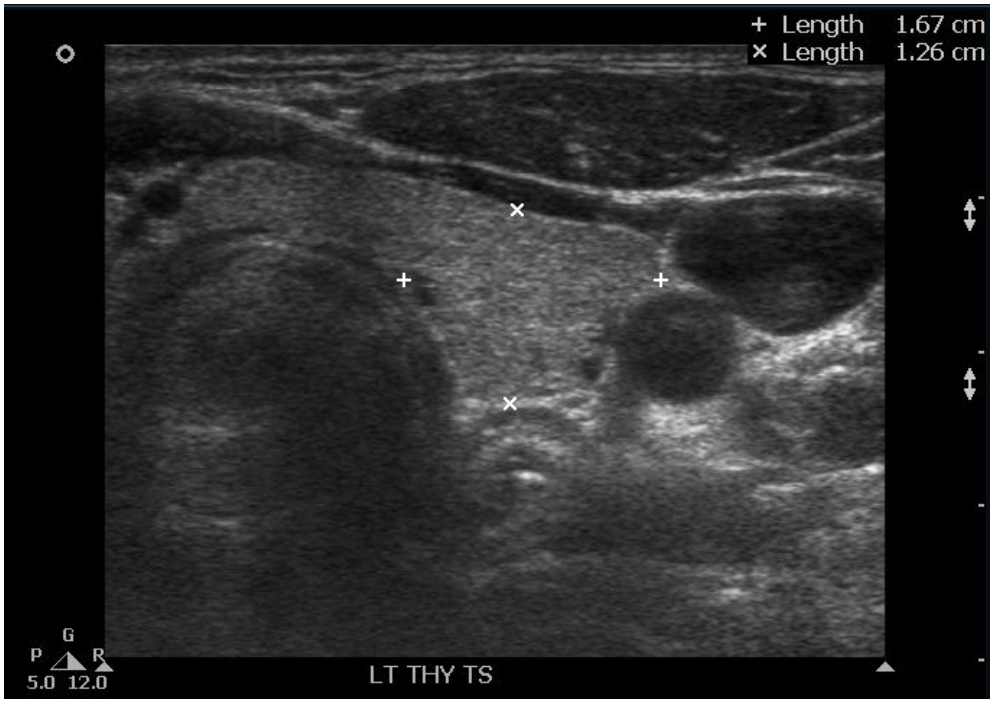

Figure 2.7: Transverse grey scale sonogram shows the measurements of the lateromedial (+) and anteroposterior (x) dimensions of the left lobe of a thyroid gland.

The accuracy and reliability of 2-D ultrasound measurement of thyroid volume are considerably high with a measurement accuracy of 80-85%, reproducibility of 85-87% and repeatability of 84.8% (Ozgen et al., 1999; Hegedüs, 2001; Ying et al., 2008). The inter-observer variation of 2-D ultrasound measurement of thyroid volume ranged from 6.6-17% (Ozgen et al., 1999; Nygaard et al., 2002; Andermann et al., 2007), while the intra-observer variation of the measurement ranged from 8.4 to 14.4% (Ozgen et al., 1999; Nygaard et al., 2002; Lyshchik et al., 2004b; Andermann et al., 2007). Although there was a good correlation (correlation coefficient, r = 70%) between the thyroid volume measured with 2-D ultrasound and the volume of surgical thyroid specimen, the measurement error of 2-D ultrasound ranged from 15% to 20% (Barraclough and Barraclough, 2000; Bruneton et al., 2002; Shabana et al., 2003; Miccoli et al., 2006). Some studies reported that the ellipsoid equation underestimated the thyroid volume as it excluded the isthmus

volume as well as for situations where there were irregular profiles of the thyroid gland or nodules within the thyroid gland (Malago et al., 2008; Ruggieri et al., 2008; Trimboli et al., 2008; Ying et al., 2008). Studies regarding the reliability of sonographic measurements of organs in marine mammals are scant (Taeymans et al., 2005; Yuen et al., 2009). Taeymans et al. (2005) investigated the repeatability of 2-D ultrasound volumetric measurement of dogs' thyroid glands, and low intra- (δ = 0.0041) and inter-observer variability (δ = 0.0019) were found in the measurements.

Another 2-D ultrasound measurement method is based on obtaining 1 or more transverse scans of the thyroid gland and then calculating its volume by the summation of the cross-sectional areas of the thyroid gland in the scan planes, multiplied by the length of the thyroid gland. This method had a smaller measurement error of 5% to 10% and was less influenced by the size and the irregular shape of the thyroid gland, but it involved a more cumbersome technique in outlining the borders of the thyroid gland in multiple transverse images (Barraclough and Barraclough, 2000; Khati et al., 2003; Shabana et al., 2003).

Although 2-D ultrasound and the ellipsoid equation are commonly used in estimating the thyroid volume, studies on human subjects have shown that this method is neither accurate nor reliable enough for a precise measurement of the thyroid volume (Szebeni and Beleznay, 1992; Ozgen et al., 1999). Previous studies have used different ways to improve the accuracy of 2-D ultrasound thyroid volume measurements. Some studies suggested adding a correction factor in the ellipsoid formula to increase the accuracy of the 2-D ultrasound thyroid volume measurement (Shabana et al., 2006; Ying et al., 2008). Other studies have suggested new mathematical formulas for a more accurate volume measurement (Shabana et al., 2003; Trimboli et al., 2008). Nonetheless, using advanced imaging techniques may be the desirable method to improve measurement accuracy.

2.6.2 Three-dimensional (3-D) sonography thyroid volume measurement

With the advancement of technology, 3-D ultrasound is readily available for more accurate and reliable measurements of thyroid volume (Riccabona et al., 1995; Vade et al., 1997). The thyroid volume measured with 3-D ultrasound has been found to be more accurate than that measured with 2-D ultrasound (Lyshchik et al., 2004a; Stephenson, 2005). Moreover, 3-D ultrasound (90% and 96.5% respectively) had higher reproducibility and repeatability than 2-D ultrasound (85% and 84.8% respectively) in thyroid volume measurements (Lyshchik et al., 2004b; Ying et al., 2005). 3-D ultrasound is more accurate in thyroid volume measurement because it outlines the area of interest in multiple image planes (Pang et al., 2006), whereas 2-D ultrasound is based on geometric assumptions and an idealized ellipsoid formula (Pang et al., 2006, Rousian et al., 2009).

Besides producing accurate and reliable volume measurements, multiplanar (MPR) views generated by 3-D reconstruction can display views that are difficult or impossible to obtain with 2-D imaging due to anatomical constraints. Another advantage of MPR views is the ability to view the anatomy from different simultaneous scan planes, providing accurate assessment of the spatial relationships between anatomical structures (Stephenson, 2005).

Other advantages of 3-D ultrasound include (Stephenson, 2005):

1. The acquisition of a single 3-D volume measurement, its subsequent reconstruction and viewing at a workstation, allows images to be acquired very quickly, freeing the remainder of the examination time to view selected areas under real-time 2-D scanning. Further manipulation of the acquired 3-D volume allows extraction of usable measurements and enhancement of the anatomic information in the images.
2. The volume acquisition of ultrasound data allows the operator to perform studies rapidly and provides offline review of the examination at a workstation.

The patient's volumetric data can also be stored for later evaluations and future comparisons.

3. The scanning protocol is more standardized than conventional 2-D ultrasound and is highly repeatable, which reduces operator dependence and increases the utility in having follow-up examinations.
4. The ability to add 3-D capabilities to existing 2-D ultrasound units, which is relatively inexpensive, should increase the availability of 3-D ultrasound in clinical settings.

2.6.3 Sonographic examination of the thyroid gland in humans

Ultrasound is a common imaging tool in the assessment of the human thyroid gland. According to the practice guideline issued by the American Institute of Ultrasound in Medicine (AIUM) in 2007, there are a number of indications for thyroid ultrasound such as evaluation of the presence, size and location of the thyroid gland, localization and characterization of palpable neck masses, evaluation of abnormalities detected by other imaging modalities or laboratory tests, evaluation for patients with increased risk of thyroid malignancies and follow-up of thyroid nodules.

Grey scale ultrasound assesses the morphology of thyroid gland and measures the thyroid volume. It could also help characterize and detect focal and diffuse abnormalities, as well as differentiating solid nodules from cysts in the thyroid glands (Hegedüs, 2001; Shapiro, 2003; Loevner et al., 2008). Colour or power Doppler ultrasound assesses the thyroid vascularity whereas spectral Doppler ultrasound evaluates the blood flow velocity and resistance within intra- and extra-thyroidal blood vessels. Ultrasound is also helpful in guiding fine-needle aspiration of thyroid nodules for tissue sampling and cytology (Hegedüs, 2001; Ota et al., 2006).

Due to the superficial location of the thyroid gland in the human neck, high

frequency (10-14MHz) linear transducers are commonly used that provide adequate ultrasound beam penetration and optimal image resolution (AIUM, 2003).

On high resolution ultrasound, normal human thyroid gland appears homogenous and is hyperechoic when compared to the adjacent muscles (Müller et al., 1985; Ying et al., 1998; Baskin et al., 2008; Loevner et al., 2008). In transverse scans of the human neck, besides the thyroid gland, other structures like the carotid arteries, internal jugular vein, sternocleidomastoid muscle, trachea, oesophagus and lymph nodes are also demonstrated. The normal limit of the LM and AP dimensions of thyroid lobe is 2 cm, while that of the CC dimension is 5.5 cm (Müller et al., 1985). The normal thyroid volume in adults ranged 5-20 cm^3 but it can be affected by the factors such as age, body weight, race and other physiological or environmental factors (Hegedüs, 2001).

Ultrasound is a useful imaging modality to identify and differentiate common thyroid pathologies such as malignancy, multinodular goiter and thyroiditis. Katagiri et al. (1994) reported that ultrasound had a specificity of 82%, a sensitivity of 78% and an overall accuracy of 80% in identifying thyroid malignancy. Benign nodules are usually characterized by hyperechogenicity, hypovascularity and lateral cervical lymphadenopathy, and the presence of a complete halo (Ahuja, 2000; Bruneton et al., 2002). In contrast, malignant nodules are suggested by the presence of microcalcifications and an incomplete halo, ill-defined margins, central hypervascularity and characteristic cervical lympadenopathy (presence of microcalcifications, hyperechogenicity, cystic necrosis and appeared round-shaped) (Ahuja, 2000; Bruneton et al., 2002). Ultrasound is also helpful in identifying multinodular goiter of the thyroid gland. Solbiati et al. (1995) reported that 70% of solitary lesions identified with scintigraphy were found to be multiple lesions with ultrasound. Tollin et al. (2000) found that the risk of cancer development for multiple and solitary nodules was similar and could be as high as 50%. Hence, ultrasound is useful in differentiating benign from malignant thyroid nodules.

Besides nodular thyroid disorders, ultrasound is also useful in the assessment of inflammatory and functional thyroid diseases such as thyroiditis, Graves' disease and hypothyroidism. Focal or diffuse hypoechogenicity and heterogeneous echopattern on grey scale ultrasound and increased vascularity on colour or power Doppler ultrasound are useful sonographic features in identifying thyroiditis (Ahuja, 2000; Bruneton et al., 2002). In Graves' disease, the thyroid gland is usually enlarged, hypoechoic and with increased vascularity (Barraclough and Barraclough, 2000; Bruneton et al., 2002). Hypothyroidism is characterized by the reduction of thyroid size, ill-defined borders and poor differentiation from adjacent structures (Ahuja, 2000; Bruneton et al., 2002). Although ultrasound may be helpful in identifying functional thyroid disorders, the extent of functional change of the thyroid gland cannot be pinpointed by ultrasound alone. Therefore, thyroid function tests assessing the levels of circulating thyroid-related hormones, in conjunction with ultrasound, are commonly used for a more accurate diagnosis.

2.6.4 Sonographic examination of the thyroid gland in domestic mammals

Ultrasound plays an important role in the assessment of thyroid gland morphology in domestic mammals. Although most studies have only concentrated on canine and feline thyroid glands, occasionally scarce attention has been directed to livestock such as cattle, horses and pigs due to their importance in agricultural settings.

Major indications for thyroid ultrasound examination in domestic mammals include evaluating the origin and location of the thyroid pathologies, and distinguishing clinically undifferentiated cervical masses that are often associated with thyroid malignancy (Senchenkov and Staren, 2004). Ultrasound also aids in the procurement of fine-needle aspiration or core-needle biopsy for cytological and histological analysis of thyroid masses. Thyroid tumors are relatively common in dogs and account for 1.2 to 3.8% of all canine tumors (Liptak, 2007). Rantanen (1998) reported that thyroid carcinoma is rare in equines, and cervical masses are

mainly attributed from other thyroid diseases. Thyroid carcinomas can be aggressive and invade surrounding soft tissues and blood vessels. Metastasis to regional lymph nodes is also common for thyroid carcinomas. Because of the aggressiveness of thyroid tumors, findings from the ultrasound examination may aid treatment planning of these animals (Rantanen, 1998).

Some other indications for thyroid gland examination, particularly in cats, include examination of the thyroid glands of hyperthyroid cats with thyroid adenomatous hyperplasia. In these cases, ultrasound may be useful in differentiating bilateral and unilateral diseases, and be useful in determining whether the animal would be more appropriately treated with unilateral thyroidectomy, or iodine therapy for bilateral diseases (Wisner and Nyland, 1998).

Hyperthyroidism is the most common endocrine disorder in cats, while hypothyroidism is more common in dogs (Ferguson, 2007; Mooney and Shiel, 2008; Taeymans et al., 2007). Hypothyroidism in neonatal horses may cause goitre and is pathognomonic, but this disease is usually investigated by thyroid function test and by monitoring the response to treatment (Frank et al., 2002). On ultrasound, canine hypothyroidism can lead to a decrease in thyroid volume along with a decrease in the echogenicity of the thyroid gland (Ferguson, 2007; Taeymans et al., 2007). Therefore, thyroid volume measurements could be beneficial in monitoring the progression of disease as well as the response of therapy. It has been reported that a combination of different investigation is required for the evaluation of thyroid function, including clinical history, clinical signs, and results of various laboratory tests; published data has revealed that thyroid ultrasound is an effective ancillary diagnostic tool to differentiate canine hypothyroidism and euthyroid sick syndrome (Reese et al., 2005).

In the ultrasound examination of the thyroid gland in domestic mammals, high resolution ultrasound (with the use of a 7.5 to 10 MHz transducer) is used. Even in large canines, the depth of the thyroid gland from the skin surface rarely exceeds 4

cm (Wisner et al., 2002) which is accessible with high resolution ultrasound.

Feline thyroid ultrasound is considered to be technically demanding and operator dependent. However, Cartee et al. (1993) and Wisner et al. (1994) evaluated the feline thyroid gland by ultrasound and concluded that the technique was feasible. On high resolution ultrasound, the feline thyroid gland appeared as hypoechoic oval structures. Each thyroid lobe measured approximately 2 cm long and 0.2 to 0.3 cm thick (Wisner et al., 2002). However, lack of advanced ultrasound imaging technology at that time resulted in poor visualization of the sonographic margin of the thyroid gland under *in vivo* conditions as well as poor visualization of physical changes after detachment of the thyroid gland from adjacent fascia during thyroidectomy, leading to the inaccurate measurement of thyroid size.

The ultrasound scanning technique and the sonographic appearance of the thyroid gland have been described in healthy dogs (Wisner and Nyland, 1998). Wisner et al. (2002) described the sonographic features of normal thyroid gland in canines. The thyroid gland appeared homogeneous, well-defined and fusiform in shape. The echogenicity of the thyroid gland is lower than that of the surrounding adventitia but greater than that of the cervical musculature. Each thyroid lobe measured approximately 2.5 to 3 cm long and 0.4 to 0.6 cm thick in medium-sized dogs. However, due to high inter-operator variability in the study, only the thyroid thickness and volume seem to be reliable in evaluating the thyroid size in dogs (Taeymans et al., 2005).

Recently, efforts have been made to establish baselines for the sonographic evaluation of different sizes of dog breeds. Brömel et al. (2006) documented the sonographic features of the thyroid gland in healthy small-, medium-, and large-breed dogs as well as the association of thyroid size with body weight and body surface area (BSA). Results showed that thyroid size was more variable than the shape, echogenicity and homogeneity of the thyroid gland. The high correlation between thyroid volume and BSA ($r = 0.74$) suggested that the size of the dog,

rather than breed, should be considered when assessing the thyroid gland sonographically.

With the recent improvements in ultrasound technology and increased availability of high-resolution transducers, ultrasound imaging of the ventral neck structures in domestic mammals is becoming popular, and the application of neck ultrasound have been expanded to different species of mammals while the diagnostic accuracy has been improved.

Primary hypothyroidism is a common endocrine disease in dogs. Destruction of the thyroid gland is usually the result of immune-medicated lymphocytic thyroiditis or idiopathic thyroid gland atrophy. Thyroid ultrasound was used as a diagnostic tool in the assessment of primary hypothyroidism and in the differentiation between euthyroid sick syndrome and primary hypothyroidism in dogs with non-thyroidal illness (Brömel et al., 2005; Reese et al., 2005; Taeymans et al., 2007). Reported ultrasound features in cases of primary hypothyroidism included hypoechoic parenchyma compared to the overlying sternothyroid muscle, heterogeneous thyroid parenchyma. Other sonographic features included an irregular outline of the thyroid lobe, decrease in thyroid size, and a more rounded contour of the thyroid lobe on transverse images (Brömel et al., 2005). One or several of these changes might be present at the same time, and the sonographic features might also differ between the left and right lobes. Taeymans et al. (2007) studied the pre- and post-treatment changes of the sonographic features of the thyroid in hypothyroid dogs, and found that a continuous decrease of thyroid volume was seen over time after treatment, while the other investigated parameters, such as echogenicity, homogeneity and capsule delineation did not change significantly during the follow-up period.

Doppler and contrast-enhanced ultrasound may be useful to determine the vascularisation and perfusion of thyroid masses (Wisner and Nyland, 1998). The common sites of metastases from thyroid carcinoma are the lungs and retropharyngeal lymph nodes. Sonography is a useful and non-invasive imaging tool

in the examination or preoperative localization of the cervical vagosympathetic trunk, which was described in dogs as a heterogeneous structure with anechoic area separated by hypoechoic bands (Reese and Ruppert, 2001).

Efforts have also been made on the assessment of the diagnostic accuracy of parathyroid gland sonography, which was used to differentiate causes of hypercalcemia in dogs and it was concluded that ultrasound is accurate in estimating parathyroid size (Wisner et al., 1997). The sonographic features of all visible parathyroid glands were similar that were round or oval in shape, well-defined and anechoic or hypoechoic as compared to the surrounding thyroid parenchyma. Some parathyroid glands also showed acoustic enhancements, especially when the parathyroid gland was large (Wisner et al., 1997).

Among the research on livestock, Braun et al. (1994) examined the left and right ventral neck regions of 30 healthy Swiss Brauvieh cows sonographically and documented the position, dimensions and morphology of the thyroid gland as well as the adjacent neck structures. The thyroid gland in cattle can be clearly identified as it is located caudal to the larynx and lateral to the trachea, and appeared as an echogenic spindle-shaped structure with a finely granular echogenic pattern on ultrasound. In the transverse plane, the thyroid gland was shown as a homogeneous, spindle-shaped structure mottled with echogenic areas of different sizes. The thyroid gland was well-defined and more echogenic than the adjacent muscles. In the transverse scans, the isthmus was seen as a narrow band running over the trachea.

The equine thyroid gland is a superficial structure found at the level of the larynx and can be visualized sonographically. It is comprised of 2 lobes that may be connected by a thin layer of connective tissue in the adult. The average thyroid gland weighs about 15 g per lobe and had a length, width and thickness of 5 cm x 2.5 cm x 2 cm. The normal thyroid gland was characterized with a homogeneous, fine “stippled” echotexture. In the transverse scans, the carotid artery and the laryngeal wall can be seen contacting the thyroid gland (Rantanen, 1998).

2.7 Effects of demographic parameters on thyroid physiology

2.7.1 Age

Delange and Fisher (1995) reported that production of TRH is regulated by environmental temperature through peripheral and hypothalamic thermal receptors. Following birth, the lower environmental temperature compared to that within the mother's body stimulates the production of TRH, which then stimulates the secretion of TSH. At this point, the thyroid gland is considered to be relatively hyperactive compared to an adult's (Fisher and Polk, 1995). However, during the first week of life, serum TSH concentrations steadily decline as a result of negative feedback mechanisms from the sufficient secretion of thyroid hormones (Foktin et. al, 2010). Serum concentrations of TBG, Tg, T4 and T3 also increase after birth, but gradually decrease over the next decade of life (Fisher and Polk, 1995). Maternal estrogens are thought to be the cause of increased TBG level at birth, and decreasing TBG is partly responsible for the decrease in serum T3 and T4 levels as a result of decreasing TSH level after birth (Delange and Fisher, 1995).

Kaloumenou et al. (2007) found that thyroid volume increased significantly with advancing age, remarking that the pubertal process was a significant determinant of thyroid volume, related to hormonal and body changes. Furthermore, the serum concentration of different thyroid hormones was the highest in the first month of age, which then decreased with advancing age (Kapelari et al., 2008). Yamada et al. (1984) found a similar result for T3, but a constant concentration for T4 and an increased concentration for basal serum TSH.

Previous findings of thyroid hormone levels in the elderly were observed. Some studies reported that the basal and TRH-induced TSH secretion decreased in the

elderly (Urban, 1992; Monzani et al., 1996). However, other studies showed that there was no significant effect of age found on the serum level of TSH in the elderly (Hegedüs et al., 1983; Sundbeck et al., 1991). In addition, there is no apparent effect of age on the serum level of Tg in the elderly; however, serum levels of T3 and T4 were observed to decline with the progression of age (Urban, 1992; Kowal and Cheng, 1994). Some other studies also found a decreasing serum T3 level in the elderly, while the serum T4 concentration is almost constant in this group of individuals (Hegedüs et al., 1983; Jacques et al., 1987). The age-related variations of thyroid hormone secretion in the elderly are suggested to be associated with chronic illnesses, medication usage, and nutritional deficiencies (Cizza et al., 1992). This may also account for the inconsistent findings of thyroid size in previous reports in which Gönczi et al. (1994) observed a decrease of thyroid size in the elderly, whereas Hintze et al. (1991) found an increase of thyroid size.

In experimental animals, Jacobs (1958) found that thyroid epithelial heights and follicle size increased with advancing age in mice, indicating decreased thyroid function with the progression of age. A study using male Wistar rats also found similar age-related changes in the structure of thyroid follicular cells and the thyroid function (Kmiec et al., 1998). Comparitively, in humans, younger people had smaller follicles and higher expression of proteins involved in iodine transport than the elderly, indicating the higher thyroid function and higher thyroid cell proliferation in the young age (Faggiano et al., 2004).

In domestic mammals, Kallfelz and Erali (1973) evaluated the serum thyroid hormone level of various species at different ages. For dogs and swine, serum T4 concentrations decreased significantly with age. T3 levels decreased with age in sheep and goats. Leyva-Ocariz et al. (1997) also found a significant decrease of T3 concentration in Carora heifers during the onset of puberty. Greco (2006) reported that puppies had a lower T3 concentration, but a higher T4, than adult dogs.

In marine mammals, phocids are reported to have a functional thyroid gland during

mid- to late-term fetal development (Harrison, 1969). At this stage of development, follicle formation was rapid, and the thyroid gland was well permeated by blood vessels. Myers et al. (2006) reported the thyroid hormone concentrations in Steller sea lions (*Eumetopias jubatus*) of different ages, and found that the concentration of free T4, total T4, free T3 and total T3 decreased as the animals matured and beyond the neonatal stage. In bottlenose dolphins, St. Aubin et al. (1996) found that the free T4 declined with age in wild dolphins whereas the reverse T3 was increased in older subjects. Rosa et al. (2007) assessed the thyroid glands of bowhead whales, and found that age did not significantly affect the total T4, free T4, total T3 and free T3 serum concentrations. However, in comparison to the adults, the juvenile whales presented an active thyroid histological appearance, suggesting a different mechanism in conversion, binding or excretion of thyroid hormones among different age groups.

Thyroid size varies with advancing age. In humans, Hegedüs et al. (1983) found that thyroid volume positively correlated with age, which may be due to the increased serum TSH levels with advancing age and also a lower iodine intake (demonstrated by falling daily urinary excretion of iodine), leading to augmentation of TSH doses. Lee et al. (2006) found that thyroid volume increased with advancing age until the fourth decade, after which it began to decrease.

Body weight may also be a factor affecting thyroid volume. Barrere et al. (2000) found that the smaller thyroid volume in older females is probably due to the decreased lean body mass in the elderly who have a reduced metabolism and thus require less thyroid hormone for regular function. It has also been suggested that body weight has also been suggested to be an important factor in thyroid volume variations (Wesche et al., 1998; Gomez et al., 2000; Harjeet et al., 2004).

In marine mammals, Turner et al. (2006) performed necropsies on 63 bottlenose dolphins and reported no significant correlation of thyroid weight with age. Morphologically, the dolphin thyroid gland was compact and homogeneous during

infancy and tended to become lobular with advancing age (Cowan and Tajima, 2006). With further histological investigation, the study also found that the variability of thyroid follicle size and colloid density tended to increase with advancing age.

2.7.2 Sex

Previous literatures regarding the influence of sex on the thyroid gland were focused on the human population. Thyroid volume in females tends to be smaller than that in males (Hegedüs et al., 1983; Berghout et al., 1987; Hintze et al., 1991; Hsiao and Chang, 1994). It has been suggested that the difference in thyroid volume between males and females is related to the difference of their physique, rather than a difference in the hormonal environment (Hegedüs et al., 1983; Berghout et al., 1987; Hintze et al., 1991; Hsiao and Chang, 1994). Previous studies showed that although there was no significant difference in thyroid volume to body weight ratio between males and females, the volume of the thyroid gland was found to positively correlate with the body weight in both sexes (Hegedüs et al., 1983; Berghout et al., 1987; Hsiao and Chang, 1994).

The mean thyroid volume of people in Denmark (Hegedüs et al., 1983) and Amsterdam (Berghout et al., 1987) is notably larger than the people in Taiwan (Hsiao and Chang, 1994) and Japan (Yamaguchi et al., 1990). Daily iodine intake was suggested to contribute to the difference in thyroid volume between people of different geographical areas (Hegedüs et al., 1983). A low iodine intake reduces thyroid hormone secretion and exerts feedback on the anterior pituitary gland, stimulating the secretion of more TSH. Increased TSH level then stimulates the thyroid gland to produce T3 and T4. Besides iodine deficiency, it has also been suggested that the difference in body size may also account for the difference in thyroid volume between Asians and Europeans (Hsiao and Chang, 1994).

Previous literature regarding the influence of sex in marine mammals was scarce. St.

Aubin et al. (1996) investigated the effect of sex, as well as other demographic parameters in the Atlantic bottlenose dolphins (*Tursiops truncatus*). Sex was found to have the most consistent influence on thyroid hormone levels, with higher total T4, free T4 and free T3 levels observed in females wild *Tursiops*, whereas higher total T3 level was found in semi-domesticated female *Tursiops*. In contrast, sex did not affect the serum total T4, free T4, total T3 and free T3 concentrations of the bowhead whales (Rosa et al., 2007).

2.7.3 Body size

In humans, the volume of the thyroid gland was measured *in vivo* in normal subjects and its correlation with body weight, age, and sex was evaluated (Hegedüs et al., 1983). Body weight was significantly and positively correlated with thyroid volume, and its influence was calculated to be 3 times that of age, however, the authors did not explain the findings.

Previous findings in investigating the correlation between body mass and thyroid level were controversial. Edén et al. (1984) studied a population of healthy elderly males, and found that there was a significant negative correlation between body mass and body mass index with the free T4, total T4, and reverse T3. Those with a higher body mass had lower levels of these hormones. However, there was no significant correlation between body mass and TSH or T3. For these findings, the authors suspected factors such as food consumption, degree of physical activity and temperature-control mechanisms. Knudsen et al. (2005) investigated the association between thyroid function and body mass index in a normal population, and found a positive association between body mass index and serum TSH, but found a negative association between body mass index and serum free T4. They found that there was no association between body mass index and serum free T3 levels. Obesity was defined as having a body mass index over 30 and there was an association between obesity and serum TSH levels (Knudsen et al., 2005). Moreover, it has been found that morbidly obese subjects had higher levels of T3, free T3, T4, and TSH

(Buscemi et al., 1997; Michalaki et al., 2006). However, another study found no association between serum TSH or free T4 and body mass index in euthyroid subjects (Manji et al., 2006). In contrast, Fox et al. (2008) found that the TSH concentration was positively associated with weight gain in both men and women. The authors suggested that modest increases in TSH concentration within the normal range may be associated with weight gain rather than pathologies.

In obese children, serum concentrations of thyroid hormones were analyzed before and after weight loss (Reinehr and Andler, 2002). TSH, T3, and T4 were found to be significantly higher in obese children when compared to children of normal weight. Weight reduction led to a significant decrease in T3 and T4, but not in TSH. The authors suspected hormonal resistance as the cause for the increased thyroid hormone concentrations, in a manner similar to obesity-related insulin resistance.

Wesche et al. (1998) investigated the possibility of lean body mass acting as a better determinant of thyroid volume than body weight. Thyroid volume was found to be larger in obese subjects than in non-obese subjects. For non-obese subjects, thyroid volume was significantly correlated to both body weight and lean body mass. However, thyroid volume was not correlated with body weight in obese subjects, although it still significantly correlated with their lean body mass. In obese subjects, the larger thyroid volume was also significantly associated with higher serum TSH and lower free T4 concentrations. In addition, Wesche and Wiersigna (2001) evaluated whether the changes of body composition and lean body mass from physical training led to changes in thyroid volume. In the group subjected to a 6-month intensive physical training, their body weight, lean body mass, fat weight, body mass index and thyroid volume were all decreased. Thus, the changes in thyroid volume correlated directly with changes in body composition and lean body mass.

An investigation of the relationship between thyroid volume and body composition was conducted on schoolchildren between 11 to 15 years of age (Boyanov et al.,

2004). In both boys and girls, thyroid volume was better correlated with their height, weight and body surface area than with their body mass index. In addition, thyroid volume significantly correlated with fat-free mass, while there was no significant correlation with body fat. In Boyanov et al. (2004), thyroid volume was concluded to be dependent on body size (measured from fat-free mass but not fat mass) and therefore it was dependent on growth variables. The main determinants of thyroid volume in schoolchildren living in an iodine-replete area have also been investigated (Kaloumenou et al., 2007). Thyroid volume was significantly correlated with body surface area in both boys and girls.

Eftekhari et al. (2007) examined the relationship between thyroid function and body mass index in adolescent girls. TSH, T4 and reverse T3 were found to correlate with body mass index. Subjects having a body mass index greater than or equal to 25 showed higher serum TSH, T4, and reverse T3. Despite the fact that thyroid function was normal in the subjects, a positive correlation in TSH and reverse T3 was found for body mass index, suggesting that TSH and reverse T3 could serve as markers of altered energy balance in overweight and obese adolescent girls. In a similar study, thyroid hormones were analyzed in female adolescents with obesity and anorexia nervosa before and after normalization of weight (Reinehr et al., 2008). For girls with anorexia nervosa, TSH and free T3 levels were significantly lower compared with girls of normal weight, while TSH and free T3 levels of obese girls were significantly higher. Obese subjects experiencing a weight loss of greater than 5% showed a significant decrease in TSH and free T3, and the subjects with anorexia nervosa having a weight gain of greater than 5% also showed a significant increase in TSH and free T3. Therefore, the study suggested that thyroid function appeared to be correlated with the weight status with increased TSH and free T3 concentrations in obesity and decreased TSH and free T3 concentrations in anorexia nervosa. Leptin was suggested as a possible link between weight status and TSH.

In female adults, Sari et al. (2003) examined the effect of body weight and weight loss on thyroid volume and function in obese women. The authors found a positive

correlation between thyroid volume and body weight, body mass index, body fat percentage, and body fat weight. A positive correlation was also found between TSH concentration and body weight and body fat weight. In a follow-up examination after 6 months of obesity treatment, the thyroid volume and TSH concentration were found to be significantly lower only in obese woman who lost over 10% of body weight. In another study, obese euthyroid women had lower free T4 levels compared to lean euthyroid women (Shon et al., 2008). Free T4 was found to be significantly and negatively correlated with body mass index after adjusting for age and smoking. However, no association was found between TSH and body mass index. These findings raised the suggestion that the low free T4 levels were associated with obese euthyroid individuals. A study of healthy premenopausal women between the ages of 42-50 also found no longitudinal changes in the TSH concentration and no association between changes in TSH and lipoproteins or body mass index (Massoudi et al., 1997). The authors concluded that healthy women could be assumed to only have slight alterations in thyroid function measures during menopause and that they had minimal effects on lipid and body mass index changes.

In a noniodine-deficient area, a study was undertaken to investigate the relationship between thyroid volume and anthropometric characteristics (Gomez et al., 2000). The study found significant correlations between thyroid volume and body weight, height, body mass index, waist-hip ratio, body surface area, total body water, free fat mass, fat mass and body fat. Multiple regression analysis with thyroid volume as the dependent variables revealed that body surface area accounted for 44% of the variation in thyroid volume, whereas other variables did not significantly affect the thyroid volume. In another study, bioelectrical impedance of the body was found to be strongly associated with the thyroid function in healthy subjects (Sartorio et al., 2002). Body resistance was the best single predictor of TSH and the authors attributed this to its direct relationship with fat-free tissues (and consequently with TSH), although the authors have noted that this relationship needs to be evaluated in under- and over-weight subjects in further studies.

In domestic mammals, changes in body mass and serum thyroid hormones were found in early weaned goat kids (Colina et al., 1993). After birth, the body weight of the goat kids increased significantly, whereas the thyroid hormones significantly decreased until the 14th day, after which an increase of the thyroid hormones was found. The authors noted a decrease in the extrathyroidal conversion of T4 to T3, possibly serving as an adaptive mechanism to protect against excessive losses of metabolically active muscle tissue.

Brömel et al. (2006) conducted a study consisting of healthy small-, medium- and large-breed dogs and sought to evaluate the relationship of thyroid volume with body weight and body surface area. Total thyroid volume correlated positively with both body weight and body surface area, and the author suggested that size of the dog, rather than breed, ought to be considered in the sonographic assessment of dogs' thyroid glands.

In dolphins, positive and significant correlations were demonstrated between thyroid weight, standard body length and body mass (Turner et al., 2006). Cowan and Tajima (2006) examined fresh thyroid glands from 60 Atlantic bottlenose dolphins and determined the effect of increasing body size on the mean thyroid weight to body length index and the mean thyroid weight to body weight index. The authors suggested that the thyroid weight lags behind as the animal increases in size, but remains constant in relation to body length throughout life.

2.7.4 Sexual maturity

In humans, thyroid volume measurement by sonography was conducted in a population of children aged between 6 – 16 years (Tajtáková et al., 1990). Thyroid volume increased slowly from the ages of 6 – 12 years, but a more remarkable increase at 13 and 14 years of age. Irrespective of body weight, the thyroid volume at 15 – 16 years of age was nearly double compared to values at 13 – 14 years of age. Thyroid growth rate was found to be significantly higher in girls than in boys.

In another study, thyroid volume increased significantly with increasing age from 4 – 9 years old in prepubertal children (Boas et al., 2009). Thyroid volume has also been shown to increase with the progression of puberty in both boys and girls (Kaloumenou et al., 2007).

2.8 Effects of reproductive status on thyroid physiology

2.8.1 Regular ovarian cycling

Cyclical variations in TSH and thyroid function were found to be correlated with the estrous cycle in females of different species (Boccabella and Alger, 1967). In humans, previous studies reported that serum T3, T4 and TSH did not alter significantly during the normal menstrual cycle (De Remigis et al., 1990; Hegedüs, 1990). However, Rasmussen et al. (1989b) found that the median serum TSH and Tg levels were significantly higher on day 23 compared with day 2 of the same menstrual cycle. A positive correlation was also found between serum Tg level and thyroid volume as measured by ultrasound (Rasmussen et al., 1989a). Moreover, a recent study showed that the cyclical changes in TSH and Tg levels are correlated to the menstrual cycle in females, whilst TSH and Tg levels are relatively constant in males (Glinoer, 2005). Although the mechanism of the TSH and Tg variations during a menstrual cycle remains unclear, the parallel increase in serum Tg and TSH levels, as well as thyroid volume, suggested possible thyroid gland stimulation by the female hormones.

Rasmussen et al. (1989a) found that the significant change of thyroid volume in women during the normal menstrual cycle, which was likely due to the stimulation by estrogen (De Remigis et al., 1990; Hegedüs, 1990). The stimulation might also be related to the peak incidence of thyroid disorders in females of reproductive age (Foktin et al., 2010). In Hegedüs (1990), the mean thyroid volume was significantly

larger on day 23 of the menstrual cycle, compared with day 9. However, in another study, the thyroid volume on day 14 was significantly larger than that on day 28 (De Remigis et al., 1990). Hegedüs (1990) also found that there was no obvious pattern of thyroid volume variation over time in a longitudinal study of men. According to Hegedüs (1990), the cyclical alteration of thyroid volume is possibly due to the altered intra-thyroidal vascularity. However, demonstration of TSH and Tg variations during the menstrual cycle have prompted Rasmussen et al. (1989a) to suggest that the changing thyroid volume might reflect the presence of an unknown thyroid stimulation, in addition to the altered vascularity of the thyroid gland.

2.8.2 Pregnancy and lactation

In humans, there is an increase in total T4 and T3 during pregnancy, due to the increase in serum TBG. Increased plasma concentration of TBG, along with increased plasma volume, results in a several-fold increase in the thyroxine pool during pregnancy. TBG synthesis is stimulated by estrogen (Glinoer et al., 1977). Weeke et al. (1982) measured the serum thyroid hormone and TSH level throughout the period of normal pregnancy in women. Serum T4 and T3 levels increased near the end of the 1st trimester and remained at high levels in the 2nd and 3rd trimester of gestation. Free T4 and T3 levels were slightly elevated in early pregnancy, and steadily decreased thereafter. TSH levels steadily increased throughout pregnancy. Reverse T3 levels elevated near the end of the 1st trimester and remained at high levels thereafter. The high level of serum thyroid hormones reflects the increased circulation of TBG during normal pregnancy.

In humans, increasing levels of serum T4 during pregnancy have been reported by Nasr et al. (1982), although serum T3 levels remain constant. Mandel et al. (1990) also found an increased level of serum T4 during pregnancy. The requirements of thyroxine are markedly enhanced during pregnancy in hypothyroid women, indicating that T4 degradation is decreased during early pregnancy and increased T4 production must occur throughout gestation. An increase of 30% - 50% in T4

production during gestation is widely accepted (Glinoer, 1997). In normal pregnancy, the stimulatory effect of human chorionic gonadotropin (hCG) on the thyroid gland induces a small and transient increase in free T4 near the end of the 1st trimester (Glinoer, 1993). Stimulation of the thyroid gland by hCG can be explained by the marked homology that exists between hCG and TSH molecules (Vassart and Dumont, 1992).

Infertile women with normal basal and stimulated TSH levels and high T4 levels were found to have the highest overall and spontaneous pregnancy rates (Gerhard et al., 1991). Pregnancy rate was the lowest in women with high stimulated TSH and low T4 levels. Women with high TBG experienced the highest delivery rate, whereas early and late abortions as well as premature deliveries were frequent among women with low TBG concentrations.

Rasmussen et al. (1989a) found that thyroid volume increased during pregnancy. The maximum thyroid volume was found at the 36^{th} week of pregnancy while the minimum thyroid volume was noted at 12 months postpartum. Thyroid volume has also been shown to be higher in women who have been pregnant compared to those who have not (Hansen et al., 2004). As the gonadal sex hormone level varied significantly during pregnancy, this was thought to be the cause of variations of thyroid volume.

The placenta has a high concentration of D3, the type 3 iodothyronine deiodinase (Roti et al., 1981). Inner ring deiodination of T4 by D3 is responsible for the high concentration of reverse T3 found in amniotic fluid. T4 deiodination may also serve to provide a source of iodine for the fetus. However, D3 may reduce the T3 and T4 concentrations in the fetal circulation. Thus, maternal thyroid hormones may represent an important source in the adequate development of the fetomaternal system (Burrow et al., 1994).

The degree of iodine intake has been reported to affect thyroid volume. For

individuals with iodine deficiency, their thyroid volume increased by 20% to 35% on average (Glinoer, 2003). The average thyroid volume of newborns from women without iodine supplement was 40% larger than those from women with iodine supplement (Glinoer, 2003). Besides the reduction of thyroid volume, the thyroid hormone level also decreased with iodine deficiency. It has been reported that in the serum free T4 concentration was progressive reduced during pregnancy in women (Elnagar et al., 1998).

Azizi (2007) measured the urinary iodine concentration of pregnant and lactating women, and found that 51% of the pregnant women had urinary iodine concentration lower than the normal limit. In lactating mothers, although the median urinary iodine concentration indicated sufficient iodine consumption of the mothers, the concentration of breast milk was inadequate in some cases (19% of the mothers had low iodine concentration in their breast milk). The study highlighted the possible alterations in the thyroid physiology leading to iodine deficiency in pregnant and lactating women.

In domestic mammals, Reimers et al. (1984) found that the serum T4 level in pregnant adult Beagle subjects was similar to that in diestrous subjects, but greater than the level found in subjects at other reproductive states. However, it was found that the T3 levels did not differ significantly between the pregnant dogs and non-pregnant dogs (Reimers et al., 1984). Thyroid function did not appear to be associated with pregnancy in broodmares; there was no significant difference in baseline and stimulated serum T3 and T4 concentrations between pregnant and non-pregnant mares (Meredith and Dobrinski 2004).

In dolphins, St. Aubin et al. (1996) found significantly higher levels of total T4, free T4, and free T3 in wild females than males, an effect possibly related to reproduction and lactation. Additional findings regarding the thyroid morphology during pregnancy were reported by Cowan and Tajima (2006), in which the authors found that the thyroid gland in pregnant and lactating females was larger than that in

non-pregnant animals. However, in baleen whales, pregnant or lactating female bowhead whales had significantly lower thyroid hormone concentrations when compared to males, as well as other female reproductive groups investigated (Rosa et al., 2007).

Glinoer et al. (1992) investigated the reversibility of pregnancy-associated thyroidal alterations during late postpartum. Six months after giving birth, the thyroid function resumed to normal. When observed during pregnancy, the increase in the T3/T4 ratio was still evident after 6 months postpartum. At 12 months after delivery, thyroid volume had not reverted to the value found in early gestation, and had increased by 54% on average. The findings of the study may indicate that pregnancy is a prolonged stimulatory effect on the thyroid and the alterations persist well after postpartum (Glinoer et al., 1992).

2.8.3 Under contraceptives

In humans, women taking oral contraceptives consisting of a combination of estrogens and progestational steroids were reported to have a greater TSH response to TRH (Ramey et al., 1975). It was suggested that estrogen may exert an effect on the hypothalamic-pituitary-thyroid axis. Estrogen has also been observed to increase the amount of TRH receptors in the anterior pituitary, thereby increasing pituitary sensitivity to TRH (Greenspan, 1994; Finke et al., 1996). This may account for the apparent effect of estrogen on the thyroid gland of adult women. Weeke and Hansen (1975) examined the serum TSH and serum T3 levels in women taking oral contraceptives consisting of progestin and estrogen. They found that the serum TSH and T3 levels were unchanged in both normal menstrual cycles and cycles of oral contraceptives. Moreover, the serum TSH, T3, and T4 levels were found to be higher in women taking oral contraceptives. Estrogen was suspected of having a direct inhibitory effect on the thyroid gland (Weeke and Hansen, 1975). In a study of a large population of women, users of estrogen oral contraceptives were found to be associated with a reduced prevalence of thyroid enlargement and goitre (Knudsen

et al., 2002). Thyroid volume was also found to be significantly lower in the users of contraceptives when compared to that in non-users.

2.9 Effects of pathology on thyroid physiology

2.9.1 Thyroid diseases

In canines, thyroid disorders, particularly lymphocytic thyroiditis and idiopathic follicular atrophy, were the most common diseases associated with clinical hypothyroidism observed in pet dogs (Gosselin et al., 1982). In a population of Beagles that lived out a full life span in a closed breeding colony, a strong association was found between progressive lymphocytic thyroiditis, hypothyroidism and thyroid follicular neoplasia, and it was believed to be related to the chronic excess TSH stimulation on the follicular epithelium (Benjamin et al., 1996).

Peterson et al. (1997) determined that the measurement of serum free T4 and TSH concentrations was useful for the diagnosis of hyperthyroidism in dogs. They found that the measurement of serum free T4 (98%, 93%, 95% respectively) concentrations had higher sensitivity, specificity and overall accuracy than the measurement of other single thyroid hormones in the diagnosis. TSH had a lower sensitivity (76%) and accuracy (84%), but had specificity matching that of free T4. In another study, Scott-Moncrieff et al. (1998) reported that the evaluation of TSH concentration in response to TRH administration could differentiate hypothyroid dogs from euthyroid dogs, with an overall accuracy of 90%. They found that hypothyroid dogs tended to have a higher TSH concentration than euthyroid dogs.

In felines, a case-control study was conducted to evaluate potential risk factors for the development of hyperthyroidism (Kass et al., 1999). A diminished risk for hyperthyroidism was found in 2 genetically related cat breeds. A 3-fold increase in

the risk of developing hyperthyroidism was found in cats that used litter compared to cats that did not. Cats consuming commercially prepared can food had a 2-fold increase in the risk of developing hyperthyroidism over those that did not. No strong association was found between hyperthyroidism and the use of commercial flea products. Based on the findings, the authors recommended further studies in the dietary and environmental factors contributing to the pathogenesis of hyperthyroidism. For hypothyroidism, the natural occurrence of the disease is rare in cats (Scott-Moncrieff, 2007).

Sonography has been found to act as an effective tool for the investigation of thyroid diseases (Reese et al., 2005). Thyroid ultrasound was conducted in healthy dogs, dogs with euthyroid sick syndrome, thyroglobulin autoantibody-positive hypothyroid dogs, and thyroglobulin autoantibody-negative hypothyroid dogs. Findings of the study suggested that ultrasound is a useful imaging tool in the assessment of thyroid gland in dogs.

2.9.2 Non-thyroidal diseases

In humans, various non-thyroidal diseases alter thyroid hormone levels (Hegedüs, 1990). Acute liver disease was found to be associated with a significantly increase in thyroid volume (Hegedüs, 1990). In chronic liver disease, the thyroid volume decreased in patients with alcoholic liver cirrhosis, however, alcohol itself was also suspected to be a cause of thyroid volume alterations (Hegedüs, 1984). Chronic renal failure (CRF) has been suggested to have a goitrogenic effect, and CRF patients tended to have a larger thyroid gland (Hegedüs, 1990).

In canines, thyroid hormone levels were evaluated and compared between healthy dogs and severely sick dogs (Torres et al., 2003). The total T4 and free T4 concentrations were found to be significantly lower than the normal limit in severely sick dogs. Therefore, evaluation of thyroid hormones may give a clue in the diagnosis of non-thyroidal diseases in dogs. It has also been suggested that

evaluation of thyroid hormone concentration is useful in the management of dogs with non-thyroidal illness (Mooney et al., 2008).

Sonographic evaluation of the thyroid volume was conducted in healthy, hypothyroid and euthyroid Golden Retriever dogs with non-thyroidal diseases (Brömel et al., 2005). There was no significant difference in the thyroid volume between healthy dogs and euthyroid dogs with non-thyroidal diseases, suggesting that non-thyroidal diseases may not alter the thyroid volume in Golden Retriever dogs. The sonographic appearance and features of the thyroid gland in hypothyroid dogs was more variable than that in the other 2 groups of animals. In hypothyroid dogs, there were a greater frequency of round to oval-shaped thyroid lobes in the transverse plane ($p < 0.05$), hypoechogenicity of the thyroid gland compared with the surrounding musculature, and decrease in the volume of the thyroid gland when compared with euthyroid dogs. Moreover, a significant difference in thyroid volume was found between hypothyroid and euthyroid dogs. The study concluded that the measurement of thyroid volume by ultrasound may be a useful adjunct in distinguishing hypothyroid dogs and euthyroid dogs with non-thyroidal diseases.

2.10 Basis of this study

The thyroid gland is an important organ in the mammals because it maintains the basal metabolism of other tissues and organs so that they can function normally. A clear understanding of the anatomy and physiology of thyroid gland aids the diagnosis of thyroid disorders and helps the monitoring of treatment response. To the best of my knowledge, there are very few detailed reports of the anatomy and physiology of the dolphin thyroid gland (Harrison, 1969; Arvy, 1970; Ridgway and Patton, 1971; St. Aubin, 2001; Shimokawa et al., 2002; Cowan and Tajima, 2006), and this limited information is not adequate for the clinical management of dolphins with thyroid abnormalities. Ultrasound has been used in the assessment of various organs in bottlenose dolphins, and it could be a useful mean for the assessment of

the thyroid gland in this species. There are only a few studies that reported the ultrasound assessment of dolphin thyroid gland (West et al., 2003; West and Ramer, 2005), but these studies were limited by their narrowed scope of study and small sample size (n = 2). In addition, essential sonographic features of the normal thyroid gland in bottlenose dolphins, which are crucial to offer a basis for the diagnosis of pathology, have not yet been documented. Possible factors that may influence the morphology and physiology of the thyroid gland in bottlenose dolphins are not fully evaluated. Therefore, a comprehensive study to assess the thyroid anatomy and physiology of bottlenose dolphins is needed. Moreover, the development of a standardized scanning protocol is essential for an accurate and reliable ultrasound assessment of the dolphin thyroid gland.

Chapter Three

Study One

Sonographic imaging of the thyroid gland and adjacent neck structures of the Indo-Pacific Bottlenose dolphin, *Tursiops aduncus*

3.1 Introduction

Sonography has been proven to be an effective imaging tool in assessing thyroid glands and screening for thyroid pathologies in humans (Barraclough and Barraclough, 2000; Hegedüs, 2001; AIUM, 2003; Khati et al., 2003). For accurate diagnosis, the normal sonographic features of the thyroid gland need to be established, as they are essential for morphological investigation, pathological evaluation and the follow-up of treatment regimens. The application of real-time sonography in the evaluation of the human thyroid gland has been reported (Brunn et al., 1981; Loevner, 1996; Hegedüs, 2001; Khati et al., 2003; Lyshchik et al., 2004a,b; Malago et al., 2008), and the normal human thyroid gland has been defined as a medium to high homogeneous echotexture structure hyperechoic to the sternomastoid muscle (Ying et al., 1998; Ahuja, 2000; Hegedüs, 2001; Bruneton et al., 2002; Khati et al., 2003). In the clinical practice of human medicine, ultrasound is usually used for detecting and diagnosing thyroid nodules, and guiding fine-needle aspiration of these nodules. With the use of Doppler ultrasound, the vascularity of thyroid nodules can also be evaluated. Diffuse thyroid disease may cause thyroid gland enlargement and thus thyroid volume measurement is a useful parameter for diagnostic purposes. Decreasing thyroid volume in serial examinations after treatment may also indicate a positive response. According to the AIUM practice guidelines for the performance of a thyroid and parathyroid sonographic examination (AIUM, 2003), the volume of each thyroid lobe should be recorded in at least 2 dimensions and preferably in 3 dimensions. Any visualized thyroid abnormalities should be documented with the location, volume, number and

sonographic features of abnormalities recorded. Abnormalities of the adjacent soft tissues, when encountered, such as enlarged lymph nodes or thrombosed veins, should also be documented (AIUM, 2003).

A number of studies have reported the normal sonographic appearance of thyroid glands in humans of different sex, race, age, weight, health conditions, dietary conditions and reproductive status within female subjects (Chan et al., 1998, 1999; Ying et al., 1998; Barraclough and Barraclough, 2000; Hess and Zimmermann, 2000; Zimmermann et al., 2000; Hegedüs, 2001; Zimmermann et al., 2001; Khati et al., 2003; Senchenkov and Staren, 2004; Sheikh et al., 2004). However, in veterinary medicine, there are only a few studies that have reported the normative thyroid volume and sonographic features in dogs, cats and horses (Cartee et al., 1993; Breuhaus, 2002; Reese et al., 2005; Brömel et al., 2006). To the best of our knowledge, the formal literature is devoid of any reference to the sonographic evaluation of normal thyroid glands and the adjacent neck structures of any marine mammal species.

Sonographic measurement of the bottlenose dolphin thyroid gland has been reported and efforts have been made to establish baseline values of parameters related to thyroid function (West et al., 2003; West and Ramer, 2005). However, the authors claimed that sonographic measurements were extremely variable because of skill differences between operators, and no detailed description on adjacent neck structures and related anatomical landmarks was made. To the best of our knowledge, there is scant information on the anatomy of the dolphin thyroid gland (Arvy, 1970; Ridgway and Patton, 1971), and no detailed reference for the sonographic anatomy of the thyroid gland and adjacent neck structures in this species. Essential sonographic features of the normal dolphin thyroid gland, which are crucial to offer a basis for the diagnosis of pathology, have not been documented. The purpose of the present study is to describe a simple scanning protocol that enables repeatable visualization of the thyroid gland of bottlenose dolphins and to describe the sonographic features of the normal dolphin thyroid gland and adjacent

neck structures.

3.2 Materials and Methods

3.2.1 Animals and management

Eighteen *Tursiops aduncus* at Ocean Park, Hong Kong (7 males and 11 females) were included in the study. Estimated age, sex, sexual maturity, and body size of all animals are provided (Table 3.1), in January 2009. Diets consisted of different proportions of capelin, sardine, herring and squid, with vitamin and mineral supplements (see Appendix 3). This population of dolphins was maintained in 2 different locations with semi-enclosed and enclosed outdoor facilities, consisting of inter-connected tanks with treated natural sea water (see Appendix 1). From August 2006 to January 2009, 1404 individual observations were performed from this captive population. All dolphins involved in the study weretrained to cooperate for neck ultrasound examination. The subjects were apparently healthy with no recent history of illnesses, and were not receiving medication that could alter thyroid gland physiology during the time of the study. Serum concentrations of thyroxine (free [fT4] and total [tT4]), triiodothyronine (free [fT3], total [tT3]) were also determined on each individual subject and the values were all within normal ranges (St. Aubin, 2001).

Table 3.1: Estimated age, sex, sexual maturity, body size and mean thyroid volume in 18 bottlenose dolphins (*Tursiops aduncus*) at Ocean Park, Hong Kong, in the beginning of 2009.

Subject	Estimated age (years)	Sex	Sexual maturity	Body weight (kg)	Body length (cm)	Mean thyroid volume (mL)
Molly	26	M	Sexual mature	126.6	218	8.31
Mini	deceased at 26	M	Sexual mature	142	233	15.29
Toto	13	M	Sexual mature	138.9	236	11.13
Perky	deceased at 7	M	Sexual mature	120.1	223	19.67
Leo	9	M	Sexual mature	112.7	216	10.63
Anson	4	M	Sexual immature	122.6	222	14.83
Ginsan	4	M	Sexual immature	130.4	222	15.96
Jessie	37	F	Sexual mature	185.1	242	21.41
Angel	25	F	Sexual mature	136.5	215	11.66
Ada	26	F	Sexual mature	106.1	201	9.37
Ester	21	F	Sexual mature	138	212	5.81
Gina	26	F	Sexual mature	132.8	208	8.22
Hicky	21	F	Sexual mature	124	196	7.00
Pinky	10	F	Sexual mature	140.3	220	20.34
Hoi Kei	7	F	Sexual mature	108.3	214	14.35
Maya	7	F	Sexual mature	147.1	244	18.82
Nona	3	F	Sexual immature	125.8	222	15.74
Mia	1	F	Sexual immature	98	198	44.06

3.2.2 Equipment

All sonographic examinations were performed with either a Philips HD-11 ultrasound unit or a Philips HD-11 XE ultrasound unit, in conjunction with a 6-2 MHz curvilinear 3-D broadband curved array transducer and a 5-2 MHz 2-D broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA) or an Aloka SSD 900 ultrasound unit (Aloka Co. Ltd, Mitakasho, Tokyo) in conjunction with a 5 MHz curvilinear transducer. All images were recorded with either direct digital capture or with a thermal printer. Due to the nature of the dolphin's skin, there being no air layer between the surface and the transducer, thus no coupling gel was required.

3.2.3 Behavioural training for neck ultrasound examination of dolphins

All dolphins in the facility were trained to cooperate for neck ultrasound examination, which made routine and repeatable examinations possible. Subjects were familiarized to a similar behaviour due to the routine body temperature measurement. Initially, the dolphins were trained to approach the poolside and position themselves in dorsal recumbence, with the tail supported by a trainer. The transducer was introduced onto the thorax region and slowly moved cranial beyond the sternum (Figure 3.1). Dolphins appeared to be sensitive to the ultrasound beam, were initially startled and would not remain in a stable position. With time and positive reinforcement, all dolphins became accustomed to the beam and would usually accept ultrasound examinations.

Figure 3.1: Dorsal recumbence position of the dolphin with the tail supported by a trainer during neck ultrasound examination. The transducer was placed at the thoracic inlet and was slowly moved cranially superior to the sternum, midway between the insertions of the pectoral flippers.

3.2.4 Protocol for sonographic examination of the thyroid gland

For the 2-D ultrasound scanning, the transducer was placed in a transverse orientation at the thoracic inlet, midway between the insertions of the pectoral flippers. The transducer was moved cranially until the brachiocephalic vein was identified (Figure 3.2). The transducer was then moved further cranially until the transverse dimension of the left and right lobes of the thyroid gland was identified. The transducer could then be rotated by 90° to visualize the longitudinal dimension of different portions of thyroid gland. The transducer could also be placed in an oblique manner to visualize the long axis of the thyroid lobes and produce measurements for thyroid volume calculations (Figure 3.3).

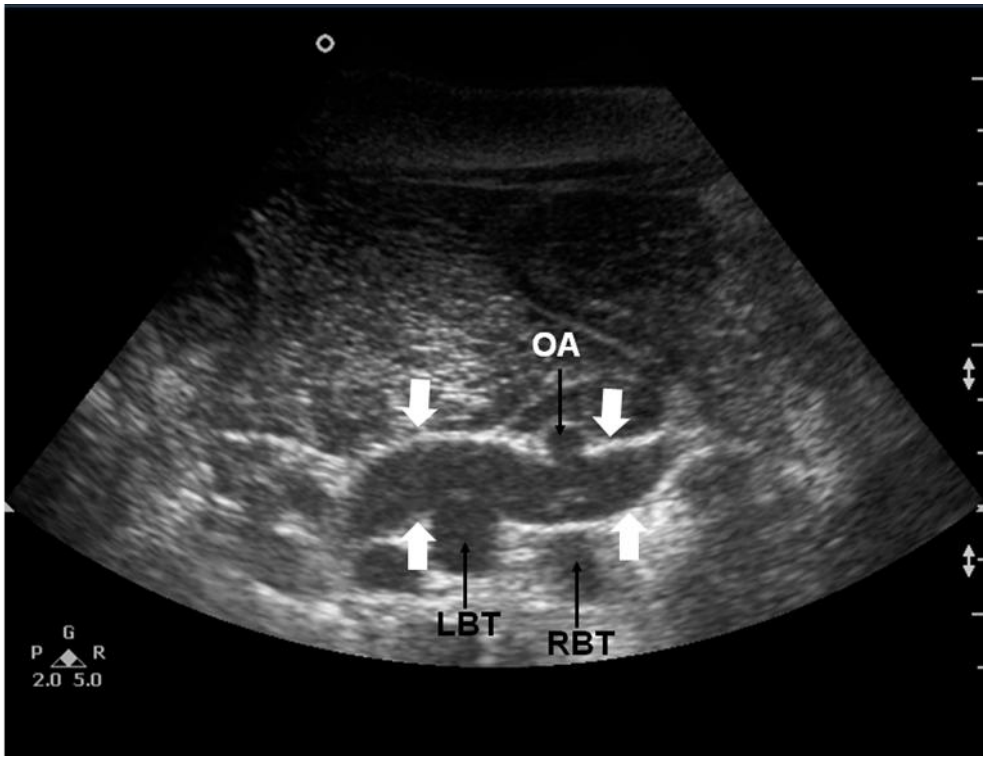

Figure 3.2: Transverse grey scale sonogram of a dolphin's neck shows the brachiocephalic vein (white arrows), left brachiocephalic trunk (LBT), right brachiocephalic trunk (RBT) and omooccipital artery (OA).

Figure 3.3: Oblique position of the transducer during neck ultrasound examination. This position allowed the visualization of the long axis of the thyroid lobes and produced measurements for thyroid volume calculations.

For the 3-D ultrasound scanning, the thyroid gland was initially identified by 2-D ultrasound as described above (Figure 3.4). After the thyroid gland was identified, the 3-D data acquisition function was activated and the mechanical scanner inside the transducer scanned through the thyroid gland in a single sweep with a sweeping angle of 50º, which was adequate to encompass the entire thyroid gland. The 3-D images were then reconstructed automatically by the built-in software, QLAB 5.0 (Philips Medical System, Bothell, Washington, 98021, USA). The software program allowed manual manipulation of reconstructed slices on 3 image scan planes: transverse plane, reconstructed longitudinal plane, and reconstructed coronal plane of the thyroid gland. The 3-D images were stored in the hard disk of the ultrasound unit. After the poolside scanning, the set of 3-D images was retrieved and the thyroid configuration was evaluated by the software, which assessed the different levels of coronal scan planes (Figure 3.5). All dolphin thyroid glands were

categorized into 4 different gross configurations (Cowan and Tajima, 2006) by evaluating different scan planes using both 2-D and 3-D sonography.

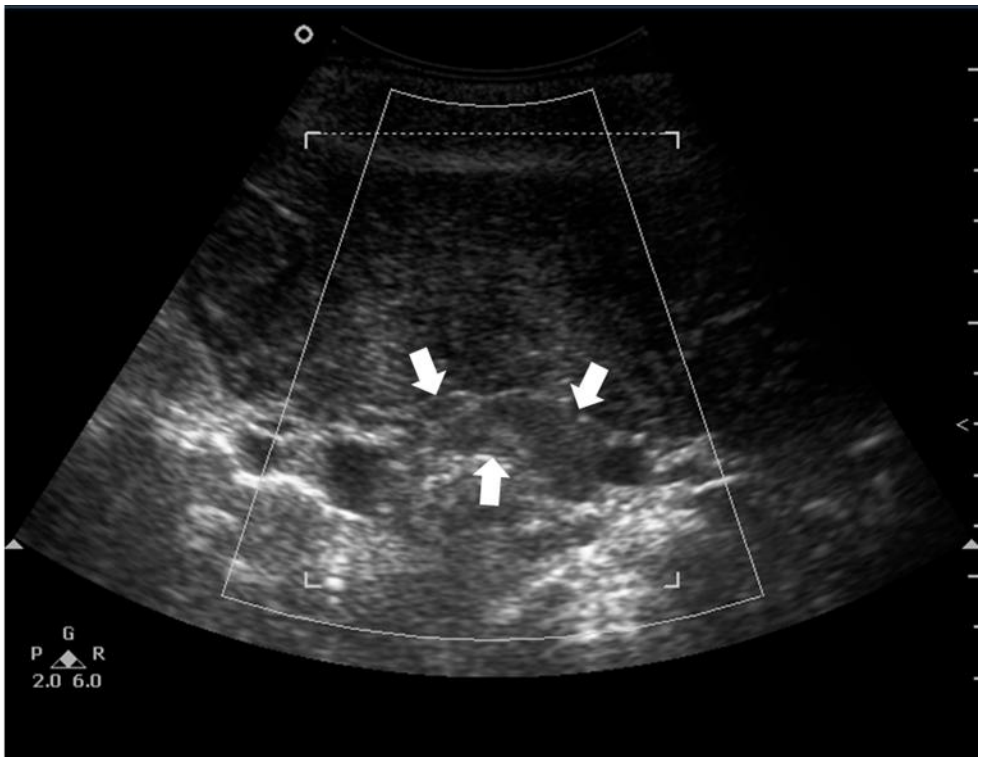

Figure 3.4: Transverse grey scale sonogram showing a dolphin thyroid gland (white arrows) prior to 3-D data acquisition. The fan-shaped box on top of the sonogram indicates the area where the 3-D data acquisition performed.

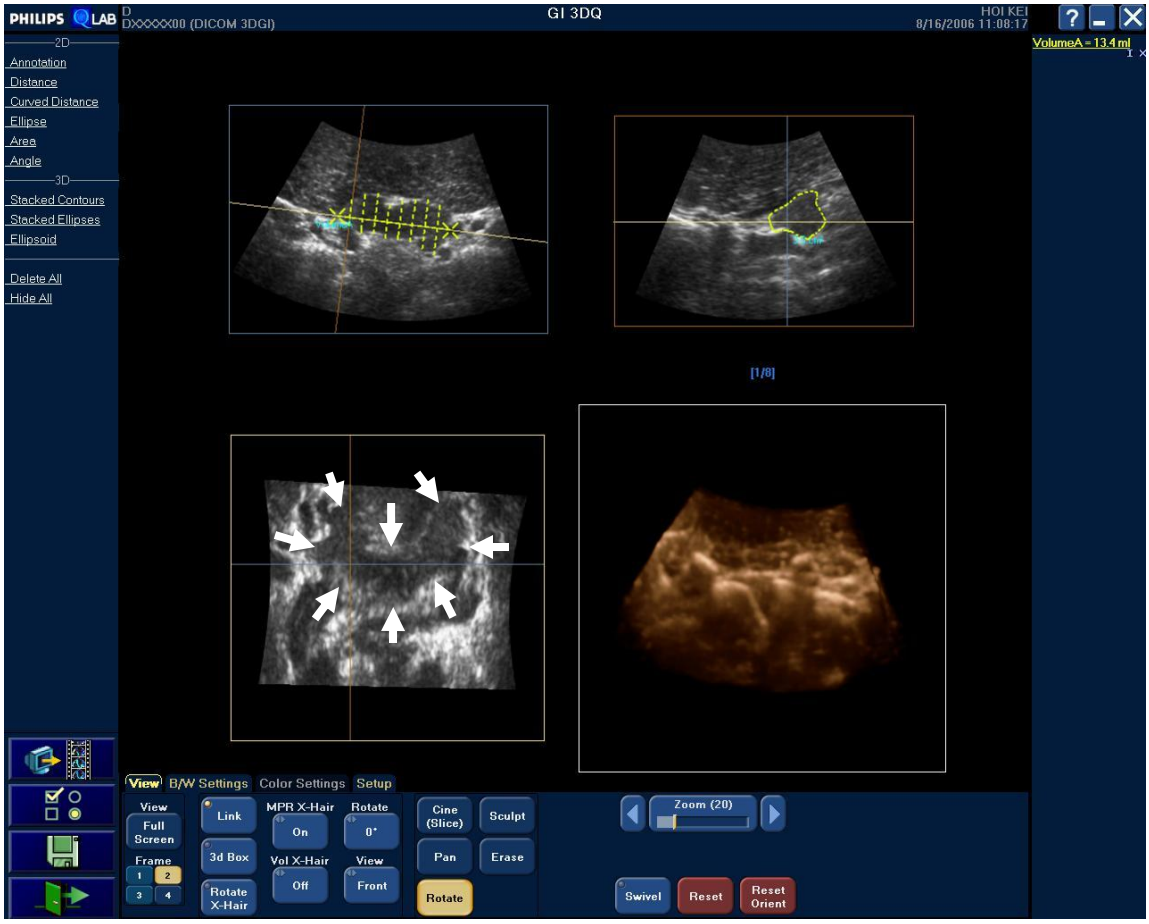

Figure 3.5: Determination of thyroid configuration with 3-D ultrasound. The upper left image demonstrates the transverse plane of the thyroid gland, with the 8 equal distance slices indicated by the yellow dotted lines. The upper right image demonstrates the reconstructed longitudinal plane of the thyroid gland. Note the boundaries of the thyroid gland are outlined with a yellow dotted line. The lower left image demonstrates the reconstructed coronal planes of the thyroid gland (white arrows). The lower right image demonstrates the reconstructed 3-D image of the thyroid gland. Different levels of the reconstructed coronal scan could be assessed and were used to evaluate thyroid configuration.

The shape, border sharpness, echogenicity and homogeneity of the thyroid gland were evaluated. The shape of each thyroid lobe was subjectively described as fusiform or elliptical in transverse scan plane, and irregular or round to oval in longitudinal scan plane. The border sharpness was assessed by the smoothness of the margin between the thyroid gland and the surrounding soft tissues; a well-

defined margin was considered to be a sharp thyroid border, whereas an ill-defined margin was considered to be a dull thyroid border. The echogenicity of the thyroid gland was compared to sternocephalicus muscle and classified into hypoechoic, isoechoic or hyperechoic. The thyroid parenchyma was categorized into homogeneous or heterogeneous on the basis of the presence or absence of any nodules or space-occupying lesions.

Adjacent neck structures such as cervical lymph nodes, musculatures and vasculatures could be identified and imaged by placing the transducer at the thoracic inlet, transversely between the insertion of the sternum and cranial pole of the thyroid lobes, and longitudinally between the insertion of the pectoral flippers, with varied scan planes and angles in the surrounding area for visualization.

3.3 Results

3.3.1 Sonographic appearance of the thyroid gland

In the 18 subjects, all thyroid glands were clearly visualized on ultrasound. The time taken to conduct a full survey of thyroid sonographic examination was approximately 3-5 minutes per subject. The shape of the thyroid lobes appeared elliptical or fusiform in the transverse scan plane, and round to oval in the longitudinal scan plane (Figures 3.6a and b). The thyroid capsule was usually echogenic and the borders of the thyroid gland were usually well-defined and smooth (82%), while ill-defined borders were observed in 3 subjects (18%) (Figures 3.7a-c). The echopattern of the thyroid parenchyma was generally uniform and homogeneous (76%), consisting of a dense agglomerate of very fine small echoes of equal size, with the presence of echogenic reticulations (Figure 3.8). In the remaining dolphins, the thyroid gland had a heterogeneous distribution of echoes caused by hyperechoic foci, isoechoic foci, hypoechoic foci, or a mottled appearance of thyroid gland (Figure 3.9). Intrathyroidal vessels appeared as small rounded structures on the scan planes perpendicular to the axis of the vessel, or

appeared as small linear structures on scan planes parallel to the axis of the vessel (Figure 3.10). The echogenicity of the thyroid gland was usually hypoechoic [8 in adult (89%); 4 in calf (80%); and 1 in juvenile (17%)] or isoechoic [5 in juvenile (83%); 1 in calf (20%); and 1 in adult (11%)] when compared to the adjacent sternocephalicus muscle. (Figure 3.11). Echogenicity of the right and left thyroid lobes, compared with the isthmus, was different in over half of the population (65%) (Figure 3.12).

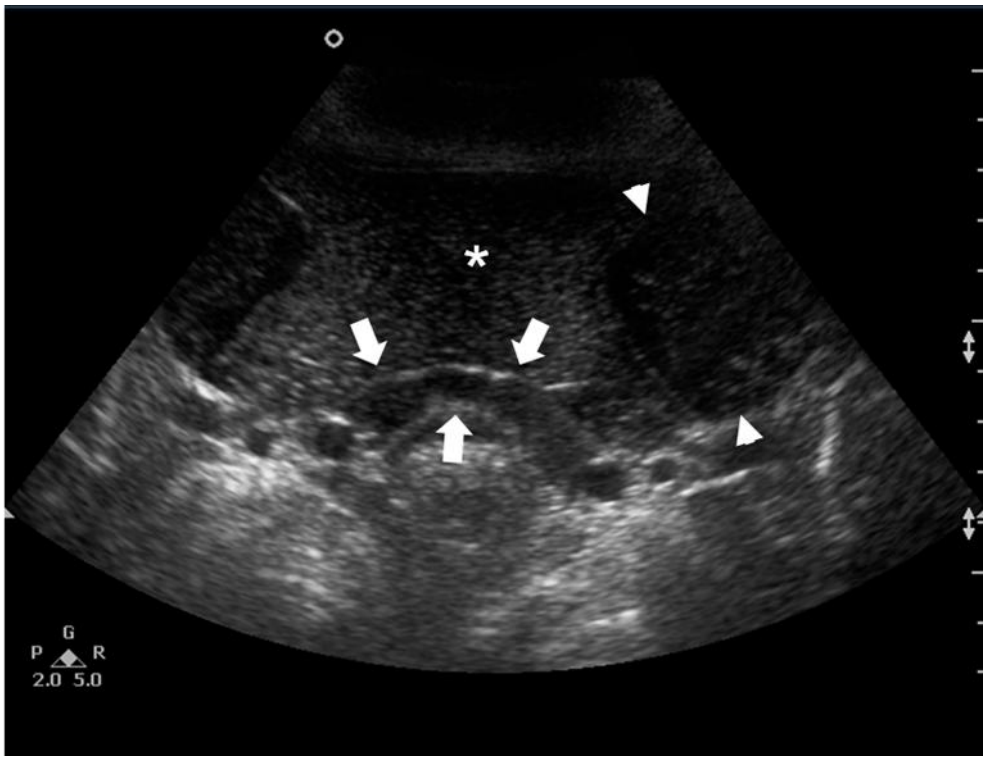

Figure 3.6a: Transverse grey scale sonogram showing a fusiform-shaped and well-defined dolphin thyroid gland with a uniform and homogenous echopattern (white arrows) and adjacent neck muscles: sternocephalicus muscle (asterisk) and sternohyoideus muscle (arrow heads).

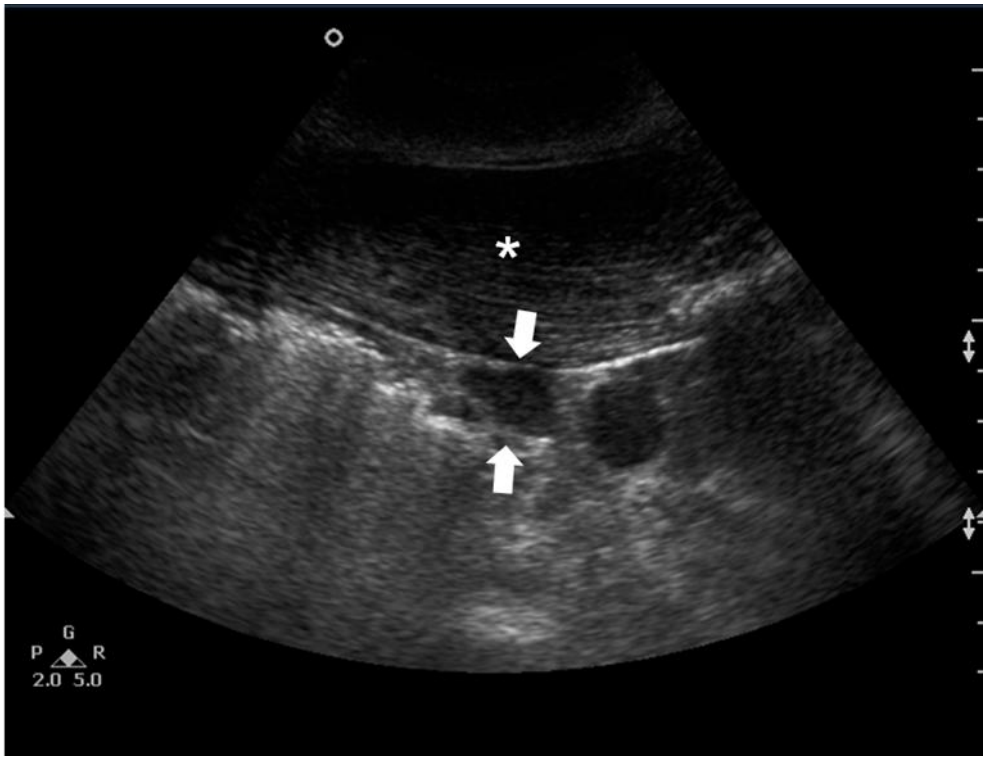

Figure 3.6b: Longitudinal grey scale sonogram of an oval-shaped and well-defined dolphin thyroid gland (white arrows) and the sternocephalicus muscle (asterisk). The thyroid gland is hypoechoic when compared with the sternocephalicus muscle (asterisk).

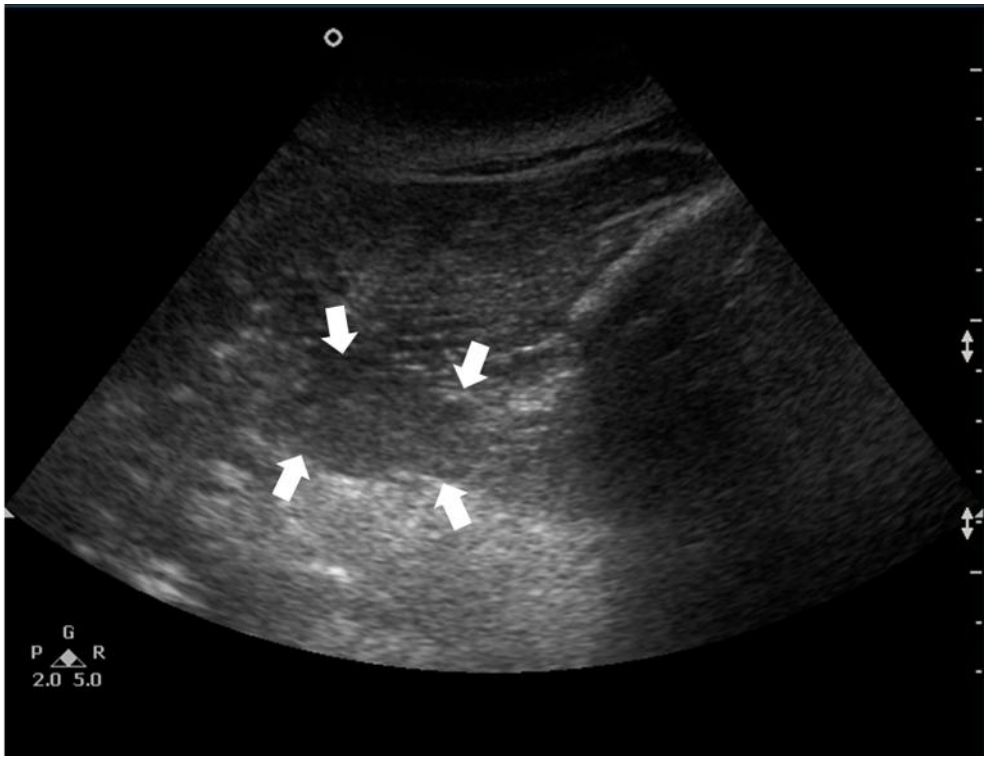

Figure 3.7a: Longitudinal grey scale sonogram of a dolphin thyroid gland with ill-defined borders (white arrows).

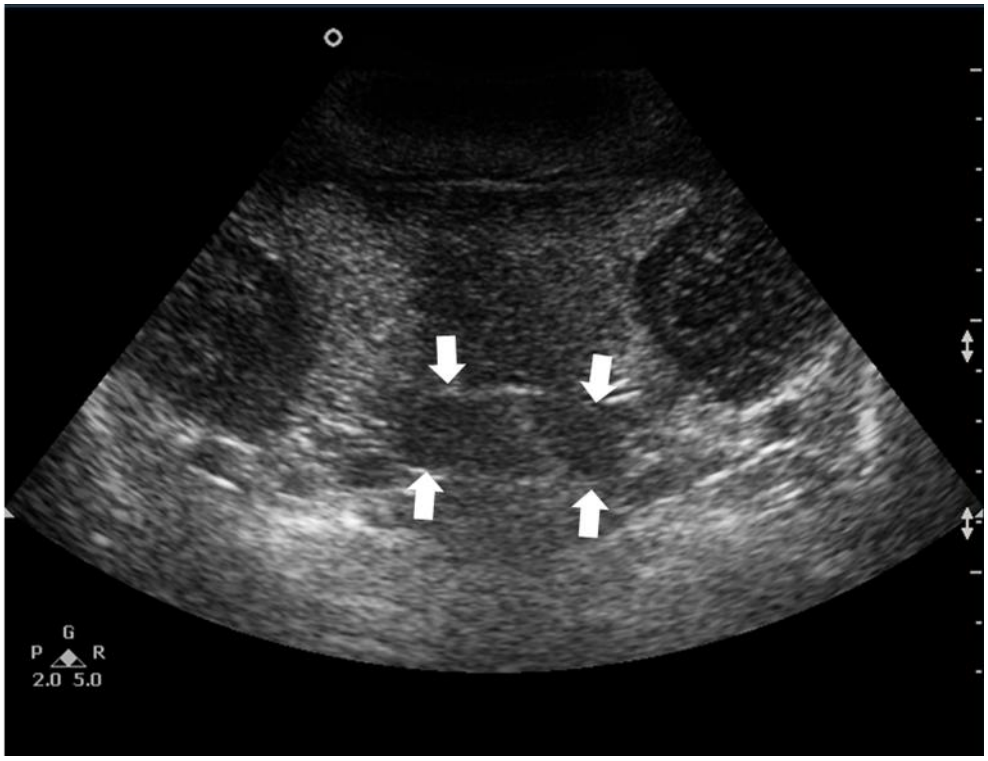

Figure 3.7b: Transverse grey scale sonogram of an ill-defined dolphin thyroid gland (white arrows).

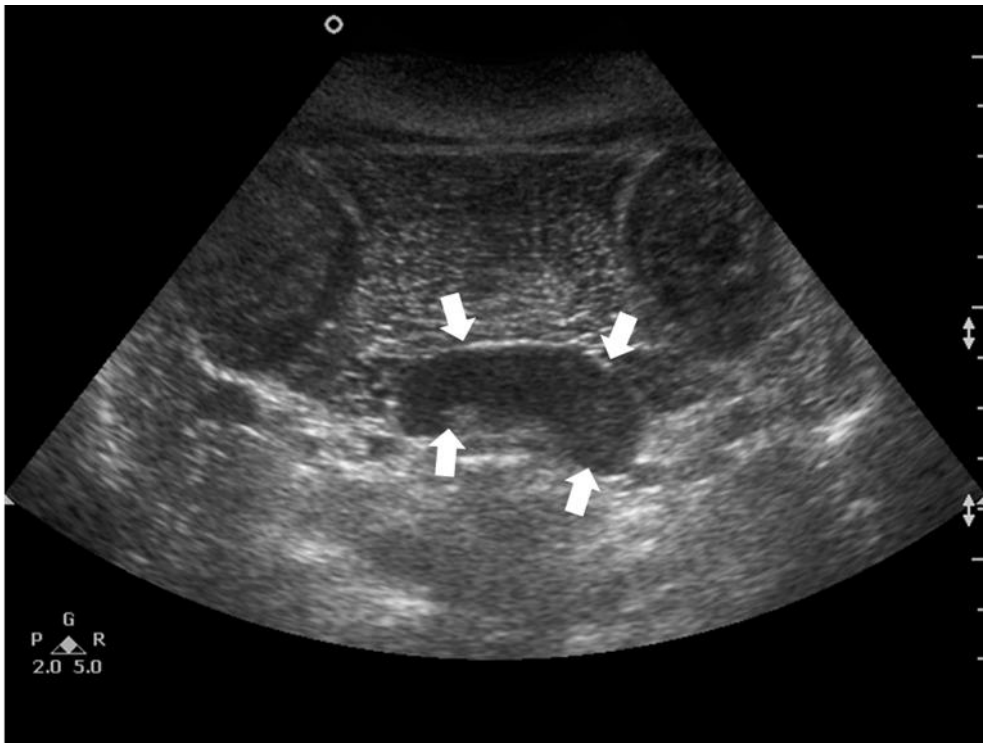

Figure 3.7c: Transverse grey scale sonogram shows dolphin thyroid gland with well-defined borders (white arrows).

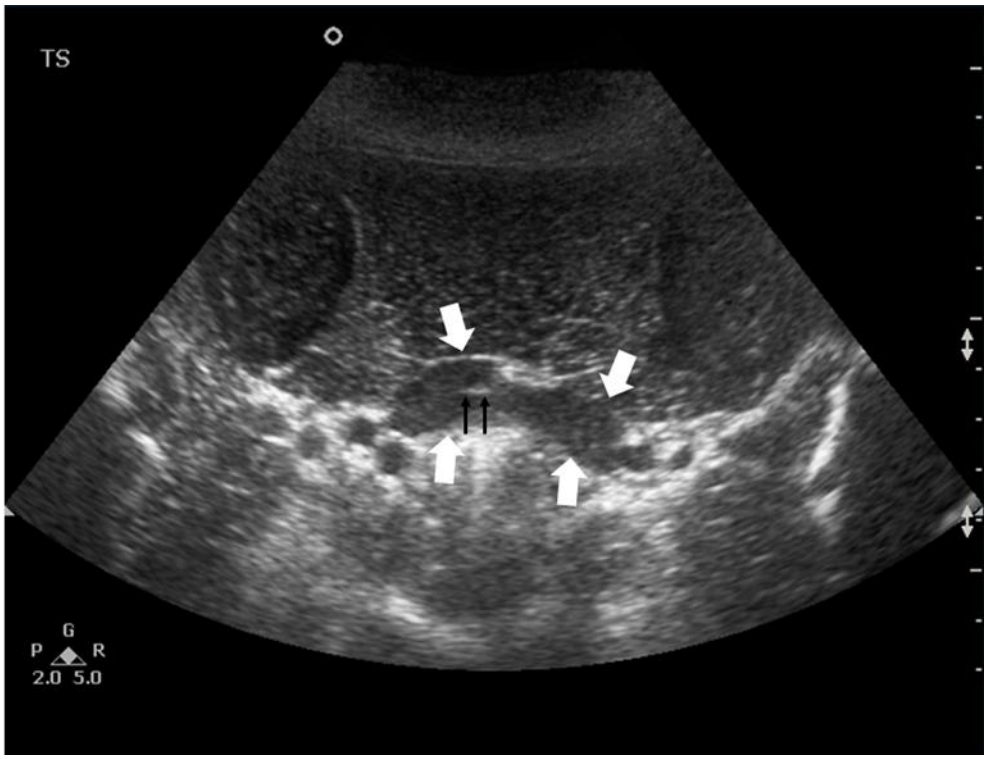

Figure 3.8: Transverse grey scale sonogram shows a dolphin thyroid gland with an uniform and homogenous echopattern (white arrows). Note the presence of echogenic reticulations within the thyroid gland (black arrows).

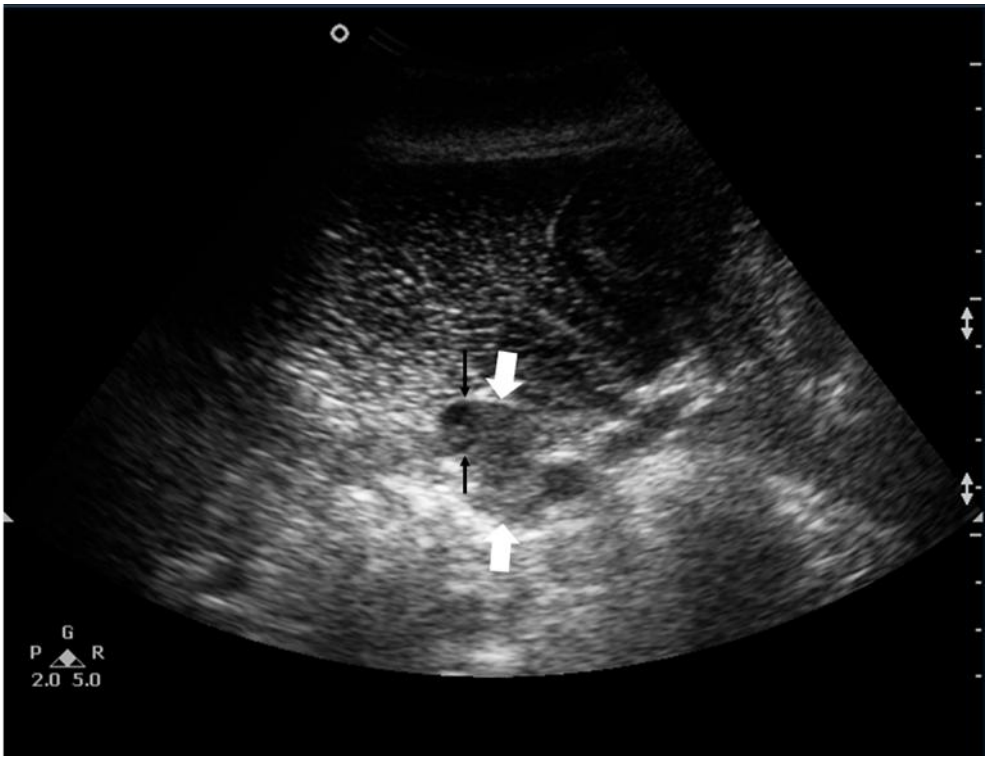

Figure 3.9: Transverse grey scale sonogram shows a dolphin thyroid lobe (white arrows) with an echolucent, ill-defined thyroid nodule (black arrows).

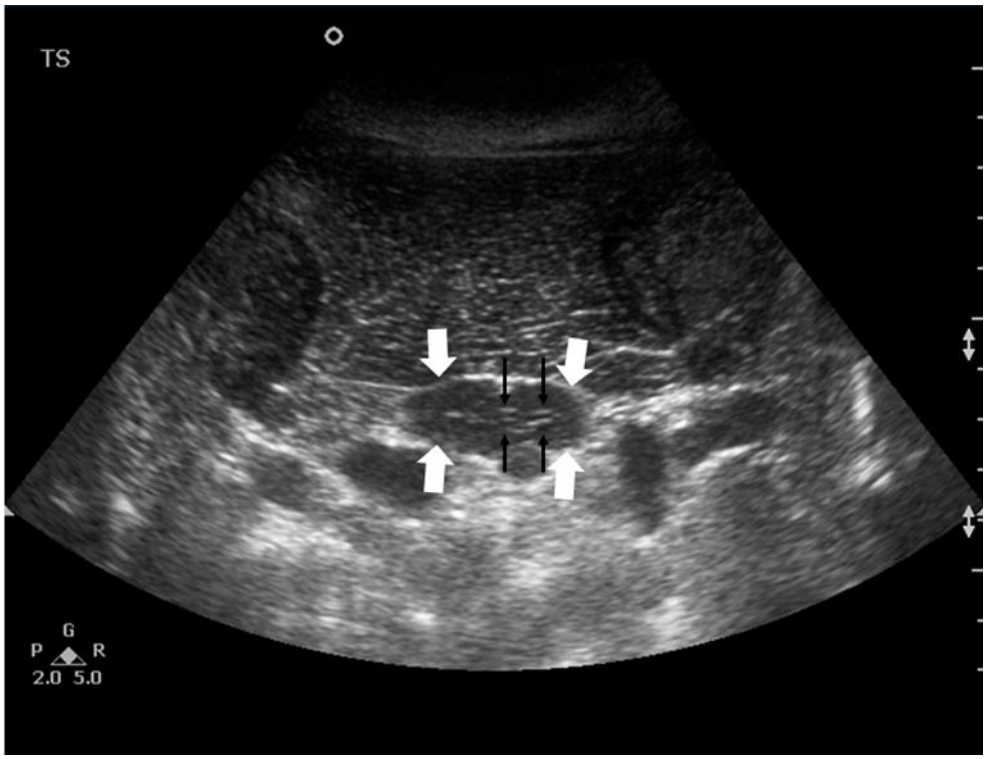

Figure 3.10: Transverse grey scale sonogram shows a dolphin thyroid gland (white arrows) with the presence of an intrathyroidal vessel, appeared as a small linear structure (black arrows).

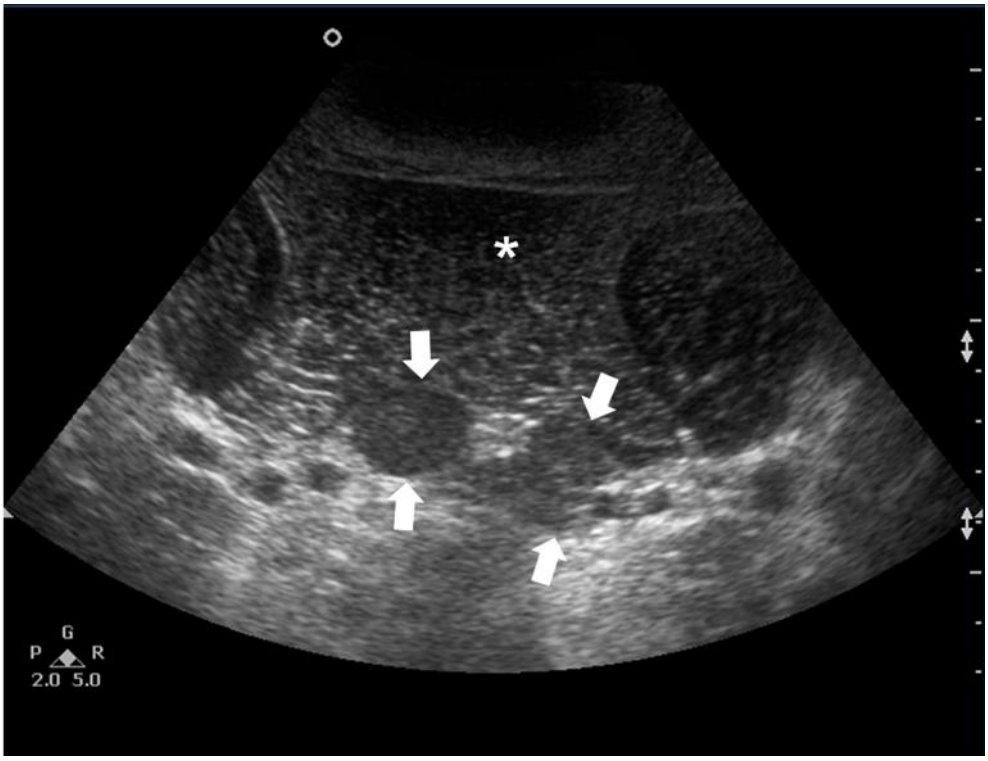

Figure 3.11: Transverse grey scale sonogram shows a dolphin thyroid gland (white arrows), which is isoechoic when compared with the sternocephalicus muscle (asterisk).

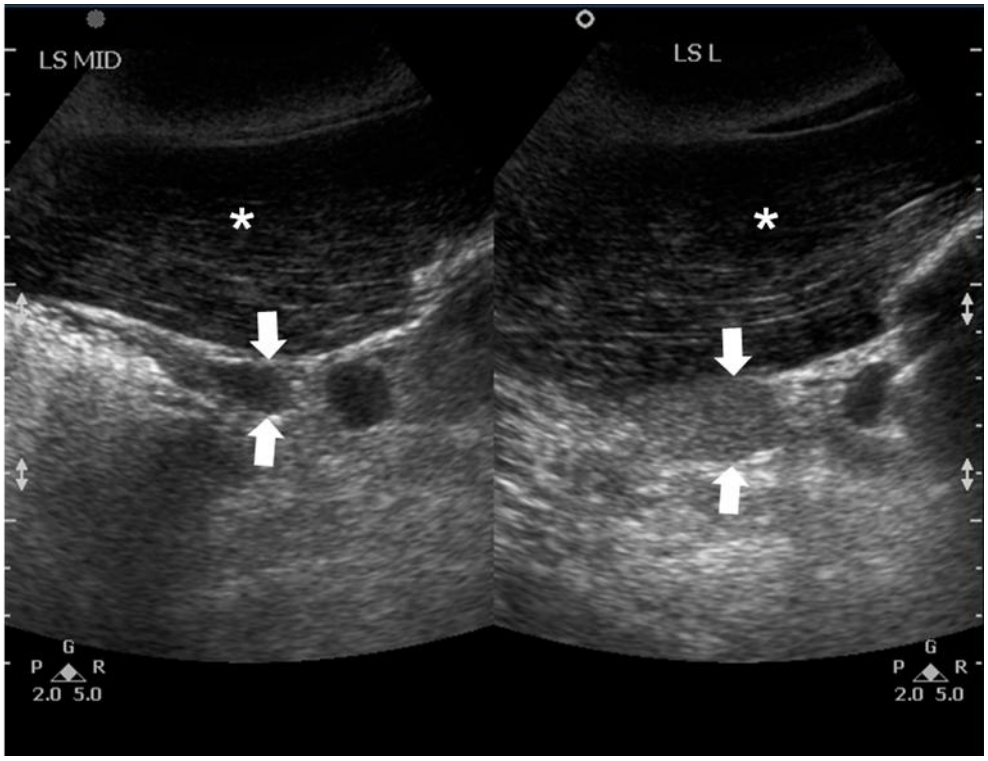

Figure 3.12: Dual longitudinal grey scale sonograms show the thyroid gland of the same dolphin. Left: Longitudinal sonogram shows the isthmus of the thyroid gland (white arrows), which is isoechoic when compared with the sternocephalicus muscle (asterisk). Right: Longitudinal sonogram shows the left thyroid lobe (white arrows), which is hyperechoic when compared with the sternocephalicus muscle (asterisk).

In the 18 subjects, 2 gross configurations of the thyroid gland, Type A: two lobes joined by an isthmus (n = 9) (Figure 3.13); and Type C: a shield-like, single mass, roughly diamond-shaped, placed ventrally on the trachea (n = 9) (Figure 3.14); were identified in this population of bottlenose dolphins. The isthmus was always well-visualized on ultrasound in both configurations. A pyramidal lobe was seen in 7 subjects and was best demonstrated in the longitudinal scan plane with a small dorsoventral diameter of 5-8 mm and craniocaudal diameter of 11-21 mm (Figure 3.15). The origin of the pyramidal lobes varied, as they were found to branch out from the right thyroid lobe (n = 3), from the isthmus (n = 3), and from the left thyroid lobe (n = 1).

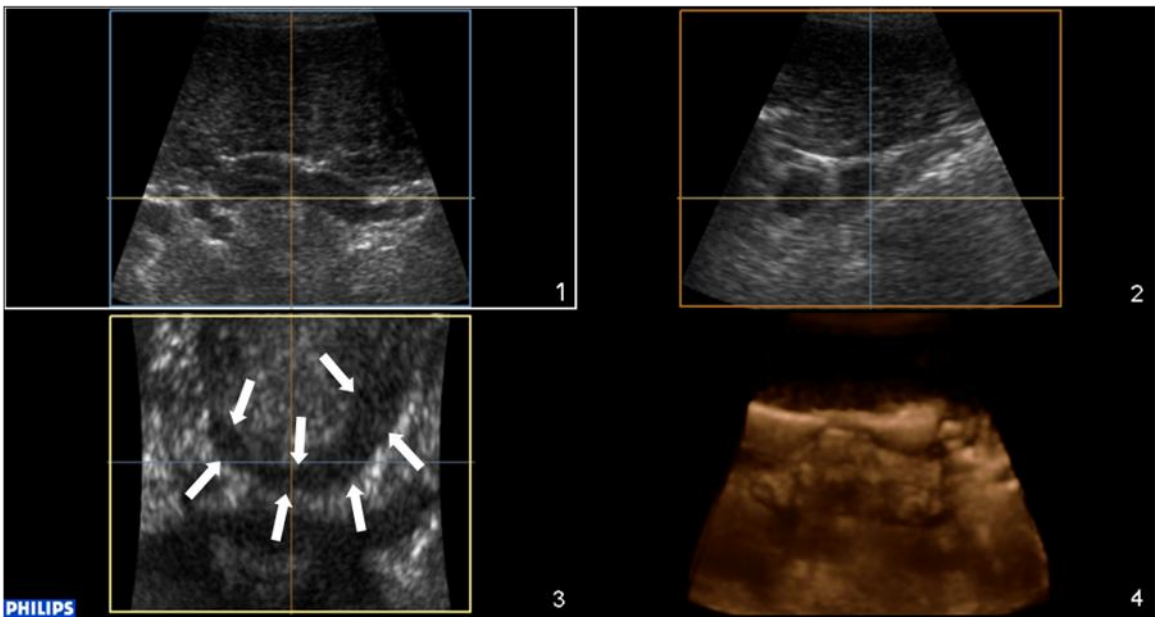

Figure 3.13: Determination of Type A thyroid configuration with 3-D ultrasound. The upper left image demonstrates the transverse plane of the thyroid gland. The upper right image demonstrates the reconstructed longitudinal plane of the thyroid gland. The lower left image demonstrates the reconstructed coronal planes of the thyroid gland (white arrows). The lower right image demonstrates the reconstructed 3-D image of the thyroid gland. The Type A configuration consists of two lobes joined by an isthmus (white arrows) and is best demonstrated in the reconstructed coronal plane.

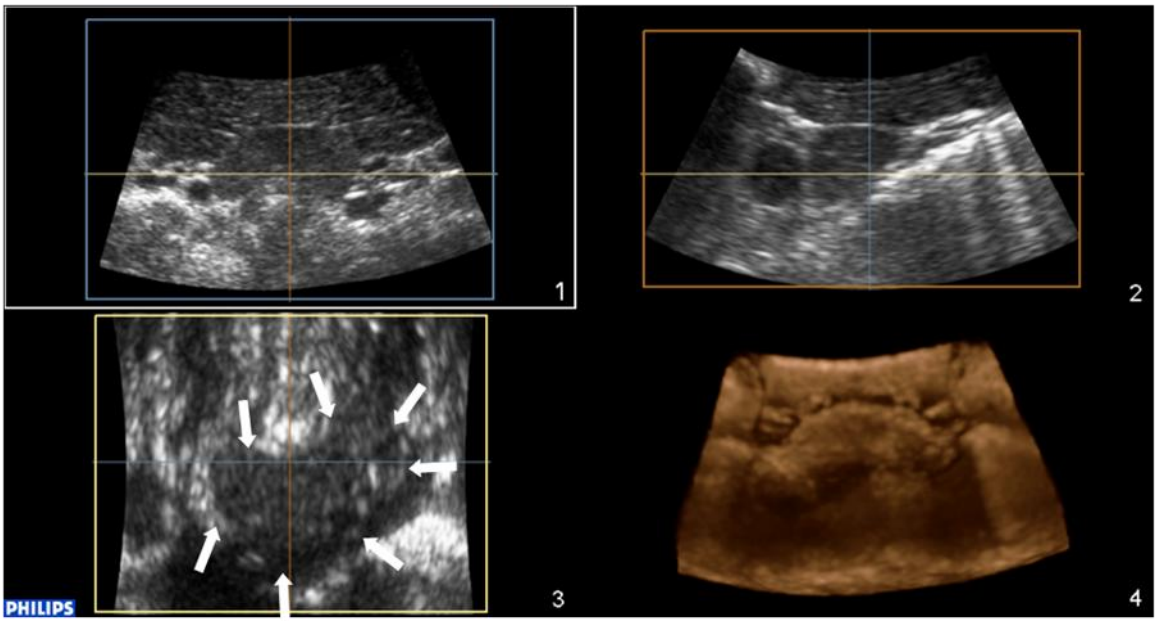

Figure 3.14: Determination of Type C thyroid configuration with 3-D ultrasound. The upper left image demonstrates the transverse plane of the thyroid gland. The upper right image demonstrates the reconstructed longitudinal plane of the thyroid gland. The lower left image demonstrates the reconstructed coronal planes of the thyroid gland (white arrows). The lower right image demonstrates the reconstructed 3-D image of the thyroid gland. The Type C configuration consists of a shield-like, single mass, roughly diamond-shaped (white arrows) and is best demonstrated in the reconstructed coronal plane.

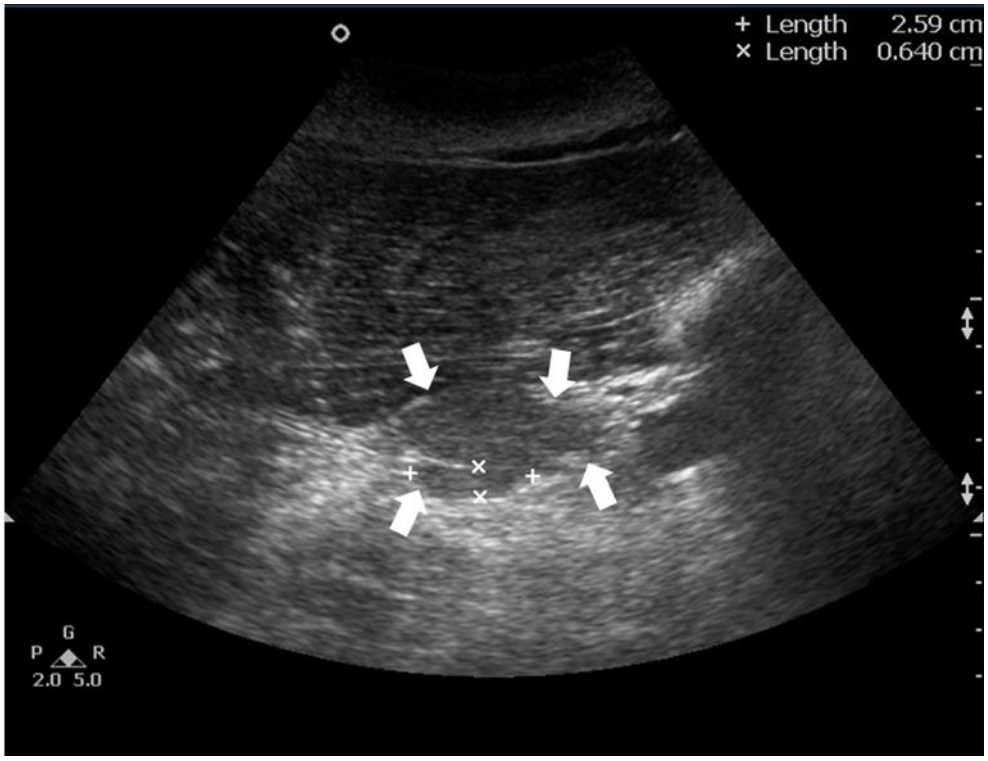

Figure 3.15: Longitudinal grey scale sonogram shows a dolphin thyroid gland (white arrows) with the presence of a pyramidal lobe and its dorsoventral and craniocaudal diameters are measured (calipers).

3.3.2 Anterior and posterolateral relationship

The superficial covering of bottlenose dolphins consisted of skin and blubber. Sonographically, this covering corresponded to a thick hypoechoic band ranging from 14.8 – 20.3 mm in thickness; exact thickness depended on the ontogeny and nutritional state of the subject (Montie et al., 2008).

The pretracheal layer of the deep cervical fascia formed a sheath encasing the sternocephalicus and sternohyoideus muscles (Kastelein et al., 1997). These muscles were always well-visualized by ultrasound. Although varying in thickness, the sternocephalicus and sternohyoideus muscles could be recognized as elongated structures at the anterior aspect of the thyroid gland. The sternocephalicus muscle was slightly hyperechoic than the normal thyroid parenchyma, whereas the

sternohyoideus muscles appeared as 2 round structures and was either isoechoic or hypoechoic when compared to the thyroid gland (Figure 3.7c).

The posterior surface of the thyroid gland is related, from front to back, to the trachea. The trachea was identified on ultrasound as a hyperechoic line, corresponding to the anterior wall of the trachea, with posterior acoustic shadowing (Figure 3.6a).

The lateral surface of the thyroid gland was related to the major cervical vessels embedded with the carotid sheath and cricoid cartilage. Major cervical vessels comprised the internal and external carotid arteries (located immediately lateral to thyroid lobes), and internal jugular vein (IJV) (located lateral to the internal and external carotid arteries and more or less spontaneously visible). Cricoid cartilage is located lateral to the carotid arteries and the IJV was identified on ultrasound as an oblique hyperechoic line (Figure 3.6a).

3.3.3 Blood supply and Lymphatics

Arteries in the neck such as the brachiocephalic trunk, subclavian arteries, internal and external carotid arteries, omooccipital artery and superior thyroid arteries were easily visualized. This also held true for veins in the neck such as the brachiocephalic vein, internal jugular veins and superior thyroid veins. The superior thyroid artery arose from the brachiocephalic trunk, supplying blood to the thyroid gland (De Kock, 1959; Galliano et al., 1966; Viamonte et al., 1968). The superior thyroid vein drained into the internal jugular vein (Galliano et al., 1966; Viamonte et al., 1968) (Figures 3.16-3.18).

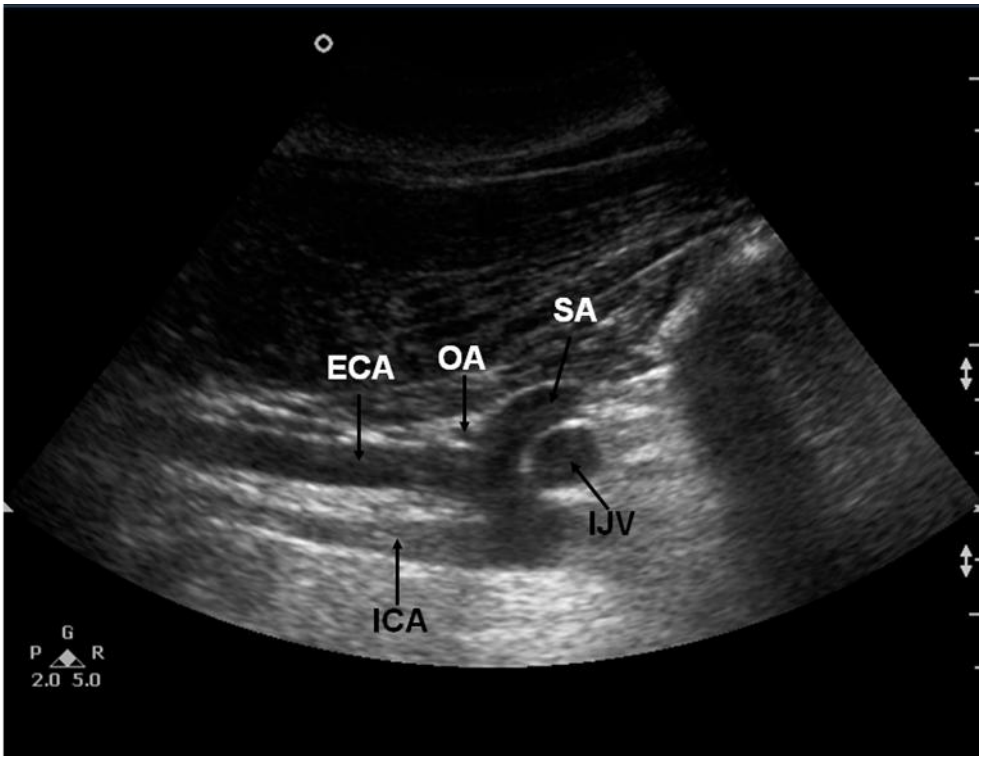

Figure 3.16: Longitudinal grey scale sonogram shows the anatomical structures of the right side of a dolphin's neck (medial to the pectoral flipper) - right subclavian artery (SA), right internal carotid artery (ICA), right external carotid artery (ECA), right omooccipital artery (OA) and right internal jugular vein (IJV).

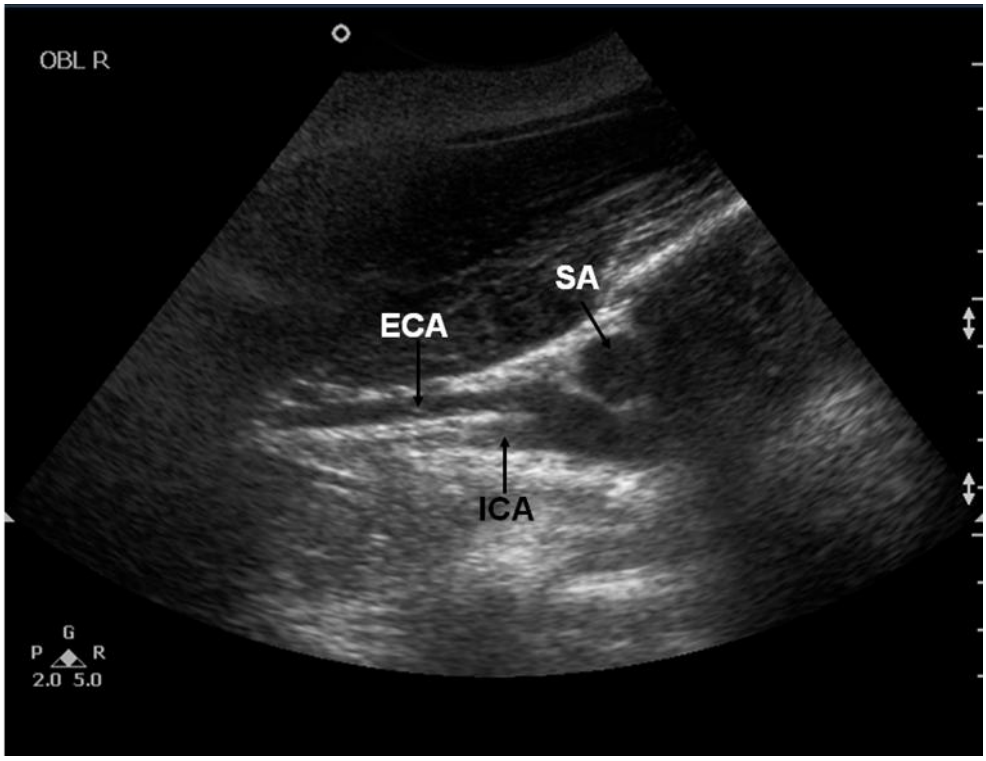

Figure 3.17: Longitudinal grey scale sonogram shows the anatomical structures the right side of a dolphin's neck (transducer placed obliquely medial to the pectoral flipper) - right subclavian artery (SA), right internal carotid artery (ICA) and right external carotid artery (ECA).

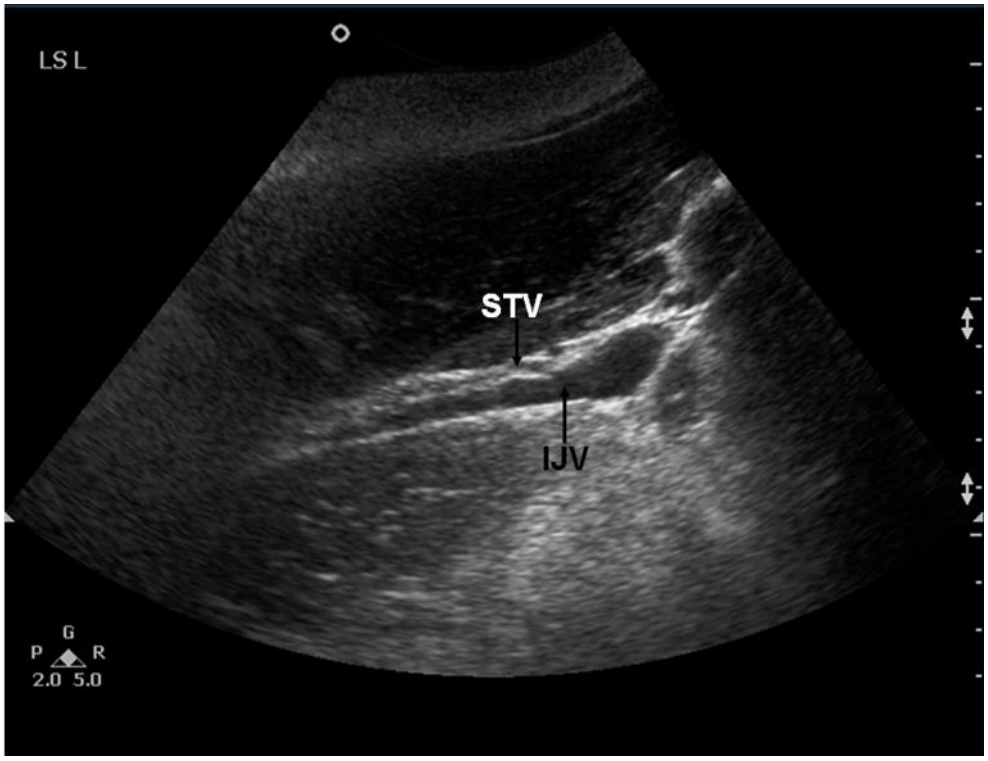

Figure 3.18: Longitudinal grey scale sonogram shows the anatomical structures of the left side of a dolphin's neck (further medial to the saggital line) - left internal jugular vein (IJV) and left superior thyroid vein (STV).

The cervical lymph nodes were visualised by ultrasound. The lymph nodes were isoechoic to hypoechoic when compared to the adjacent sternocephalicus muscle, round or oval in shape, and with an echogenic hilus (Figure 3.19). The cervical lymph nodes were usually found between the sternocephalicus and sternohyoideus muscles, cranial and lateral to the thyroid gland, and around the mediastinum and brachiocephalic veins, at the ventral depth of 40 - 70 mm. Approximately 2 - 3 lymph nodes could be identified in each subject. In the 18 subjects, 44 lymph nodes were identified with sonography. The mean long axis measurement of the lymph nodes was 24 mm (ranged from 20 to 35 mm).

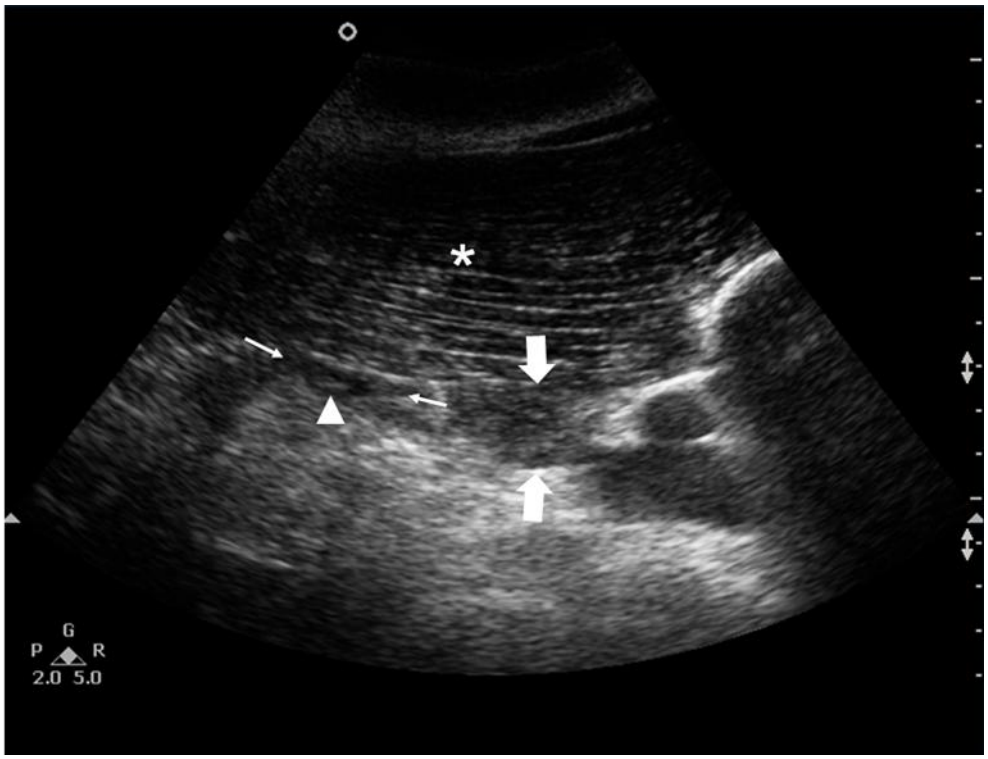

Figure 3.19: Longitudinal grey scale sonogram shows a dolphin thyroid gland (big white arrows) and a cervical lymph node (small white arrows). The lymph nodes appear hypoechoic when compared to the adjacent sternocephalicus muscle (asterisk), oval in shape, and has an echogenic hilus (arrow head).

3.4 Discussion

Assessment of dolphin thyroid gland and adjacent neck structures is usually performed in post-mortem studies (Galliano et al., 1966; Viamonte et al., 1968; Cowan and Smith, 1999; Cowan and Tajima, 2006). Clinical examination of the dolphin thyroid gland is not possible due to the thick blubber and strong neck muscles. Although serum thyroid hormones are useful for studying thyroid function, frequency of blood sample monitoring may be limited due to the invasiveness of the procedure (Brook, 1997). To date, there is scant information on non-invasive, real-time assessment of dolphin thyroid morphology and adjacent neck structures. West and Ramer (2005) used ultrasound to assess the thyroid gland of 2 *Tursiops truncatus*. However, no details were given on the normal sonographic appearances of the dolphin thyroid gland as well as the adjacent neck structures. The present

study applied a simple scanning protocol that demonstrated the scanning techniques for repeatable thyroid ultrasound examination and documented the sonographic features of the normal dolphin thyroid gland and adjacent neck structures.

In the present study, a 5-2 MHz transducer was used. This was different from the ultrasound examination of the human thyroid gland in which a high frequency transducer (≥ 7.5 MHz) was used. The use of different transducers was because the presence of thick blubber on the animals requires an ultrasound beam with higher penetration and thus low frequency ultrasound was used. Moreover, curvilinear transducer is preferred in dolphin thyroid ultrasound because a larger field of view can be demonstrated in the thyroid gland with a single image scan plane, and allows the operator to see the relationship between the thyroid gland and adjacent neck structures.

The sonographic appearance of the dolphin thyroid gland correlated well with the gross anatomy previously reported (Cowan and Tajima, 2006) and was similar in all subjects. The borders of the thyroid gland were usually well-defined, since the dolphin thyroid gland was encapsulated, which provided a uniform surface for ultrasound beam reflection. An increase in age corresponds with a possible increase in adipose tissue deposition and connective tissue proliferation surrounding the thyroid gland, leading to a decrease in the acoustic impedance difference between the thyroid parenchyma and the adjacent soft tissues (Burroughs and Shenkman, 1982; Das et al., 2006), which may explain the ill-defined thyroid borders found in 2 older subjects.

Echogenicity of the thyroid varied from hypoechoic to isoechoic when compared with sternocephalicus muscle. The echogenicity also varied in different lobes or portions of a single thyroid gland. In the human thyroid, it appeared hyperechoic compared to the sternomastroid muscle (Ying et al., 1998). Documenting the relative echogenicity difference from the adjacent neck musculatures is of practical use for comparative analysis of the thyroid parenchyma, in particular of cases

concerning diffuse thyroid hypoechoenicity such as thyroiditis in human pathology. Changes in echogenicity could represent different thyroid abnormalities such as subclinical hypothyroidism and Hashimoto's thyroiditis (Schiemann et al., 2003; Vejbjerg et al., 2006). The normal echogenicity of the thyroid parenchyma is determined by the typical follicle structures (Müller et al., 1985). The interface between thyroid cells and the colloid exhibits high acoustic impedance, causing more ultrasound waves to be reflected back to the transducer. From a histological point of view, the normal dolphin thyroid cells appeared to be smaller than the normal human thyroid cells (Ridgway, 1972), resulting in a lower echogenicity. Thus dolphin thyroid glands with hypoechoic and isoechoic appearances may be considered to be normal. In addition, varied echogenicity at different lobes or portions of a single thyroid gland may be due to the uneven distribution of various sizes of follicles and colloid density within the single thyroid gland. A substantial amount of echogenic reticulations usually appeared in normal dolphin thyroid could be owed to the presence of fibrous bands which are considered to be normal and increasing in frequency with advancing age (Cowan and Tajima, 2006).

In the present study, 2 gross configurations of the thyroid gland were found in the studied population of dolphins. The first was Type A, in which both lobes of the thyroid gland were connected by an isthmus, and the second was Type C, in which there was a shield-like, diamond-shaped single mass placed ventrally on the trachea. Out of a possible 4 different configurations of the thyroid gland, only Type A and C were observed in the studied population of dolphins. It is unknown whether the configuration is determined by local environmental influences or possible progressive changes throughout thyroid development. Cowan and Tajima's study (2006) reporting the 4 different configurations of the dolphin thyroid gland used wild stranded specimens for investigation whereas the present study used a captive population of apparently healthy dolphins, with a mixture of captive-born and wild-caught subjects living in captivity for as long as approximately 30 years. Longitudinal studies should be performed on the thyroid gland of captive-born dolphins to investigate the possible factors affecting the configurations.

Major blood vessels in the neck region were visualized and identified by sonography. The significance of documenting these vessels lies in further applications. The dolphin's well-developed brain and its associated specialized anatomical and physiological adaptations to an aquatic environment have long attracted research interest. With the application of Doppler ultrasound, the hemodynamic studies such as vascular pattern, vascular resistance and blood flow velocity of these major blood vessels in the dolphin can be assessed without using invasive techniques such as angiography.

Besides the vasculatures, the cervical lymph nodes were also recognized by ultrasound. The lymph nodes were isoechoic to hypoechoic when compared to the adjacent sternocephalicus muscle due to the presence of loose lymphatic tissue. The lymph nodes were encapsulated by dense connective tissue with a number of elastic fibers, which maintained their round or oval shape (Vukovic et al., 2005). Most of the lymph nodes possessed an echogenic hilus due to the numerous interfaces from medullary cords and nodal vessels. These features all corresponded to those found in the majority of terrestrial mammals (Ying et al., 1996; Nyman and O'Brien, 2007). Cowan and Smith (1999) reported that as many as 10 nodes (6 – 8 nodes on average) could be found at the aortic arch region, and are always associated with the thymus and the thyroid gland. In the present study, only 2 – 3 nodes on average were found in each dolphin, possibly because of their echogenic similarities with surrounding fatty tissue. Ultrasound can aid in the differentiation of normal lymph nodes from abnormal lymph nodes in humans and companion animals by assessing their sonographic features and parameters such as size, shape, margin, echogenicity, homogeneity, vascular flow presence and distribution, and measurement of vascular flow indices (Ahuja and Ying, 2005; Nyman and O'Brien, 2007). Data regarding the cetacean lymph nodes are scarce (Romano et al., 1993; Cowan and Smith, 1999; Vukovic et al., 2005) and all studies were performed post-mortem. Further ultrasound study on the cervical lymph nodes in more living bottlenose dolphins is essential, so as to obtain a clear understanding of the distribution and sonographic

appearance of normal cervical lymph nodes. From this, a basis for the monitoring of disease progression or response to therapy over time can be established.

It is important to note that although the present study described the sonographic appearances of neck structures in the bottlenose dolphin over a 3 year period of time, all examinations were collected from the same population of bottlenose dolphins (n = 18) and therefore the findings of this study may be localized to this particular group of animals. Further study with a larger pool of subjects is suggested.

3.5 Conclusions

In conclusion, sonography provides a reliable and repeatable method in assessing the thyroid morphology of dolphins. With practice, locating the thyroid gland for routine clinical and follow-up examinations is quick and easy. The technique can also be applied to other cetacean species, including Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) and beluga whales (*Delphinapterus leucas*). Established knowledge of the normal sonographic anatomy of the dolphin thyroid gland and adjacent neck structures will provide a normative reference to clinically recognize and treat thyroid gland abnormalities in living dolphins. This information would also be helpful for facilities in attaining self-sustainability and allowing optimal captivity management. Developed imaging techniques with consistent and reproducible monitoring of the cetacean thyroid gland during treatment could provide valuable insight to better understand thyroid disorders in wild cetaceans.

Chapter Four

Study Two

Evaluation of 2-D and 3-D ultrasound in the assessment of the thyroid volume of the Indo-Pacific Bottlenose dolphin, *Tursiops aduncus*

4.1 Introduction

The mammalian thyroid gland produces thyroid hormones to maintain the basal metabolism of the tissues and homeostasis in response to environmental demands (Hegedüs, 2001; Myers et al., 2006). Therefore, thyroid illness may cause abnormalities in other organs of the body. In veterinary medicine, thyroid disorders have been reported in many species of wildlife (Wallach, 1970; Kaptein et al., 1994; Colborn, 2002; Reese et al., 2005), but rarely in marine mammals. Environmental contaminants and local environmental influences have been implicated in thyroid hormone imbalances (Cowan and Tajima, 2006) and the development of morphological and histological abnormalities (Schumacher et al., 1993; Mikaelian et al., 2003; Das et al., 2006) associated with calf mortality (Garner et al., 2002). The gross and histological features of the thyroid in stranded Atlantic bottlenose dolphins have been previously described and it was found that 31 out of 60 animals suffered from different thyroid abnormalities (Cowan and Tajima, 2006). To the best of our knowledge, the formal literature is devoid of any reference to the diagnosis of thyroid abnormalities in living dolphins. Clinically, the diagnosis of thyroid abnormalities in dolphins is usually based on biochemical test of thyroid hormones and clinical examination. However, the assessment of thyroid morphology may provide additional information in the diagnosis and management of thyroid diseases. In the morphological evaluation of thyroid glands, the assessment of thyroid volume plays an indispensable role in the diagnosis and management of different thyroid diseases (Berghout and Wiersinga, 1998;

Miyakawa et al., 1992). In order to accurately diagnose and monitor thyroid diseases, reliable methods for assessing the thyroid size must be developed to correlate with existing biochemical and clinical data.

Ultrasound is a useful imaging tool in the assessment of thyroid morphology and physiology in humans (Hegedüs, 2001; AIUM, 2003; Khati et al., 2003) and companion animals (Cartee et al., 1993; Wisner et al., 2002; Reese et al., 2005; Brömel et al., 2006). High resolution ultrasound measurement of thyroid volume is a non-invasive, repeatable and readily accessible method in the assessment of thyroid size (Loevner, 1996; Barraclough and Barraclough, 2000; Khati et al., 2003). In human clinical practice, the ultrasound thyroid volume assessment is usually performed by measuring the craniocaudal (length), mediolateral (width) and dorsoventral (thickness) dimensions of the thyroid lobe, and calculating the volume by an ellipsoid formula (Brunn et al., 1981). However, from a pool of stranded Atlantic bottlenose dolphins, the thyroid gland has been categorized into 4 different gross configurations (Cowan and Tajima, 2006). Thus, it is essential to customize the ultrasound thyroid volume measurement method, with respect to different dolphin thyroid configurations, for the sake of measurement accuracy.

The only published report of dolphin thyroid ultrasound stated that measurements were extremely variable “because of skill differences between operators” (West and Ramer, 2005). Previous human studies have shown that ultrasound appears to largely underestimate thyroid volume compared with the actual volume of an excised gland specimen (Malago et al., 2008). There is scant information in the literature about ultrasound measurement of thyroid size in dolphins. Previous research focused on determining anatomical landmarks and fewer measurements for the assessment of thyroid size rather than establishing a standardised method for dolphin thyroid volume measurement (West and Ramer, 2005). Thus, an accurate and reliable method is needed for the ultrasound measurement of the dolphin thyroid gland.

With the advancement of imaging technology, three-dimensional (3-D) ultrasound is available for the volumetric assessment of the thyroid gland. Previous studies on humans revealed that 3-D ultrasound volume measurement of the thyroid gland yielded higher correlation with the volume of a dissected thyroid gland, than the results obtained from the 2-D ellipsoid method (Lyshchik et al., 2004a; Malago et al., 2008). Although 3-D ultrasound demonstrated higher accuracy and reliability in volumetric measurements, both *in vivo* and *in vitro* (Riccabona et al., 1995; Vade et al., 1997; Kot et al., 2009), ultrasound units with 3-D capacity are often not available in aquarium settings.

The present study compared the accuracy of dolphin thyroid volume as determined by four 2-D ultrasound measurement methods, with the standard of reference determined by the 3-D ultrasound measurement method. The present study also assessed the inter- and intra-operator variability of the four 2-D ultrasound and the 3-D ultrasound measurement methods. The accuracy of different 2-D ultrasound methods in measuring thyroid glands with various configurations was also investigated.

4.2 Materials and Methods

4.2.1 Part I: *In vivo* measurement of the dolphin thyroid glands

A total of 16 *Tursiops aduncus*, at Ocean Park, Hong Kong (6 males between 3-26 years and 10 females between 2-35 years) were included in the study. At the beginning of the study, 11 subjects were sexually mature and 5 were immature. The sexual maturation of the female dolphins was determined by the first observation of ovarian activity, while sexual maturation of male dolphins was determined by the first presence of spermatozoa in the semen, indicating the onset of spermatogenesis. All dolphins involved in the study were trained to cooperate for thyroid ultrasound examination. The subjects were apparently healthy with no recent history of

illnesses, and were not receiving medication known to alter thyroid gland physiology during the time of the study. Serum concentrations of thyroxine (free [fT4] and total [tT4]), triiodothyronine (free [fT3] and total [tT3]) were also determined on each individual subject and the values were all within normal ranges (St. Aubin, 2001).

All ultrasound examinations were performed with a Philips HD-11 or a Philips HD-11 XE ultrasound unit, in conjunction with a 6-2 MHz curvilinear 3-D broadband curved array transducer and a 5-2 MHz 2-D broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA). All images were recorded digitally. Because of the nature of the dolphin's skin, there was no air layer between the surface and the transducer, so no coupling gel was required.

During the period of August 2006 and December 2008, a total of 856 individual ultrasound scans were conducted in 16 *Tursiops aduncus* with the use of the 2-D and 3-D ultrasound thyroid volume measurement methods. All 2-D and 3-D ultrasound measurements were performed by the same operator (BK). Each session of ultrasound examination begun with the 2-D ultrasound measurements (Methods A – D), followed by the 3-D ultrasound measurement (Method E). Images were acquired at poolside scanning. All images required for measurements of a single subject were collected at the same session. After the poolside scanning, archived images were retrieved from the hard disk of the ultrasound unit for the measurement of thyroid volume.

All ultrasound examinations of the thyroid gland were performed with the dolphin positioned in dorsal recumbence close to the poolside, with its tail supported by a trainer. The transducer was initially placed in a transverse orientation at the thoracic inlet, midway between the insertions of the pectoral flippers. The transducer was moved cranially until the brachiocephalic vein was identified. The transducer was then moved further cranially until the left and right lobes of the thyroid gland were identified. Five methods (A – E) were used to measure the thyroid volume.

4.2.1.1 Scanning technique, volumetric measurement and calculation using Methods A and B

Once the location of the thyroid gland was identified, the transducer was moved cranially and caudally until the scan plane showing the maximum transverse dimension of the thyroid gland (TS_MAX) was obtained and the TS_MAX was then measured (Figure 4.1). The transducer was then rotated 90°, to show the longitudinal scan planes of the thyroid gland. A full survey of the thyroid gland was performed in the longitudinal scan with the transducer moved from the left lobe to the right lobe. Images of 3 longitudinal scan planes were recorded (Figures 4.2a-c): 1. scan plane showing the midline of the thyroid gland (LS_MID); 2. scan plane showing the maximum longitudinal dimension of the left lobe (LS_L); 3. scan plane showing the maximum longitudinal dimension of the right lobe (LS_R). The scanning technique was identical for Methods A and B. The dorsoventral dimension and the craniocaudal dimension were measured (for Method A), and the cross-sectional area of the thyroid lobe were measured (for Method B) in each longitudinal scan plane, respectively.

The thyroid volume was calculated using a proposed ellipsoid equation (Brunn et al., 1981), i.e. volume = $\pi/6$ x craniocaudal x mediolateral x dorsoventral dimensions (for Method A); and its derivative using the cross-sectional area (for Method B) (Shabana et al. 2003) (Table 4.1).

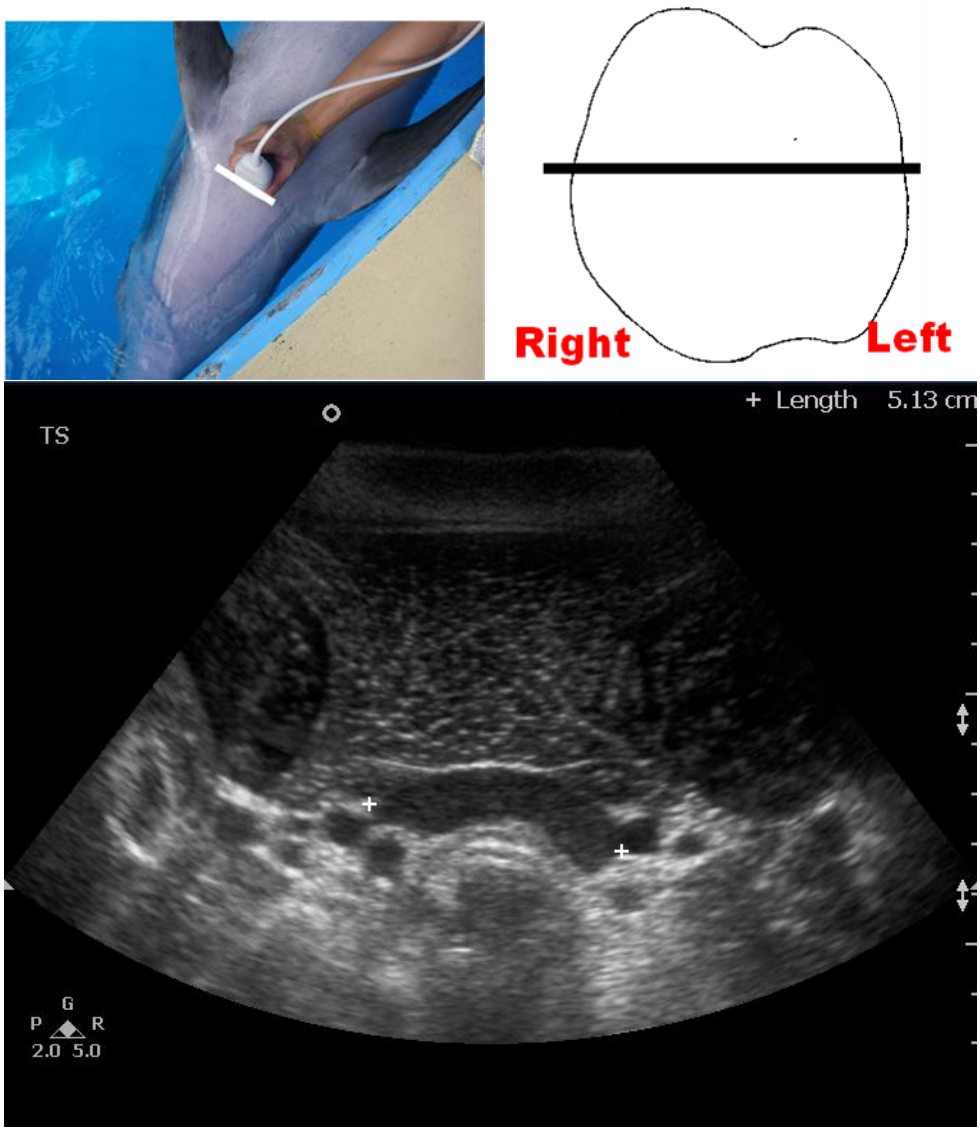

Figure 4.1: Images show the ultrasound measurement of the maximum transverse dimension of the dolphin thyroid gland (TS_MAX). Top left picture shows the position of the transducer at the neck region. Top right picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows a transverse grey scale sonogram of the thyroid gland of a bottlenose dolphin. Note the maximum transverse dimension of the thyroid gland is measured (calipers +).

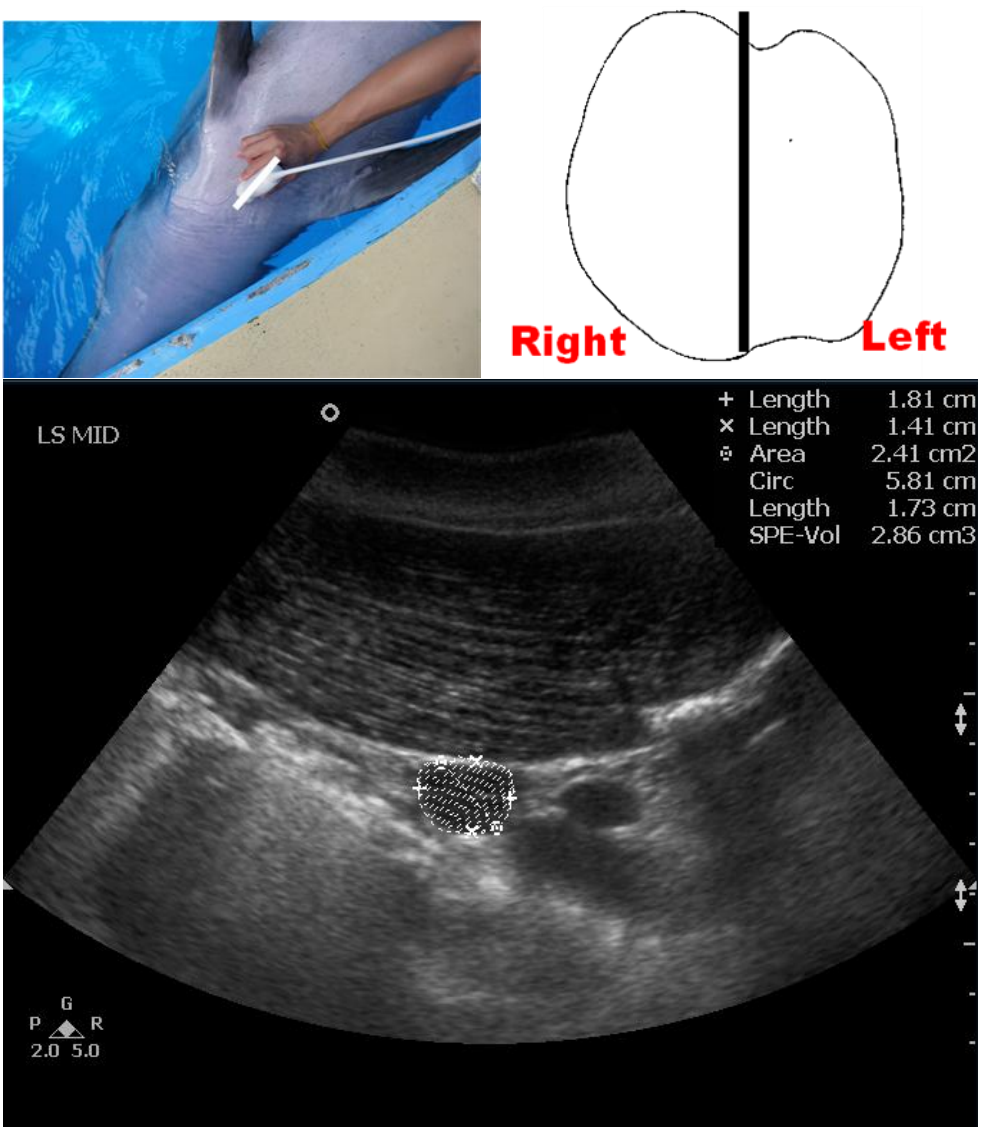

Figure 4.2a: Images show the ultrasound measurement of the longitudinal dimension of the dolphin thyroid gland at the midline (LS_MID). Top left picture shows the position of the transducer at the neck region. Top right picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows a longitudinal grey scale sonogram of the thyroid gland of a bottlenose dolphin. Note the dorsoventral dimension (calipers x), the craniocaudal dimension (calipers +) and the cross-sectional area (dotted line) are measured respectively.

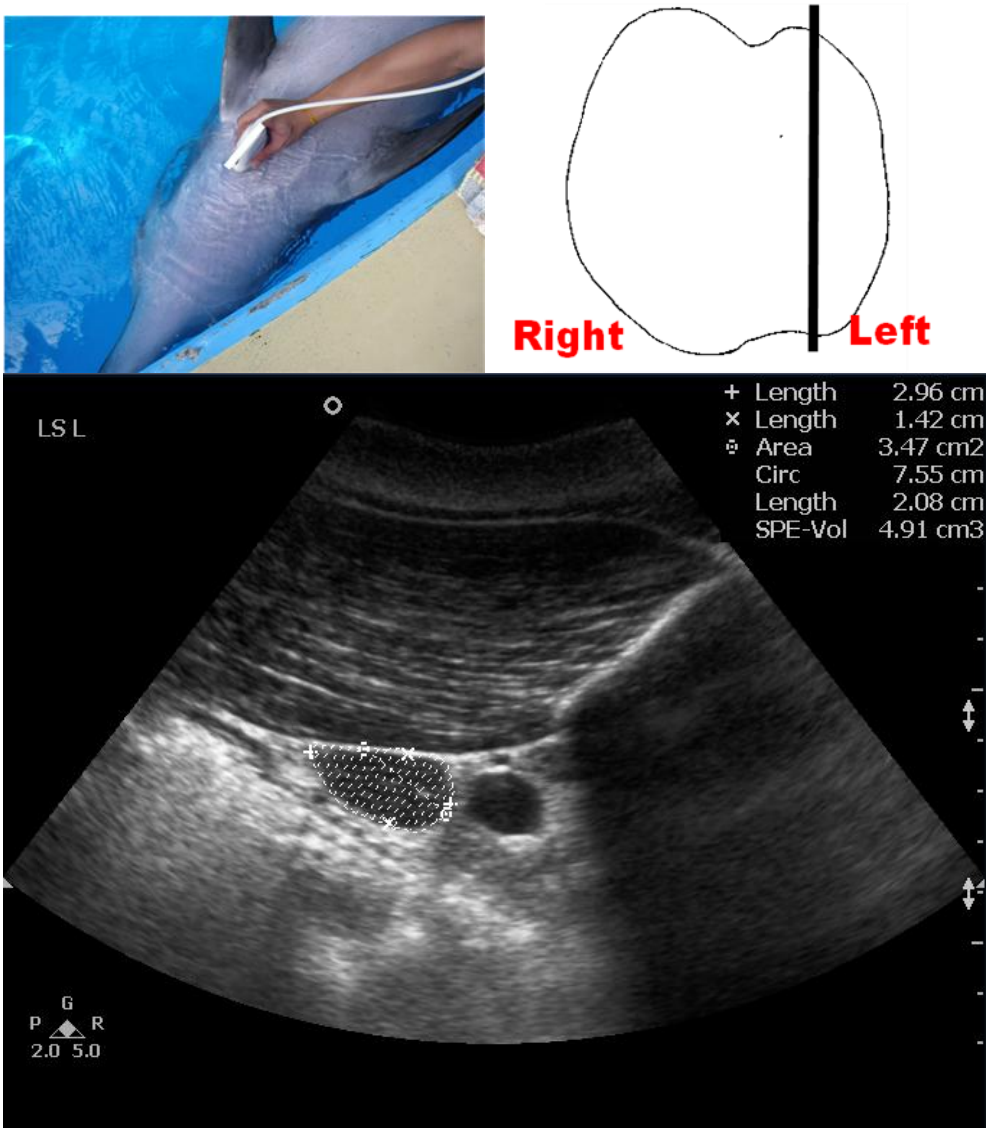

Figure 4.2b: Images show the ultrasound measurement of the maximum longitudinal dimension of the left thyroid lobe of a dolphin (LS_L). Top left picture shows the position of the transducer at the neck region. Top right picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows a longitudinal grey scale sonogram of the left thyroid lobe of a bottlenose dolphin. Note the maximum longitudinal dimension of the left thyroid lobe is demonstrated, and the dorsoventral dimension (calipers x), the craniocaudal dimension (calipers +) and the cross-sectional area (dotted line) are measured respectively.

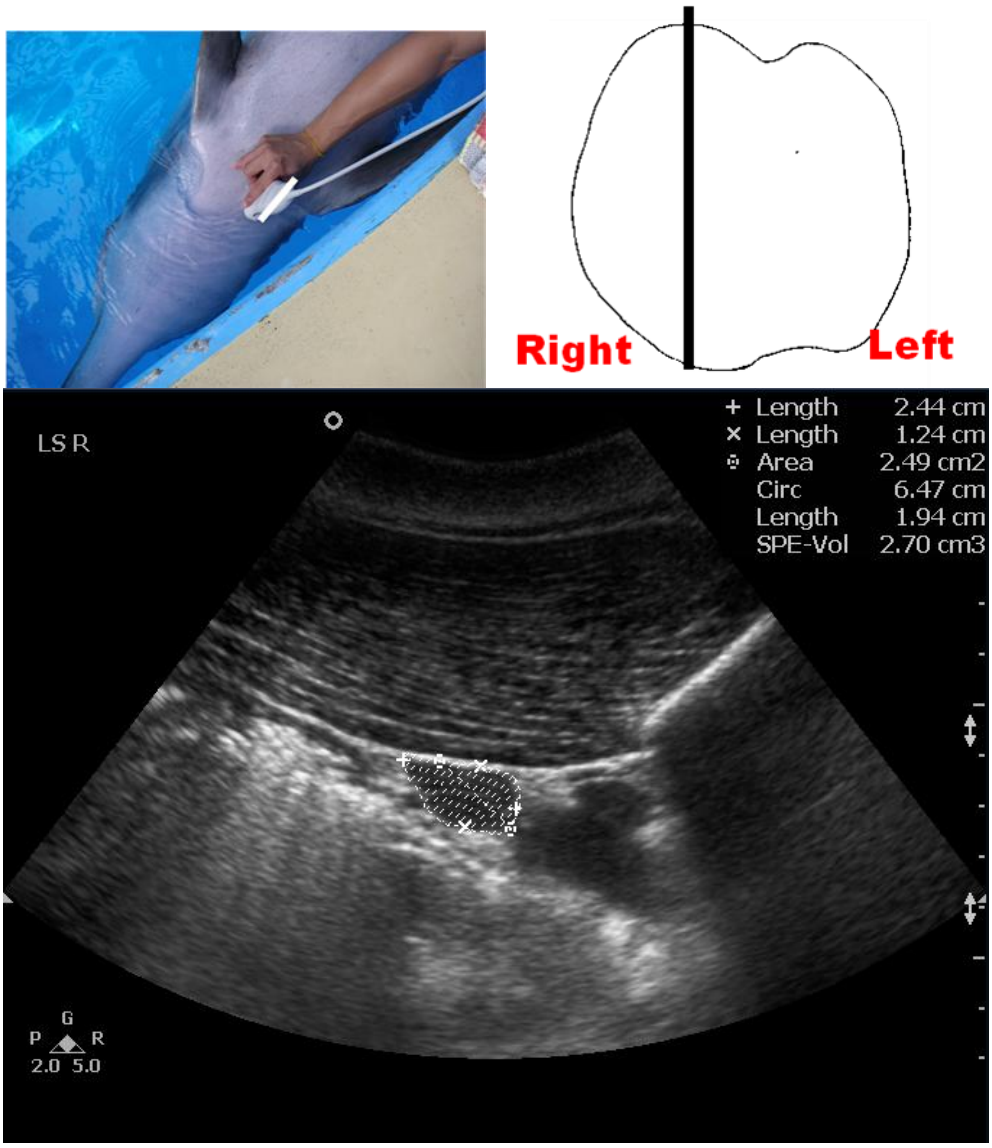

Figure 4.2c: Images show the ultrasound measurement of the maximum longitudinal dimension of the right thyroid lobe of a dolphin (LS_R). Top left picture shows the position of the transducer at the neck region. Top right picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows a longitudinal grey scale sonogram of the right thyroid lobe of a bottlenose dolphin. Note the maximum longitudinal dimension of the right thyroid lobe is demonstrated, and the dorsoventral dimension (calipers x), the craniocaudal dimension (calipers +) and the cross-sectional area (dotted line) are measured respectively.

4.2.1.2 Methods C and D

The transducer was initially placed obliquely on one side of the thyroid gland and then the transducer was slightly rotated clockwise and counterclockwise until the image showing the longest axis of the thyroid lobe was identified and recorded. The long axis of the thyroid lobe was then measured (Figure 4.3). The transducer was then rotated 90° to show the cross-sectional image of the thyroid lobe. A full survey of the cross-sectional image of the thyroid lobe was performed by scanning from the upper to lower poles of the thyroid gland. The scanning technique was identical for Methods C and D. The dorsoventral dimension and the mediolateral dimension were measured (for Method C), and the cross-sectional area of the thyroid lobe was measured (for Method D) (Figure 4.4), on the scan plane showing the maximum cross-sectional area of the thyroid lobe, respectively. The same scanning protocol was repeated for the contralateral thyroid lobe

The thyroid volume was calculated using a proposed ellipsoid equation (Brunn et al., 1981), i.e. volume = $\pi/6$ x craniocaudal x mediolateral x dorsoventral dimensions (for Method A); and its derivative using the cross-sectional area (for Method B) (Shabana et al. 2003) (Table 4.1).

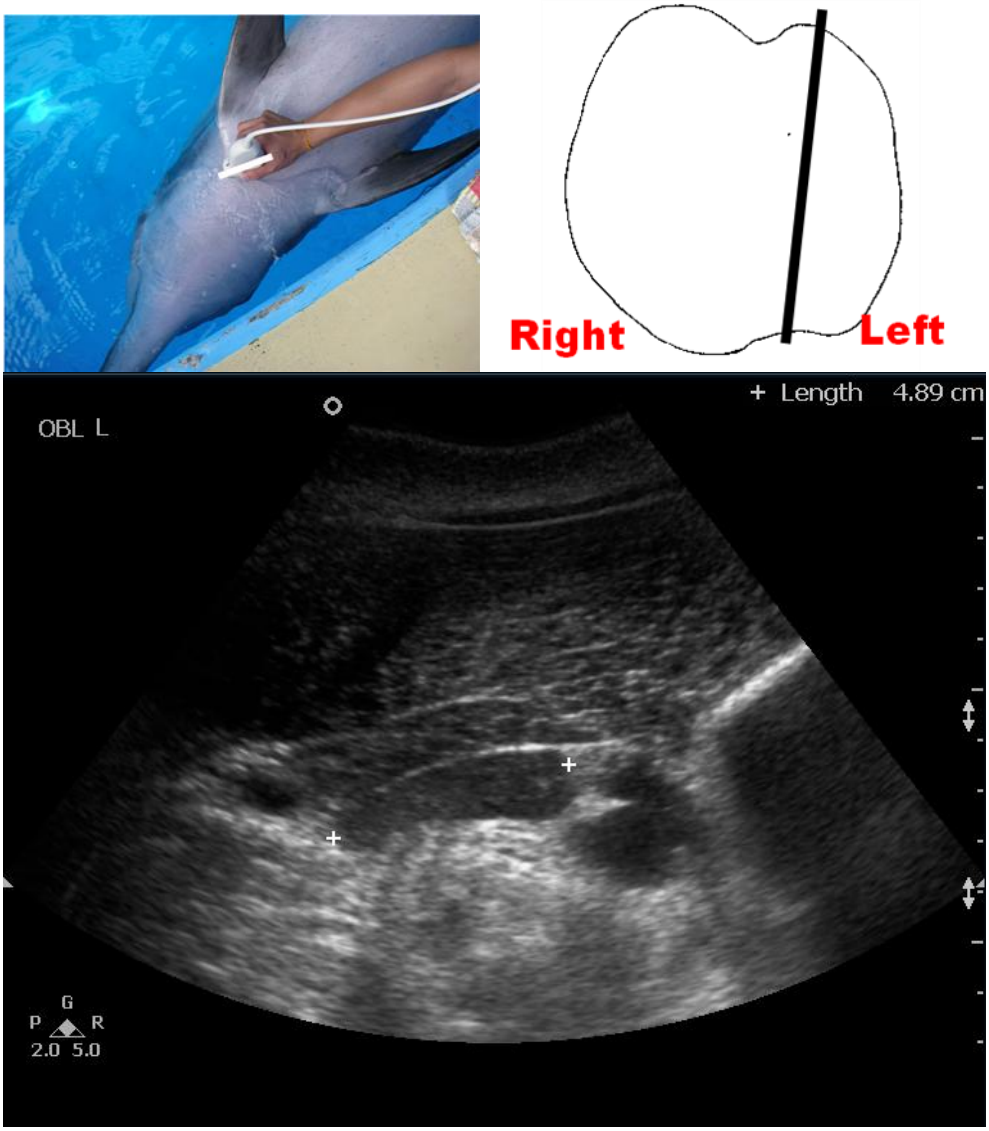

Figure 4.3 Images show the ultrasound measurement of the long axis of the left thyroid lobe of a dolphin. Top left picture shows the position of the transducer at the neck region. Top right picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows an oblique grey scale sonogram of the left thyroid lobe of a bottlenose dolphin. Note the long axis of the left thyroid lobe is measured (calipers +).

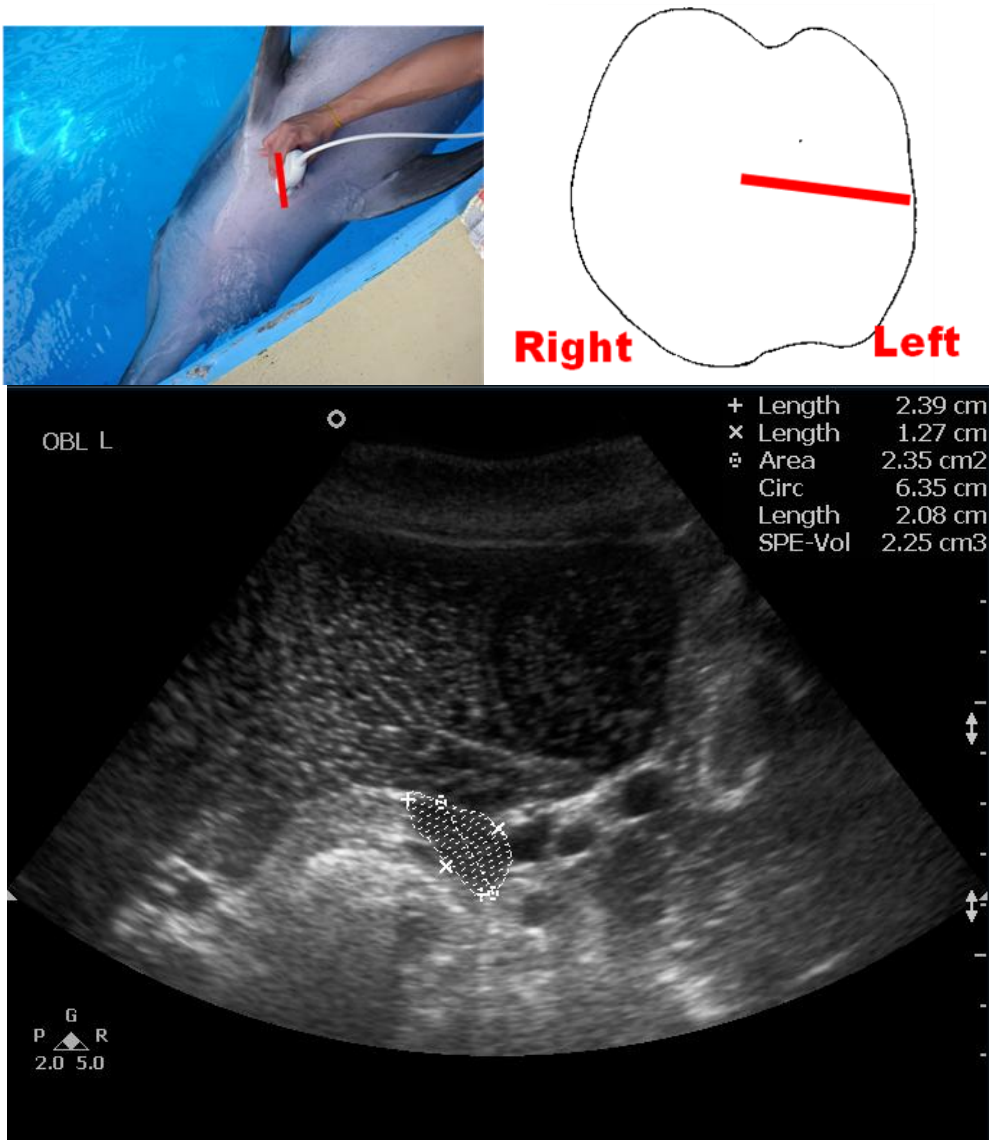

Figure 4.4 Images show the ultrasound measurement of the maximum cross-sectional area of the left thyroid lobe of a dolphin. Top left picture shows the position of the transducer at the neck region. Top right picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows an oblique grey scale sonogram of the left thyroid lobe of a bottlenose dolphin. Note the maximum cross-sectional area of the left thyroid lobe is demonstrated, and the dorsoventral dimension (calipers x), the mediolateral dimension (calipers +) and the cross-sectional area (dotted line) are measured respectively.

Table 4.1: Equations of each method for calculating the thyroid volume

Method	Equation for calculation of thyroid volume
A (2-D US[f])	π/6 x TS_MAX[a] x mean of craniocaudal dimension in 3 planes (LS_L[b] ,LS_MID[c] and LS_R[d]) x mean of dorsoventral dimension in 3 planes (LS_L[b] ,LS_MID[c] and LS_R[d])
B (2-D US[f])	2/3 x TS_MAX[a] x mean of cross-sectional area of 3 planes (LS_L[b] ,LS_MID[c] and LS_R[d])
C (2-D US[f])	π/6 x craniocaudal x mediolateral x dorsoventral
D (2-D US[f])	2/3 x craniocaudal x maximum cross-sectional area[e]
E (3-D US[g])	Calculated by built-in software (QLAB, Philips)

[a]The maximum transverse dimension of the thyroid gland.
[b]The maximum longitudinal scan plane of the left thyroid lobe.
[c]The longitudinal scan plane of the midline of the thyroid gland.
[d]The maximum longitudinal scan plane of the right thyroid lobe.
[e]π/4 x mediolateral x dorsoventral .
[f]Two-dimensional ultrasound.
[g]Three-dimensional ultrasound.

4.2.1.3 Method E

For the 3-D ultrasound measurement method (Method E), the thyroid gland was initially identified by 2-D ultrasound as described above (Figure 4.5). The 3-D data acquisition function was activated and the mechanical scanner inside the transducer scanned through the thyroid gland in a single sweep with a sweeping angle of 50º, which was adequate to encompass the entire thyroid gland. The 3-D images were then reconstructed automatically by the built-in software. The 3-D image set was stored in the hard disk of the ultrasound unit. After the poolside scanning, the 3-D image set was retrieved and the thyroid volume was measured with the specific quantification software program, QLAB 5.0 (Philips Medical System, Bothell, Washington, 98021, USA). The quantification software program utilized the manual planimetry volume measurement technique (the 'parallel planes' approach) (Kot et al., 2009). In this volume measurement approach, the 3-D image was sliced to obtain 8 image planes, which were both parallel and equally spaced along the transverse axis of the thyroid gland. The boundaries of the thyroid gland were then outlined manually in each image plane (Figure 4.6). The areas measured in each image plane were summed and multiplied by the inter-slice distances to calculate the thyroid volume.

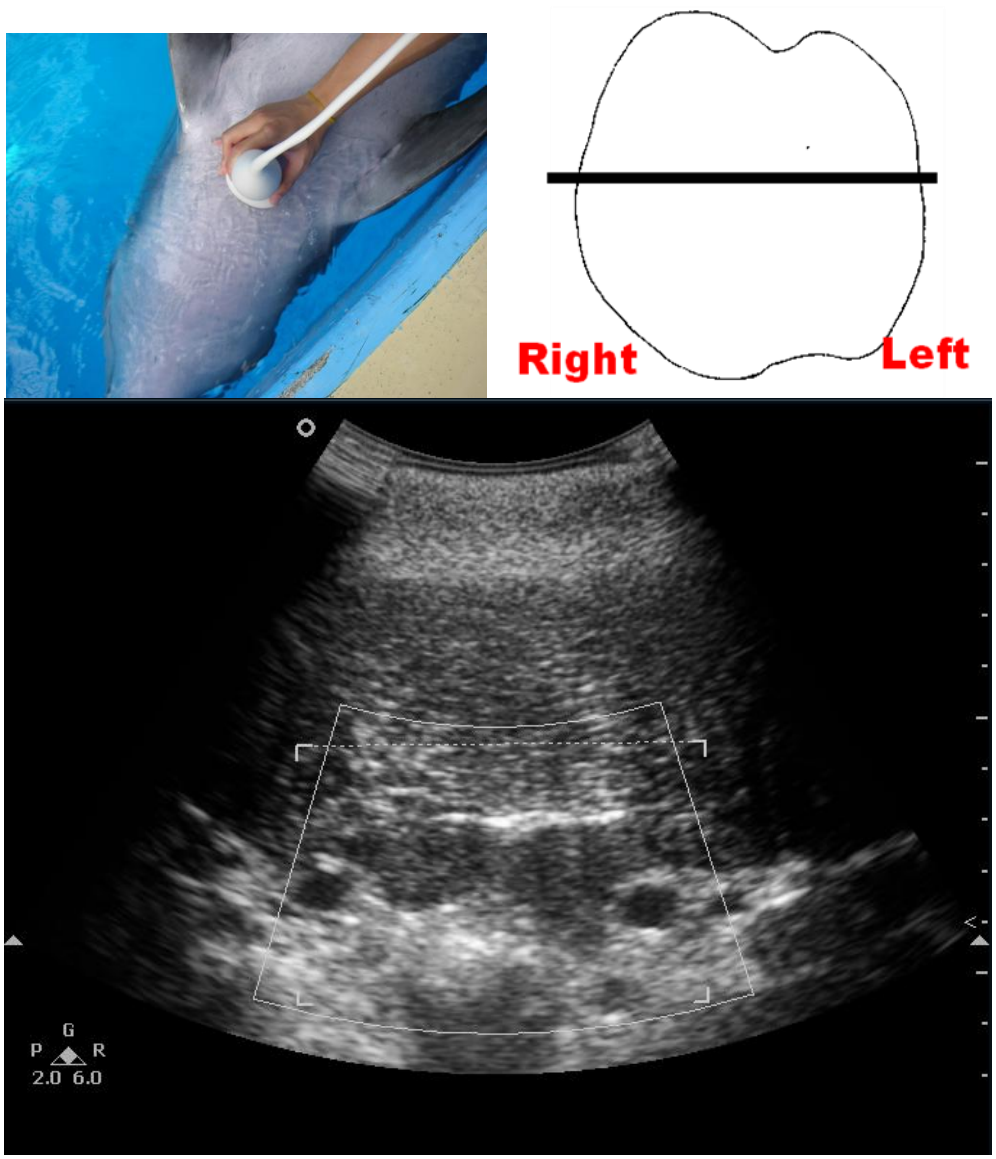

Figure 4.5 Images show the 3-D ultrasound measurement of the thyroid volume of a dolphin. Top left picture shows the position of the transducer at the neck region. Top corner picture shows the schematic diagram of the thyroid gland in a coronal orientation with the straight line representing the position of the transducer. Bottom image shows a transverse grey scale sonogram of the transverse scan plane of the thyroid gland of a bottlenose dolphin before the 3-D data acquisition, with the white sector-shaped box outlining the area for 3-D data acquisition.

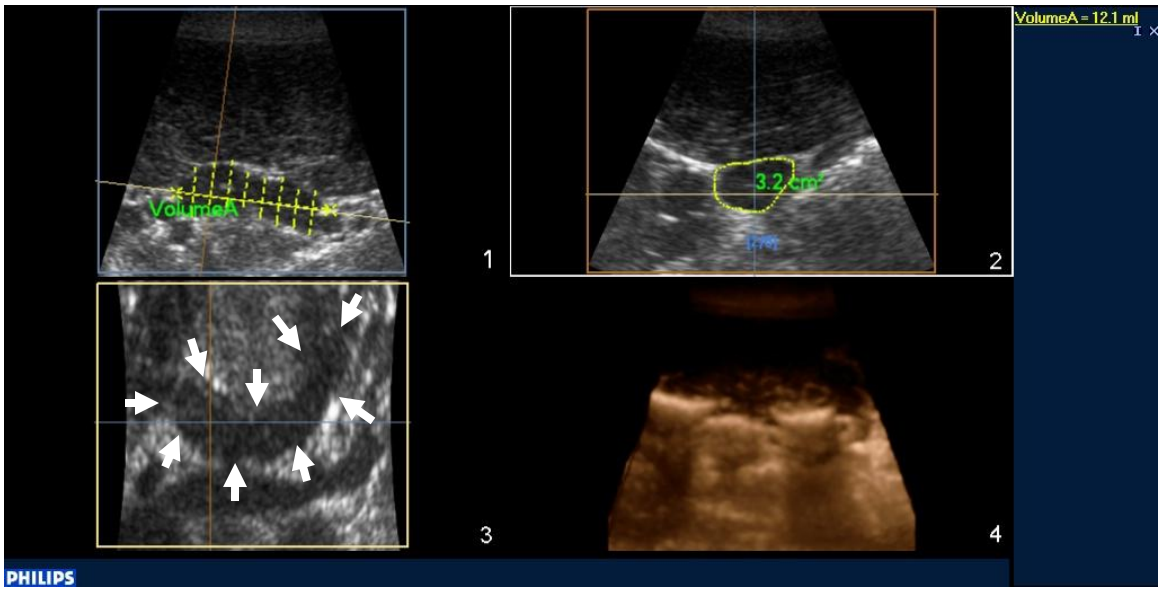

Figure 4.6 Thyroid volume measurement with 3-D ultrasound (Method E). 1. Image demonstrates the transverse plane of the thyroid gland, with the 8 equal distance slices indicated by the yellow dotted lines. 2. Image demonstrates the reconstructed longitudinal plane of the thyroid gland. Note the boundaries of the thyroid gland are outlined with a yellow dotted line. 3. Image demonstrates the reconstructed coronal planes of the thyroid gland (white arrows). 4. Image demonstrates the reconstructed 3-D model of the region of interest by the 3-D transducer, with a sweeping angle of 50°. The volume of the thyroid gland was calculated by the built-in software and is shown in the upper right corner in milliliters (mL).

To evaluate the accuracy of different 2-D ultrasound methods in measuring thyroid glands with different configuration, the thyroid glands of the 16 subjects were categorized into 1 of 4 different gross configurations as defined by Cowan and Tajima (2006):

Type A: two lobes joined by an isthmus

Type B: two separate lobes, usually of equal size, one on each side of the trachea, with no connecting isthmus

Type C: a shield-like, single mass, roughly diamond-shaped, placed ventrally on the trachea

Type D: irregular, multilobular grape cluster-like mass, with adjacent but separate lobules.

4.2.2 Part II: *In vitro* measurement of the dolphin thyroid glands

Evaluation of the accuracy of the 3-D ultrasound thyroid measurement method was also performed using fresh dolphin thyroid specimens. 3-D ultrasound examinations were performed on 2 dolphin thyroid specimens shortly after the subjects deceased. The glands were carefully dissected away from the trachea. A water displacement method was used to determine the actual volume of the dissected thyroid glands. The glands were then immersed into a glass beaker filled with distilled water, and the same 3-D ultrasound measurement technique for the live dolphins (Method E) was used.

4.2.3 Part III: Reliability of ultrasound measurements

Three operators (BK, MY, RK) scanned the thyroid gland of the 12 subjects twice in the same session to investigate the measurement reliability of Methods A – E. Standardized scanning and measurement protocols as described above were used. The 3 operators have extensive experience in ultrasound scanning of either humans or zoo animals. Each operator was blinded to the results of the other operators. The operators were also blinded to their previous measurements when they were performing the measurement of the same subject. There was a time interval of at least 30 minutes between measurements of the 2 sets of images on the same subject. Therefore, recall bias of the results on the same subject was negligible.

4.2.4 Statistical Analysis

To evaluate the accuracy of the four 2-D ultrasound measurement methods (Methods A-D) in measuring the thyroid volume, the measured volumes were compared with the volumes measured by 3-D ultrasound (Method E) and the absolute percentage error was calculated. The absolute percentage error of each 2-D

ultrasound measurement method was defined as the percentage difference between the volume measured with the 2-D ultrasound method and volume measured by 3-D ultrasound:

Absolute percentage error (%) =

$$\frac{\left| \text{Volume measured by 3-D ultrasound} - \text{Volume measured by 2-D ultrasound} \right|}{\text{Volume measured by 3-D ultrasound}} \times 100\%$$

The accuracy of the 2-D ultrasound measurement method was defined as:

Accuracy (%) = 1 - Absolute percentage error

The systematic error of each of the four 2-D ultrasound measurement methods was defined as the mean percentage difference between the 2-D and 3-D ultrasound measurements [100 x (3-D ultrasound measured volume – 2-D measured volume)/ 3-D ultrasound measured volume], whereas the standard deviations of the percentage differences were defined as the random errors.

Repeated measures analysis of variance (ANOVA) was used to calculate the level of significance of the variation of percentage difference in measurements between the 3-D and four 2-D ultrasound measurement methods and Tukey's multiple comparison test was used as the post hoc test.

The inter-operator (reproducibility) and intra-operator (repeatability) variability of Methods A - E were evaluated by the intraclass correlation coefficient (ICC) and limits of agreement. An ICC > 0.7 indicates sufficient reliability of the measurements (Khan and Chien, 2001a; 2001b).

All statistical analyses were performed using the SPSS 16.0 software (Statistical Package for the Social Sciences Inc., Chicago, Illinois, 60606, USA).

4.3 Results

4.3.1 Part I: *In vivo* measurement of the dolphin thyroid glands

All 2-D ultrasound thyroid volume measurement methods yielded similarly high accuracies (79.9% - 81.3%) (Table 4.2). There was no significant difference in absolute percentage error among the four 2-D ultrasound thyroid volume measurement methods ($F = 2.202$, $p > 0.05$). The mean and SD of the 3-D ultrasound thyroid volume measurement method, Method E was 15.7 mL and 5.1 mL, respectively.

Table 4.2: Comparison of the accuracy of different 2-D ultrasound thyroid volume measurement methods including the significance level among Methods A - D, using repeated measures ANOVA with post hoc.

Methods[a]	Mean thyroid volume ± 1 SD (mL)	Mean absolute error (%)	Measurement accuracy compared with Method E (%)	Systematic error (%)	Random error (%)
A	13.36 ± 4.66	18.70	81.30	14.07	11.86
B	13.22 ± 4.49	19.12	80.88	14.83	11.89
C	17.55 ± 6.48	20.12	79.88	-12.73	16.75
D	17.20 ± 5.87	18.89	81.11	-11.19	15.35
E	15.73 ± 5.08	-	-	-	-

Methods Compared	p value
A vs B	$p > 0.05$
A vs C	$p < 0.01$
A vs D	$p > 0.05$
B vs C	$p < 0.05$
B vs D	$p > 0.05$
C vs D	$p > 0.05$

[a] Refer to Table 4.1 for the equations of each method for calculating the thyroid volume

In the 16 subjects, 9 subjects had a thyroid gland in type A configuration, and 7 subjects had a thyroid gland in type C configuration. For the thyroid glands in type A configuration, Methods A and B had a higher measurement accuracy (83.7% and 82.9% respectively) than Methods C and D (77.5% and 79.4% respectively), using Method E as the standard of reference. There were significant differences in absolute percentage error among four 2-D ultrasound thyroid volume measurement methods ($F = 11.073$, $p < 0.0001$). The mean absolute percentage error of the measurements of Methods A and B was significantly smaller than that of Methods C and D ($p < 0.01$). However, no significant difference in absolute percentage error was found between Method A and Method B ($p > 0.05$) (Table 4.3).

Table 4.3: Type A Configuration-based analysis of the methods' accuracy of the volume measurements using four 2-D ultrasound dolphin thyroid measurement methods, including the significance level among Methods A – D.

Methods[a]	Mean thyroid volume ± 1 SD (mL)	Mean absolute error (%)	Measurement accuracy compared with Method E (%)	Systematic error (%)	Random error (%)
A	11.48 ± 3.80	16.31	83.69	5.80	19.97
B	11.58 ± 4.16	17.15	82.85	5.77	20.82
C	14.18 ± 4.22	22.53	77.47	-17.36	22.15
D	14.04 ± 4.31	20.63	79.37	-15.73	20.64
E	12.44 ± 4.13	-	-	-	-

Methods Compared	p value
A vs B	$p > 0.05$
A vs C	$p < 0.001$
A vs D	$p < 0.001$
B vs C	$p < 0.001$
B vs D	$p < 0.01$
C vs D	$p > 0.05$

[a] Refer to Table 4.1 for the equations of each method for calculating the thyroid volume

Table 4.4: Type C Configuration-based analysis of the methods' accuracy of the volume measurements using four 2-D ultrasound dolphin thyroid measurement methods, including the significance level among Methods A – D

Methods[a]	Mean thyroid volume ± 1 SD (mL)	Mean absolute error (%)	Measurement accuracy compared with Method E (%)	Systematic error (%)	Random error (%)
A	13.77 ± 4.73	19.22	80.78	15.89	15.86
B	13.58 ± 4.48	19.56	80.44	16.81	15.30
C	18.29 ± 6.65	19.59	80.41	-11.71	22.93
D	17.89 ± 5.94	18.51	81.49	-10.19	21.72
E	16.46 ± 4.99	-	-	-	-

Methods Compared	p value
A vs B	$p > 0.05$
A vs C	$p > 0.05$
A vs D	$p > 0.05$
B vs C	$p > 0.05$
B vs D	$p > 0.05$
C vs D	$p > 0.05$

[a] Refer to Table 4.1 for the equations of each method for calculating the thyroid volume

For the thyroid glands in type C configuration, all 2-D ultrasound thyroid volume measurement methods (Methods A - D) yielded similar high accuracies (80.4% – 81.5%), using Method E as the standard of reference. There was no significant difference in absolute percentage error among the four 2-D ultrasound thyroid volume measurement methods ($F = 1.098$, $p > 0.05$) (Table 4.4).

4.3.2 Part II: *In vitro* measurement of the dolphin thyroid glands

One of the 2 dissected dolphins suffered from severe acute cardiomyopathy, while the other had viral infection of the liver leading to acute hepatic necrosis. Pathological reports revealed that both dolphin thyroid glands appeared active and normal cellular architectures were demonstrated. The actual thyroid volumes measured 20 mL and 32 mL respectively, whereas the volumes measured with the 3-D ultrasound measurement method were 18.70 mL and 30.94 mL respectively, indicating a mean measurement accuracy of 95.1% for Method E *in vitro*.

4.3.3 Part III: Reliability of ultrasound measurements

The inter-operator variability (reproducibility) of the different ultrasound thyroid volume measurement methods is shown in Table 4.5. Three-dimensional ultrasound (Method E, ICC = 0.862) had a higher reproducibility than 2-D ultrasound (Methods A-D, ICC = 0.776 - 0.811). For the different 2-D methods, Method D had the highest reproducibility (81.1%) (ICC = 0.811, upper bound 95% C.I. = 0.935; lower bound 95% C.I. = 0.589).

The intra-operator variability (repeatability) of the different ultrasound thyroid volume measurement methods is shown in Table 4.6. All methods yielded high repeatability (ICC = 0.781 - 0.997), indicating that all methods were repeatable by the 3 operators.

Table 4.5: Reproducibility of the different ultrasound thyroid measurement methods.

Method[a]	ICC (2,1)	95% C.I. of ICC (Lower - Upper)
A	0.776	0.455 - 0.926
B	0.808	0.449 - 0.941
C	0.782	0.538 - 0.924
D	0.811	0.589 - 0.935
E	0.862	0.688 - 0.954

[a] Refer to Table 4.1 for the equations of each method for calculating the thyroid volume.

Table 4.6: Evaluation of the repeatability of the four 2-D ultrasound dolphin thyroid measurement methods (Methods A-D) and one 3-D ultrasound dolphin thyroid measurement method (Method E).

Operator 1

Method[a]	ICC (3,1)	95% C.I. (Lower - Upper)	Range of measurement error (mL)	95% limit of agreement for difference in measurement
A	0.96	0.867 - 0.988	-1.82 - 2.34	-2.48 - 3.12
B	0.971	0.904 - 0.992	-1.23 - 2.00	-1.94 - 2.60
C	0.925	0.762 - 0.978	-2.98 - 4.26	-4.76 - 4.82
D	0.94	0.805 - 0.982	-2.35 - 3.86	-3.97 - 4.32
E	0.997	0.991 - 0.999	-0.20 - 1.40	-0.74 - 0.99

Operator 2

Method[a]	ICC (3,1)	95% C.I. of ICC	Range of measurement error (mL)	95% limit of agreement for difference in measurement
A	0.878	0.632 - 0.963	-6.19 - 2.90	-6.49 - 3.13
B	0.888	0.663 - 0.967	-6.50 - 2.50	-6.31 - 3.20
C	0.949	0.833 - 0.985	-3.37 - 3.82	-4.35 - 4.13
D	0.96	0.867 - 0.988	-2.21 - 5.26	-3.88 - 4.43
E	0.992	0.974 - 0.998	-1.10 - 1.60	-1.27 - 1.34

Operator 3

Method[a]	ICC (3,1)	95% C.I. of ICC	Range of measurement error (mL)	95% limit of agreement for difference in measurement
A	0.945	0.821 - 0.984	-1.68 - 2.06	-2.36 - 2.18
B	0.941	0.81 - 0.983	-1.96 - 1.92	-2.14 - 2.31
C	0.781	0.401 - 0.932	-3.48 - 11.11	-6.55 - 8.74
D	0.89	0.662 - 0.967	-2.60 - 4.42	-4.49 - 5.80
E	0.963	0.876 - 0.989	-2.60 - 2.00	-3.11 - 2.51

[a] Refer to Table 4.1 for the equations of each method for calculating the thyroid volume.

4.4 Discussion

Different imaging modalities are commonly used for thyroid volume measurement in human medicine, but are rarely applied in veterinary medicine due to animal's physical constraints and different living habitats (King, 2006). Extensive medical imaging investigations have been performed on domestic and companion animals' thyroid gland, but rarely on marine mammals. Ultrasound is advantageous for being non-invasive, real-time, relatively low-cost and readily accessible, and would be a useful imaging tool for the assessment of thyroid glands..

The results of this study indicated that both 2-D and 3-D ultrasound can be used to evaluate the dolphin thyroid volume. Access to 3-D ultrasound equipment may be limited in most aquariums; however, results showed that the four 2-D ultrasound measurement methods (Methods A – D) achieved high accuracy and reliability in the measurement of dolphin thyroid volume. Of these, Method A and Method B had relatively higher accuracy in both overall and configuration-specific analyses among different 2-D ultrasound thyroid volume measurement methods.

Methods A and B were developed for dolphin thyroid volume measurement in which the concept of segmentation was applied in the volume calculation. This volume calculation technique required the operator to outline the boundaries of the thyroid lobe in the longitudinal scan planes (Kot et al., 2009). The more segments that are included, the higher the accuracy; however, the segmentation process will be more time consuming (Pang et al., 2006). For the sake of simplicity and standardisation, 3 major representative longitudinal scan planes with maximum longitudinal dimension were selected from the major portions of the thyroid gland correspondingly (left lobe, right lobe and isthmus), : 1. scan plane showing the midline of the thyroid (LS_MID); 2. scan plane showing the maximum longitudinal dimension of the left lobe (LS_L); and 3. scan plane showing the maximum longitudinal dimension of the right lobe (LS_R). The 3 major longitudinal scan planes were recorded and a summation of these 3 scan planes resulted in fulfilling the assumption that the dolphin thyroid gland itself models an ellipse. This volume

calculation technique is similar to that of the 3-D ultrasound volume measurement method applied in the present study, with 8 image planes generated from the 3-D image set for volume calculation.

In contrast, Methods C and D were measurement methods originally derived from human thyroid volume measurement in which each thyroid lobe was modeled as an ellipse. The length, width and thickness of each thyroid lobe were measured and multiplied together with a constant, as described by Brunn et al. (1981). The only published report on dolphin thyroid ultrasound, which adopted this method for thyroid volume measurement, reported that measurements were extremely variable "because of skill differences between operators" (West and Ramer, 2005). However, the reliability test results of the present study disagreed with West and Ramer. Methods C and D yielded a high reproducibility (ICC = 0.782 – 0.811) and repeatability (ICC = 0.781 – 0.960). The possible reason that may account for the lower accuracy yielded by Methods C and D is that these volume measurement methods virtually ignored the volume estimation of isthmus, which led to various degrees of over- and under-estimation of the total thyroid volume. Also, the lower accuracy yielded may also be explained by considering the difficulty in judging the maximal dimension of the craniocaudal dimension of the dolphin thyroid lobes due to the difference in obiquity in different subjects, inherent in the thyroid volume measurements with Methods C and D. Therefore the accuracies yielded by Methods A and B were higher than those of Methods C and D.

For Methods B and D, the cross-sectional area of the image plane was measured and the thyroid volume was calculated using the ellipsoid equation developed by Shabana et al. (2003). Measurement of the cross-sectional area of the thyroid gland allowed a more precise reflection of the actual contour of the thyroid lobe, and thus errors in the thyroid volume calculation could be minimized. As such, the operator should measure the cross-sectional area of the image plane when they encounter difficulties in judging the maximum linear dimension of the thyroid gland. The results yielded by Methods B and D have no significant differences with their

corresponding volume measurement methods, i.e. Methods A and C, indicating that Method A is interchangeable with Method B, and Method C is interchangeable with Method D.

When taking thyroid configuration into consideration, Methods A and B yielded a higher accuracy than Methods C and D for Type A thyroid configuration, whereas all 4 methods showed similar accuracy for Type C thyroid configuration. This could be explained by the anatomical difference between Type A and Type C thyroid configuration. A distinct observable isthmus connecting the left and right thyroid lobe is present in Type A configuration, whereas Type C configuration is described as a single shield-like mass without a distinguishable isthmus. Thus, when encountering thyroid volume measurement in Type A configuration, Methods A and B demonstrated higher accuracy due to the incorporation of all 3 major portions of the thyroid gland for volume measurement, contrary to Methods C and D, which only considered the left and right lobe, excluding the isthmus for volume measurement. However, when encountering thyroid volume measurement in Type C configuration, the absence of isthmus made the inherent measurement error of Methods C and D insignificant.

In human medicine, 3-D ultrasound is recognized as the standard of reference for thyroid volume measurements (Ng et al., 2004; Ying et al., 2005; Andermann et al., 2007). Previous studies have reported that 3-D ultrasound is more accurate and reliable in volume measurements than 2-D ultrasound (Chou et al., 1997; Tong et al., 1998; Matre et al., 1999; Fenster and Downey, 2003; Raine-Frenning et al., 2003a, 2003b; Landry et al., 2004; Lyshchik et al., 2004a; Ng et al., 2004; Park et al., 2004; Benacerraf et al., 2006). A previous study on the evaluation of the accuracy and reliability of the 3-D ultrasound in volume measurements *in vitro* using tissue phantoms showed that the 3-D ultrasound with manual planimetry volume measurement technique applied in the present study, yielded high accuracy (81.5 – 83.4%), reproducibility (91.1%) and repeatability (98.8 – 99.1%) in volume

measurements (Kot et al., 2009). Thus, the thyroid volume measured with 3-D ultrasound was used as the standard of reference in the present study.

The present study evaluated the accuracy of the 3-D ultrasound thyroid volume measurement method on 2 fresh dolphin thyroid specimens *in vitro*. Results showed that the mean absolute percentage error of 3-D ultrasound was 4.9%, indicating a high accuracy of volume measurement, i.e. 95.1%, compared to the actual volume obtained by the water displacement method. Results also demonstrated that 3-D ultrasound thyroid volume measurement method has low inter-operator and intra-operator variability, which is comparable to the findings in a previous phantom study (Kot et al., 2009). The higher measurement accuracy for 3-D ultrasound volume measurements may be due to the fact that the thyroid specimens were submerged into the water *in vitro*, demonstrated clear boundaries on ultrasound, and hence allowed the operator to outline the boundaries easily. However, the accuracy of 3-D ultrasound volume measurements may be different for *in vivo* scanning, owing to a thin fibrous capsule enveloping the thyroid gland, rendering it to be clearly distinguished from adjacent neck tissues and musculatures. Besides, in order to shorten the long measurement time, 8 image planes were suggested in the segmentation process in the present study. A previous study demonstrated that the more segments that are included, the higher the accuracy; however, the measurement time will be longer (Pang et al., 2006). Further study in evaluating the accuracy of the 3-D ultrasound thyroid volume measurement method *in vivo* with increased number of image planes for evaluation is suggested to reinforce confidence towards the measurement of the dolphin thyroid volume.

The present study shows that there is a considerably high reproducibility (77.6% - 86.2%) and repeatability (78.1% - 99.7%) in thyroid volume measurement using both 2-D and 3-D ultrasound. The findings are comparable with previous human studies with the application of Methods C and D, with reproducibility of 85 - 87% and repeatability of 84.8% (Ozgen et al., 1999; Hegedüs, 2001; Lyshchik et al., 2004a; Ying et al., 2005; Ying et al., 2008). Riccabona et al. (1996) reported that 3-

D ultrasound is more accurate than conventional 2-D ultrasound in the volume measurement of objects. Method E utilizes an automated 3-D mechanical transducer to perform a single sweep on the thyroid gland, followed by the tracing the boundaries of the thyroid gland to yield the cross-sectional area in each segment, without any involvement of linear measurement in any dimension of the thyroid gland. However, as pointed out by Malago et al. (2008), even a small difference between each dimension of the thyroid gland may increase the error separately between the 2 calculated volumes of the thyroid gland by 2 different operators. When each measurement by an operator is 10% more than that of another, the total volume of the thyroid gland calculated from the measurements of the first operator would be 33% more than the total volume of the thyroid gland calculated from the measurements of the other. This may explain why 3-D ultrasound is more reliable than any 2-D ultrasound measurement methods investigated. Similarly, this could also explain why Methods B and D yielded slightly better reproducibility and repeatability compared to Methods A and C, which part of the linear measurements were substituted by the cross-sectional area measurements. Nevertheless, studies to further improve the reliability of 2-D ultrasound measurement of the dolphin thyroid gland are needed.

This study has indicated that ultrasound is a useful diagnostic tool in assessing the thyroid volume of the bottlenose dolphin. Standard reliable ultrasound thyroid volume measurement methods have been established for the first time. These provide a means of data collection in further studies for investigating the dolphin thyroid volume in relation to different demographic parameters, physiological cycles and both thyroidal and non-thyroidal illness. The scanning techniques may be applied to other marine mammals and provide information about thyroid physiology of other species, allowing optimal captive management. Such techniques may also aid conservation of wild animals in the future. 3-D ultrasound findings are reported in marine mammal research for the first time. There is enormous potential in future applications towards veterinary medicine, due to the higher measurement accuracy and reliability of 3-D ultrasound, as well as its reduced examination time owing to

the higher post-processing capability. Although 3-D ultrasound demonstrated higher measurement accuracy and reliability in both *in vivo* and *in vitro* studies of various regions of interest (Riccabona et al., 1996; Vade et al., 1997), 3-D ultrasound is not available in most of the aquarium settings. The largest obstacle appears to be access in using 3-D ultrasound equipment since it is relatively expensive and it has not yet supplanted 2-D ultrasound equipment in most zoological and aquarium settings.

Despite the large number of individual examinations in the evaluation of the accuracy of the four 2-D ultrasound measurement methods, there are limitations in the present study. All examinations were collected from the same population of bottlenose dolphin (n = 16) and therefore the findings of this study may be localized to this particular group. Also, the lack of dolphin thyroid glands in Types B and D configurations in the presently studied population prevented the evaluation of accuracy of different 2-D ultrasound measurement methods on these 2 configurations. Further study with a larger pool of subjects is suggested. Also, since the dolphin thyroid specimens were scanned *in vitro* in this study, the results may be different in an *in vivo* study. Further study is needed to compare the accuracy and reliability of the 3D ultrasound volumetric measurements *in vivo*.

4.5 Conclusions

Both 2-D and 3-D ultrasound can be used to evaluate the dolphin thyroid volume. The methods A and B described are considered to be more accurate and reliable methods for 2-D ultrasound dolphin thyroid measurement, regardless of the dolphin thyroid configuration. For the first time, a reliable ultrasound scanning protocol for measuring dolphin thyroid volume was developed, which provides a means to establish a normative reference for the diagnosis of thyroid pathologies and to monitor the thyroid volume during the course of treatment in living dolphins.

Chapter Five

Study Three

Sonographic evaluation of the thyroid gland in association with the serum thyroid hormones concentrations of the captive Indo-Pacific Bottlenose dolphin, *Tursiops aduncus*

5.1 Introduction

Thyroid gland disorders have been reported in many different species of wildlife (Wallach, 1970; Kaptein et al., 1994; Colborn, 2002; Reese et al., 2005), but rarely in marine mammals. Pathological changes in the cetacean thyroid gland have been described in both stranded and captive animals, although the majority of them are associated with other clinical problems (Greenwood and Barlow, 1979). Environmental contaminants and local environmental influences have been implicated in thyroid hormone imbalances (Cowan and Tajima, 2006), the development of morphological and histological abnormalities (Schumacher et al., 1993; Mikaelian et al., 2003; Das et al., 2006), and calf mortality (Garner et al., 2002; West et al., 2002; West et al., 2003; West and Ramer, 2005). Cowan and Tajima (2006) described the gross and histological features of thyroid glands in stranded Atlantic bottlenose dolphins, and found that 31 out of 60 animals suffered thyroid gland abnormalities or pathologies. Mikaelian et al. (2003) reported that 17 out of 30 beluga whale specimens collected from highly contaminated waterways were found to have hyperplastic and cystic thyroid lesions, and these thyroid abnormalities were correlated with the high tissue concentration of chemical contaminants. However, the assessment of the cetacean thyroid gland has been limited to post-mortem studies (Galliano et al., 1966; Viamonte et al., 1968; Ridgway and Patton, 1971; Cowan and Tajima, 2006).

In human and companion animals studies, clinical examination of the thyroid gland is usually the initial investigation for thyroid dysfunction. However, clinical examination, by means of palpation and inspection, is insensitive and extremely observer-dependent for detecting any enlargement or nodularity of the thyroid gland (Sheikh et al., 2004). Due to the fact that thyroid dysfunction usually arises from primary disorders of the thyroid gland, the measurement of serum thyroid stimulating hormone (TSH) is the most widely employed test to assess thyroid dysfunction. However, application of serum TSH assay alone may overlook thyroid dysfunction in cases of high clinical suspicion of hypothyroidism (Foktin et al., 2010). Consequently, simultaneous evaluation of both the serum TSH and free thyroxine (T4) would be required to further substantiate or exclude uncertain diagnoses.

Clinical examination of the dolphin thyroid gland is difficult due to the thick blubber and strong neck muscles of the animals. Baseline values for serum thyroid hormones have been established in some common captive cetacean species (St. Aubin, 2001), but discrepancies exist due to the different methodologies and assays applied. Although analyses of serum concentrations of thyroid hormones can give valuable insight to any alternation in thyroid function, repeated serial measurements in follow-up examinations may result in the loss of voluntary behaviour of the dolphin subject, and repeated needle insertions may damage skin and blood vessels (Brook, 1997). An important consideration in the evaluation of thyroid function is the degree to which thyroid hormones are bounded by circulating carrier proteins (Cunningham, 2002). However, efforts to demonstrate the binding protein concentrations in belugas and bottlenose dolphins have proved unsuccessful when using methodologies established for other mammals (St. Aubin, 2001). The fraction that remains free is considered as the physiologically active portion of the hormone. The mechanisms regulating thyroid function have a direct effect on the concentration of this free fraction, which explains its independence of the concentration of carrier proteins. In view of this, a number of tests are necessary in order to fully describe thyroid gland activity. Concentrations of free and total

triiodothyronine (T3), free and total T4, as well as TSH should be evaluated together as a panel (Cunningham, 2002). Although different types of thyroid hormone test kits are available, they have been developed for humans and may not be compatible for use in other species due to the mismatch of corresponding antibodies. St. Aubin (2001) stated that commercially available reagents for measuring human TSH were not effective in detecting the hormone in Atlantic bottlenose dolphins (*Tursiops truncatus*). Development of a new assay specific to a particular species, as well as the evaluation of the whole panel of thyroid hormones, is costly and time consuming. A successful evaluation of serum TSH using the radioimmunoassay (RIA) technique in bottlenose dolphins has not yet been published. Although TSH analysis in dolphins does not seem to be feasible, measurements of other thyroid hormones such as T3 and T4 may be more promising due to their structural simplicity when compared to TSH (Arvy, 1970; Ridgway et al., 1970; Ridgway and Patton, 1971; St. Aubin et al., 1996; St. Aubin, 2001).

Ultrasound is a useful, real-time, safe and relatively low-cost imaging tool in the assessment of thyroid morphology and physiology in humans (Ahuja, 2000; Hegedüs, 2001; AIUM, 2003) and companion animals (Cartee et al., 1993; Kaptein et al., 1994; Wisner et al., 2002; Brömel et al., 2006). Studies have suggested that ultrasound may be a more sensitive index of thyroid disturbance than serum TSH concentrations (Stewart et al., 1989). If ultrasound is more sensitive in the detection of morphological abnormalities in comparison to serum concentrations of thyroid hormone analysis, then ultrasound could possibly be considered as an initial investigation before conducting more expensive and invasive procedures such as blood sampling for thyroid hormone analysis.

The purpose of the study were to determine any potential variation in serum concentrations of thyroid hormones among different age groups, sex and degree of sexual maturity; establish reference ranges of serum concentrations of thyroid hormones in the investigated population; and determine the possible association of

thyroid morphology and serum concentrations of thyroid hormones in a healthy captive population of Indo-Pacific bottlenose dolphins.

5.2 Materials and Methods

5.2.1 Animals and management

Seventeen *Tursiops aduncus* at Ocean Park, Hong Kong (7 males and 10 females) were included in the study. Estimated age, sex, sexual maturity, and body size of all animals obtained in July 2008 are tabulated (Table 5.1).

Animals' diets consisted of different proportions of capelin, sardine, herring and squid air-freighted from USA, with vitamin and mineral supplements (See Appendix 3). Caloric requirement and total daily intake were formulated according to individual requirements. This population of dolphins was maintained in 2 different locations with semi-enclosed and enclosed outdoor facilities, consisting of inter-connected tanks with treated natural sea water (see Appendix 1). All dolphins involved in the study were being trained to cooperate for neck ultrasound examination.

Table 5.1: Estimated age, sex, sexual maturity and body size in 17 bottlenose dolphins (*Tursiops aduncus*) at Ocean Park, Hong Kong, in July 2008.

Subject	Estimated age (years)	Sex	Sexual maturity	Body weight (kg)	Body length (cm)
Molly	25	M	Sexual mature	126.3	218
Mini	deceased at 26	M	Sexual mature	142.0	233
Toto	12	M	Sexual mature	143.0	236
Perky	deceased at 7	M	Sexual mature	120.1	223
Leo	8	M	Sexual mature	112.0	216
Anson	3	M	Sexual immature	115.6	219
Ginsan	3	M	Sexual immature	125.0	219
Jessie	36	F	Sexual mature	190.7	239
Angel	24	F	Sexual mature	141.7	214
Ada	25	F	Sexual mature	98.0	203
Ester	20	F	Sexual mature	134.5	212
Gina	25	F	Sexual mature	131.1	209
Hicky	20	F	Sexual mature	130.4	196
Pinky	9	F	Sexual mature	143.6	220
Hoi Kei	6	F	Sexual mature	104.6	209
Maya	6	F	Sexual mature	148.0	241
Nona	2	F	Sexual immature	125.0	219

5.2.2 Equipment

All sonographic examinations were performed with either a Philips HD-11 ultrasound unit or a Philips HD-11 XE ultrasound unit, in conjunction with a 6-2 MHz curvilinear 3-D broadband curved array transducer and a 5-2 MHz 2-D broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA) or an Aloka SSD 900 ultrasound unit (Aloka Co. Ltd, Mitakasho, Tokyo) in conjunction with a 5 MHz curvilinear transducer. All images were recorded with either a direct digital capture or a thermal printer. Because of the nature of the dolphin's skin, there is no air layer between the surface and the transducer, thus no coupling gel was required.

5.2.3 Protocol for sonographic examination of the thyroid gland

Sonographic examination of the thyroid gland was conducted once a month for 2 years (August 2006 to July 2008) on the same day that routine blood collection on this population was performed. All ultrasound examinations of the thyroid gland were conducted with the dolphin positioned in dorsal recumbence close to the poolside, with its tail supported by a trainer. The transducer was initially placed in a transverse orientation at the thoracic inlet, midway between the insertions of the pectoral flippers. The transducer was moved cranially until the brachiocephalic vein was identified. The transducer was then moved further cranially until the left and right lobes of the thyroid gland were identified. The thyroid volume was measured using the same protocol of Method A as used in Chapter 4 (see Section 4.2.1.1).

5.2.4 Thyroid hormones evaluations and assay protocols

Blood sample were collected monthly whenever possible during the same morning that the sonographic examination was conducted. Medical behavior training enabled blood sampling procedures to be carried out at the poolside. Approximately 10 mL of blood was collected in a non-heparinized, disposable syringe after dorsal venipuncture of tail fluke vessels, using a 22 G butterfly needle. The blood was left

to stand to clot in a plain tube for 30 minutes at room temperature, and serum was harvested by centrifugation at 4500 RPM for 10 minutes. For the evaluation of serum concentrations of thyroid hormones, commercial test kits VIDAS free T3, free T4, total T3, total T4, (bioMérieux sa, France), coupled with an automated VIDAS analyzer (bioMérieux sa, France) were used. For total T3, free T3, free T4 tests, 100 µL of serum sample was required for each test; whereas 200 µL of serum sample was required for total T4 test. The standards are of human serum origin.

The assay principle of this test kit combines an enzyme immunoassay sandwich method with a final fluorescent detection (ELFA). Assays for each hormone were performed as a single run, with the intra- and inter-assay coefficients of variation at less than 10%, calculated by assaying human serum samples with low to slightly high hormone concentration by the manufacturer. The measurement range of the VIDAS free T3, free T4, total T3, total T4 kit extends between 0.7 and 45 pmol/l, 1 and 70 pmol/l, 0.4 and 9 nmol/l and 6 and 320 nmol/l respectively. The detection limit of these hormones was defined as the smallest concentration of free T3, free T4, total T3, total T4 which is significantly different from the zero concentration with a probability of 95%: ≤ 0.7 pmol/l, ≤ 1 pmol/l, < 0.4 nmol/l and 6nmol/l respectively (Manufacturer specifications were described in Appendix 4).

At the time of sample collection, for the basis of age, each dolphin was categorized into 1 of the following 3 age groups: < 5 years (calf), > 5-10 years (juvenile) or > 10 years (adult). On the basis of sexual maturity, each dolphin was categorized as sexually immature or sexually mature. Sexual maturation of the female dolphin was determined by the first observation of the ovarian cycle or activity, while for the male dolphin, sexual maturation was determined by the first presence of spermatozoa in the semen, indicating the onset of spermatogenesis, using ultrasound.

5.2.5 Statistical analysis

The Kruskal-Wallis test, followed by Dunn's test, was used to determine whether serum concentrations of thyroid hormones differed among age groups of this dolphin population. The Mann-Whitney U test was used to determine whether serum concentrations of thyroid hormones differed among sex and sexual maturity of this dolphin population. Reference ranges for serum concentrations of thyroid hormones based on different age groups and sex were established by use of the nonparametric method of percentile estimates with confidence intervals to determine the central 95^{th} percentile interval (i.e. 2.5 to 97.5 percentile rank) of the data (Ramsey et al., 1997; Peterson et al., 2001). The correlation between serum concentration of thyroid hormones and thyroid volume was determined by calculating Spearman's rank correlation coefficient. For all statistical analyses, a value of $p < 0.05$ was considered to be significant.

5.3 Results

A concurrent total of 241 blood samples and ultrasound examinations were obtained from the 17 subjects from August 2006 to July 2008. There were significant differences in serum thyroid hormones among different age groups, sex and sexual maturity of the investigated dolphin subjects ($p < 0.05$) (Table 5.2). The reference ranges of the thyroid hormones were established based on different age groups and sexes and are shown in Table 5.3.

Table 5.2: Comparison of serum thyroid hormones concentrations among different age groups, sex and sexual maturity with corresponding p values.

Age	**Free T3 (pg/mL)**	**Free T4 (ng/dL)**	**Total T3 (ng/dL)**	**Total T4 (µg/dL)**
Calf	3.31	1.93	118.64	9.65
Juvenile	2.59	1.82	87.86	8.72
Adult	2.03	1.40	63.27	7.17
P value	<0.05 for all	<0.05 for Calf vs. Adult, Juvenile vs. Adult	<0.05 for all	<0.05 for all
Sex	**Free T3 (pg/mL)**	**Free T4 (ng/dL)**	**Total T3 (ng/dL)**	**Total T4 (µg/dL)**
Male	2.48	1.73	85.98	8.97
Female	2.25	1.49	71.24	7.27
P value	<0.05	<0.05	<0.05	<0.05
Sexual Maturity	**Free T3 (pg/mL)**	**Free T4 (ng/dL)**	**Total T3 (ng/dL)**	**Total T4 (µg/dL)**
Immature	3.31	1.93	118.64	9.65
Mature	2.20	1.53	70.81	7.64
P value	<0.05	<0.05	<0.05	<0.05

Conversion table:
Free T3: pmol/L *0.651 = pg/mL
Free T4: pmol/L * 0.0777 = ng/dL
Total T3: nmol/L * 65.1 = ng/dL
Total T4: nmol/L * 0.0777 = µg/dL

Table 5.3: Reference ranges of the serum thyroid hormones concentrations based on different age groups and sex.

Age group	**Free T3 (pg/mL)**	**Free T4 (ng/dL)**	**Total T3 (ng/dL)**	**Total T4 (µg/dL)**
Calf	2.65 - 4.27	1.71 - 2.38	93.09 - 158.52	8.62 - 11.38
Juvenile	1.94 - 3.85	1.34 - 2.33	52.34 - 161.58	6.41 - 11.23
Adult	1.34 - 2.89	0.77 - 2.07	39.52 - 104.88	4.19 - 9.95
Sex	**Free T3 (pg/mL)**	**Free T4 (ng/dL)**	**Total T3 (ng/dL)**	**Total T4 (µg/dL)**
Male	1.32- 3.92	1.30 - 2.36	47.00 - 151.94	7.13 - 11.46
Female	1.55 - 3.39	0.78 - 2.27	39.63 - 121.41	4.20 - 10.41

Conversion table:
Free T3: pmol/L *0.651 = pg/mL
Free T4: pmol/L * 0.0777 = ng/dL
Total T3: nmol/L * 65.1 = ng/dL
Total T4: nmol/L * 0.0777 = µg/dL

In the assessment of the correlation between serum concentrations of thyroid hormones and thyroid volume, results showed that there was a weak correlation between free T3 and thyroid volume, and total T3 and thyroid volume ($r = 0.21$ and 0.16 respectively, $p < 0.05$) (Table 5.4).

Table 5.4: Correlation between serum concentrations of thyroid hormones and thyroid volume based on different age groups, sex and sexual maturity.

	All Subjects				Male Subjects Alone				Female Subjects Alone			
	Free T3	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**
Spearman's Rho	0.21	0.09	0.16	0.10	0.57	0.45	0.64	0.57	0.03	-0.06	-0.04	-0.06
P value	<0.05	0.19	<0.05	0.13	<0.05	<0.05	<0.05	<0.05	0.75	0.43	0.63	0.47
n	241	241	241	241	87	87	87	87	154	154	154	154
	All Subjects Age <5 years				**All Subjects Age 5 – 10 years**				**All Subjects Age >10 years**			
	Free T3	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**
Spearman's Rho	0.55	0.54	0.35	0.39	-0.25	-0.31	-0.17	-0.21	-0.28	-0.39	-0.35	-0.30
P value	<0.05	<0.05	0.07	<0.05	<0.05	<0.05	0.17	0.10	<0.05	<0.05	<0.05	<0.05
n	29	29	29	29	65	65	65	65	147	147	147	147
	Sexually Immature				**Sexually Mature**			
	Free T3	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**
Spearman's Rho	0.55	0.54	0.35	0.39	0.04	-0.05	-0.02	-0.05
P value	<0.05	<0.05	0.07	<0.05	0.61	0.47	0.76	0.44
n	29	29	29	29	212	212	212	212
	Male Age ≤10 years				**Male Age >10 years**			
	Free T3	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**
Spearman's Rho	0.57	0.53	0.31	0.29	-0.11	0.02	0.07	0.21
P value	<0.05	<0.05	0.13	0.17	0.47	0.88	0.63	0.18
n	40	40	40	40	45	45	45	45
	Female Age ≤10 years				**Female Age >10 years**			
	Free T3	**Free T4**	**Total T3**	**Total T4**	**Free T3**	**Free T4**	**Total T3**	**Total T4**
Spearman's Rho	-0.40	0.80	-0.95	0.80	-0.33	-0.57	-0.46	-0.51
P value	0.60	0.20	0.05	0.20	<0.05	<0.05	<0.05	<0.05
n	52	52	52	52	98	98	98	98

Since significant differences in serum thyroid hormones were found among age groups, sex and sexual maturity ($p < 0.05$), the results were stratified by age groups, sex and sexual maturity for further investigation. For the analysis of age groups, a moderate to strong positive correlation was found between free T3, free T4, total T4 and thyroid volume in calves ($r = 0.39$ to 0.55, $p < 0.05$). A weak to moderate negative correlation was found between free T3, free T4 and thyroid volume in juveniles ($r = -0.31$ to -0.25, $p < 0.05$). A moderate negative correlation was found between all serum concentrations of thyroid hormones and thyroid volume in adults ($r = -0.39$ to -0.28, $p < 0.05$). For the analysis of sex, there was a strong positive correlation between all serum concentrations of thyroid hormones and thyroid volume in males ($r = 0.45$ to 0.64, $p < 0.05$). For the analysis of sexual maturity, a moderate to strong positive correlation was found between free T3, free T4, total T4 and thyroid volume in the sexually immature subjects ($r = 0.39$ to 0.55, $p < 0.05$) (Table 5.4).

5.4 Discussion

The metabolic rate of cetaceans is commonly believed to be higher than that of terrestrial species with comparable body size, and is suggested to be related to the larger thyroid volume in cetaceans (Quiring, 1950; St. Aubin, 2001). However, other studies evaluated the metabolic rate of cetaceans by measuring the thyroid hormones, and found that there was no association between thyroid volume and metabolic rate (Harrison and Young, 1970; Ridgway and Patton, 1971; St. Aubin and Geraci, 1989). Previous studies concentrated on the evaluation of thyroid hormones and the possible factors influencing the thyroid hormones concentrations (Harrison and Young, 1970; Ridgway and Patton, 1971; St. Aubin and Geraci, 1989; St. Aubin et al., 1996). However, a recent study suggested that morphological examination may be more sensitive for the assessment of thyroid disturbance, rather than a traditional functional examination such as serum TSH concentration assessment (Stewart et al., 1989). Previous thyroid morphological research works were exclusively performed in deceased cetaceans, of captive, stranded and wild

status (Harrison, 1969; Arvy, 1970; Cowan and Tajima, 2006). To the best of our knowledge, the literature is devoid of any documentation regarding the association of the thyroid volume and serum concentrations of thyroid hormones in living dolphin subjects. Since ultrasound is non-invasive, understanding of the association of ultrasound thyroid morphology and serum concentrations of thyroid hormones may potentially allow ultrasound to be used as an initial investigation before conducting more expensive and invasive procedures such as blood sampling for thyroid hormone analysis.

In the present study, a weak positive correlation was found between both serum free T3 concentration, total T3 concentration and thyroid volume, independently of age, sex and sexual maturity, indicating a weak association between the thyroid function and morphology in the studied group of Indo-Pacific bottlenose dolphins. In humans, T3 is a hormone produced from thyroidal secretion (20%) and from the peripheral deiodination mechanism which converts T4 to T3 (80%). T3 is physiologically much more active than T4, and plays an essential role in maintaining euthyroidism (Cunningham, 2002; Foktin et al., 2010). Since the function of T3 in dolphins does not deviate largely from other mammals, and there was no significant difference in thyroid histology between dolphins and other mammals, the result of the present study is consistent with the previous studies speculating that T3 partially influences the morphological changes in dolphin thyroid glands (Ridgway, 1972; St. Aubin et al., 1996).

A unique trend was observed on the correlation between thyroid function and morphology when we stratified the analyses by age groups. For the calves, there was a positive correlation between all serum concentrations of thyroid hormones and thyroid volume. In juveniles and adults, the correlation between all serum concentrations of thyroid hormones and thyroid volume was found to be negative.

The positive correlation observed in the calves was possibly due to the active metabolism of the young, fast-growing subjects. Thyroid hormones are important

mediators of metabolism, which maintains homeostasis in response to environmental demands (St. Aubin et al., 1996; Cunningham, 2002). The higher serum concentrations of thyroid hormones and the larger thyroid volume observed in young subjects coincide with higher energy requirements at this stage of life, fully contributing to the somatic growth and development of each individual.

Following the process of the life cycle, the group of juveniles showed a weak negative correlation for free T3 and T4. The weak association in the adolescent subjects may be due to the fact that the subjects are undergoing a 'transitional period' - from purely somatic growth and development, their energy expenditures are refocused to the onset of reproductive organ maturity, reproductive events and cycling.

Unlike the juveniles, there was a moderate negative correlation for all thyroid hormones with the thyroid volume in the adults. In several marine mammals, concentrations of thyroid hormones decrease with postnatal development and maturation (Hall et al., 1998; Haulena et al., 1998; Woldstad and Jenssen, 1999; Myers et al., 2006). St. Aubin et al. (1996) found that free T4 declined with maturation in wild dolphin populations. Combined with the increase in thyroid volume during normal growth, this may attribute to the moderate negative correlation that was found between all serum concentrations of thyroid hormones and thyroid volume in adults.

Reproduction is an important event in the life cycle of adults. Hormonal changes associated with reproduction can lead to variations in thyroid hormones concentrations, by altering the thyroid hormone binding capacity in plasma (Ramey et al., 1975; Huang et al., 1995; St. Aubin et al., 1996; Sekulić et al., 2007). St. Aubin et al. (1996) proposed that hormonal changes associated with reproduction in female dolphins can indirectly lead to elevations in total T4 and T3, by increasing hormone binding capacity in plasma. Female sex steroids such as estrogen and progesterone may alter the thyroid hormones concentrations. Estrogens have been

shown to conflictingly exert stimulatory (Kuhl et al., 1985; Furlanetto et al., 1999), inhibitory (Sosić-Jurjević et al., 2005; Sekulić et al., 2007), or no effects (Ceresini et al., 2008) on thyroid activity, while progesterone was found to be not in synchronization with any oscillations of human thyroid flow velocity (Krejza et al., 2004), although a vasoconstrictor effect on the blood vessels was suggested (Miyamoto et al., 2005). In diestrous dogs, it has also been postulated that progesterone may also enhance the binding affinity of plasma proteins for thyroid hormones, resulting in an increase in serum concentrations of total T4 and T3 (Reimers et al., 1984). Therefore it is possible that female sex steroids would have similar effects on serum concentrations of thyroid hormones in dolphins. In the present study, further investigation was made on adults by categorizing them according to sex. The female subjects yielded a stronger negative correlation than the male subjects between all serum concentrations of thyroid hormones and thyroid volume. This finding may illustrate the possible interactions caused by different concentrations of female sex steroids during different reproductive events.

When we stratified the analyses by sex, all investigated serum concentrations of thyroid hormones correlated well with the thyroid volume of the male subjects, whereas there was no correlation between serum concentrations of thyroid hormones and thyroid volume in the female subjects. The result is different from a previous study which reported that there was no significant difference in concentrations of thyroid hormones between captive male and female bottlenose dolphins in both natural and artificial sea water settings (St. Aubin et al., 1996). The discrepancy of the findings may be due to the bias presentation of the subject group in the present study. Approximately half of the samples (42 out of 87) in males were obtained from calves and juveniles, and therefore the finding may be more influenced by age, rather than sex. Moreover, the lack of correlation between serum concentrations of thyroid hormones and thyroid volume in the female subjects may reflect the diversity of female subjects at different reproductive stages, as well as for the sexually immature subjects. The thyroid function and morphology in females are affected by the cyclic change of the hormonal variation during the normal menstrual

cycle, and are varied according to different reproductive events (Hegedüs, 1990; Chan et al., 1998, 1999; Hegedüs, 2001; Krejza et al., 2004). Therefore, it would be difficult to establish a strong correlation between thyroid function and morphology when categorizing the samples as a function of sex.

When we stratified the analyses by sexual maturity, all serum concentrations of thyroid hormones were found to have a mild to strong positive correlation with the thyroid volume in the sexually immature group than the sexually mature group. This discrepancy can be accounted by the difference in metabolism and energy expenditure between the 2 groups, in which the subjects in the sexually immature group are focused on somatic growth that has higher metabolism and energy consumption than reproductive maturation in sexually mature subjects.

The concentrations of thyroid hormones have been reported in a number of marine mammal species; however, variation in the methodology, captive environment and diets of the animals in previous literature rendered it difficult to perform direct comparisons of the results (Greenwood and Barlow, 1979; St. Aubin and Geraci, 1989; St. Aubin et al., 1996; Haulena et al., 1998; St. Aubin, 2001; Myers et al., 2006). Although it is not possible to globally establish valid reference ranges for thyroid volume and concentrations of thyroid hormones of bottlenose dolphins due to differences in hereditary and environmental factors, establishing reference ranges for thyroid volume and concentrations of thyroid hormones is still very important in this group of subjects for the purpose of insight and also to enable correct evaluation of thyroid morphology and function. To date, the present study is the first to establish reference ranges across different age groups and sex in a captive population of Indo-Pacific bottlenose dolphins.

Attempts were made in the present study to evaluate serum TSH concentration of the bottlenose dolphins using commercially available enzyme-linked immunosorbent assay (ELISA) kits for measuring human TSH concentration. However, it was abolished due to the mismatch of the specific TSH antibodies of

bottlenose dolphins, and the limited resources to develop a new hormone testing assay for this species of animals. TSH is considered as a glycoprotein hormone which is highly variable in configuration across different species, whereas all thyroid hormones are considered as amine-derived hormones that are structurally identical across different species. The present study is the first to document the application of the ELISA technique in the detection and determination of dolphin serum concentrations of thyroid hormones. St. Aubin (2001) stated that commercially available reagents for measuring human TSH were not effective in detecting the hormone in Atlantic bottlenose dolphins (*Tursiops truncatus*). RIA technique for the evaluation of TSH concentration in bottlenose dolphins have been attempted but are not yet published; thus the feasibility of using RIA in evaluating TSH concentration in bottlenose dolphins should be carefully considered.

There are limitations to the present study. Confounding variables such as serum thyroglobulin concentrations, hormone binding affinity to carrier proteins, iodine intake, serum TSH concentrations and serum TRH concentrations were not evaluated in this study. The present study also did not include the analysis of female sex steroids for elucidating their possible association with serum concentrations of thyroid hormones. Previous literature has suggested that dolphins may have different iodine turnover rates, which vary the concentrations of thyroglobulin at different life stages and thus influence the serum concentrations of thyroid hormones. Further studies in evaluating thyroid hormones with the consideration of these variables, in conjunction with the morphological investigation using ultrasound, are suggested.

5.5 Conclusions

To conclude, results of the present study showed various degrees of correlation between the serum concentrations of thyroid hormones and thyroid volume among age groups, sex and sexual maturity in a captive population of Indo-Pacific bottlenose dolphins. The most prominent association was identified when we

stratified the analyses by age. This association is suspected to be related to the energy requirements of somatic growth and development transitioning to reproductive events and the estrus in females.

Chapter Six

Study Four

Determinants of thyroid morphology investigated by sonography in a captive Indo-Pacific Bottlenose dolphin (*Tursiops aduncus*) population

6.1 Introduction

Assessment of thyroid morphology and function is one of the diagnostic challenges in cetacean clinical endocrinology (St. Aubin et al., 1996; St. Aubin, 2001) Different factors affect cetacean thyroid function and morphology, such as demographic parameters, physiological cycles, and health status (St. Aubin and Geraci, 1989; Docter et al., 1993; St. Aubin et al., 1996; Berghout and Wiersinga, 1998; St. Aubin, 2001; Cowan and Tajima, 2006; Myers et al., 2006; Mooney et al., 2008). Many of these factors may alter the baseline thyroid function and morphology, potentially causing misdiagnosis of thyroid abnormalities.

Studies in several marine mammals have identified age-related changes in thyroid function and morphology (St. Aubin et al., 1996; Haulena et al., 1998; Hall et al., 1998; Myers et al., 2006). As animals age (from postnatal development to maturation), the common trend is for thyroid function to decrease. St. Aubin et al. (1996) reported the decrease in free T4 with the onset of maturity in a wild dolphin population. Thyroid hormones are essential for growth and organ development (Feldman and Nelson, 2004; Foktin et al., 2010), and overt hypothyroidism and hyperthyroidism may be associated with weight gain or weight loss (Eftekhari et al., 2007; Fox et al., 2008; Shon et al., 2008). Studies in humans and companion animals have identified correlations between thyroid morphology, thyroid function, body weight and lean body mass (Hegedüs et al., 1983; Reimers et al., 1990; Wesche et al., 1998; Gomez et al., 2000; Sari et al., 2003; Brömel et al., 2006). When specific stages of the female dolphin reproductive cycle are not considered

and experimental data is merely classified as male or female, sex has no apparent effect on thyroid function and morphology (Greenwood and Barlow, 1979). However, the prevalence of thyroid disorders is different between males and females in both humans and companion animals (Feldman and Nelson, 2004; Farahati et al., 2006; Foktin et al., 2010), suggesting that there may be sex-specific determinants and mechanisms that promote or prevent thyroid disorders. Females' thyroid function and morphology are likely to be affected by the cyclic change of hormonal variation during the estrous cycle and different reproductive events (Chan et al., 1998; Krejza et al., 2004; Fister et al., 2009). Moreover, seasonal influence on thyroid function and morphology has been described for belugas (St. Aubin and Geraci, 1989) and some pinnipeds, which may possibly be associated with the annual molt of these animals. A previous study investigated the seasonal difference of thyroid hormones in a group of wild bottlenose dolphins (*Tursiops truncates*), and found that reverse T3, an inactive metabolite of T4, in male dolphins had a significantly higher level in the summer (St. Aubin et al., 1996).

Ultrasound is a real-time, non-invasive and inexpensive imaging tool that is useful in the assessment of thyroid morphology and physiology in humans (Ahuja, 2000; Hegedüs, 2001; AIUM, 2003) and companion animals (Cartee et al., 1993; Kaptein et al., 1994; Wisner et al., 2002; Brömel et al., 2006). It has been suggested that ultrasound is more sensitive than serum thyroid stimulating hormone measurements in the assessment of thyroid disturbance (Stewart et al., 1989). To the best of our knowledge, the formal literature is devoid of any reference to possible determinants such as age, sex, sexual maturity, body size, and season on thyroid morphology in bottlenose dolphins. Recognizing any associated changes on the thyroid gland of these determinants in bottlenose dolphins will help in the diagnosis of pathologies and monitoring of the thyroid gland during the course of treatment. It is important to recognize the potential influence of these determinants when interpreting physical thyroid changes and serum thyroid hormone levels. Since an imaging project was conducted in this bottlenose dolphin population for a length of over 2 years, with a regularly updated storage of their health reports and known demographic parameters,

a unique opportunity was provided to investigate the determinants of thyroid morphology in a captive environment. Given this collection of longitudinal data, the present study aimed to investigate the possible variations in thyroid morphology of a group of Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) under human care with different demographic factors using sonography. Findings of this study provide baseline information for better understanding of the thyroid morphology in somatic growth (different age, sex, degree of sexual maturity), reproductive development (sex and degree of sexual maturity) and different body sizes of Indo-Pacific bottlenose dolphins, as well as examine potential seasonal variations of thyroid morphology. We hypothesized that the thyroid morphology in Indo-Pacific bottlenose dolphins would vary with age, sex, sexual maturity and body size, but may not vary with season.

6.2 Materials and Methods

6.2.1 Subjects

Seventeen *Tursiops aduncus*, at Ocean Park, Hong Kong (7 males and 10 females) were included in the study. From August 2006 to January 2009, 1384 ultrasound scans were performed in this captive population for the present study. All dolphins involved in the study were trained to cooperate for neck and ovarian sonographic examinations. The estimated age, sex, sexual maturity and body size of the 17 subjects upon completion of the study are shown in Table 6.1.

Table 6.1: Estimated age, sex, sexual maturity and body size in 17 bottlenose dolphins (*Tursiops aduncus*) at Ocean Park, Hong Kong, upon completion of the study.

Subject	Estimated age (years)	Sex	Sexual maturity	Body weight (kg)	Body length (cm)
Molly	26	M	Sexual mature	126.6	218
Mini	deceased at 26	M	Sexual mature	142.0	233
Toto	13	M	Sexual mature	138.9	236
Perky	deceased at 7	M	Sexual mature	120.1	223
Leo	9	M	Sexual mature	112.7	216
Anson	4	M	Sexual immature	122.6	222
Ginsan	4	M	Sexual immature	130.4	222
Jessie	37	F	Sexual mature	185.1	242
Angel	25	F	Sexual mature	136.5	215
Ada	26	F	Sexual mature	106.1	201
Ester	21	F	Sexual mature	138.0	212
Gina	26	F	Sexual mature	132.8	208
Hicky	21	F	Sexual mature	124.0	196
Pinky	10	F	Sexual mature	140.3	220
Hoi Kei	7	F	Sexual mature	108.3	214
Maya	7	F	Sexual mature	147.1	244
Nona	3	F	Sexual immature	125.8	222

6.2.2 Equipment

All sonographic examinations were performed with either a Philips HD-11 ultrasound unit or a Philips HD-11 XE ultrasound unit, in conjunction with a 6-2 MHz curvilinear 3-D broadband curved array transducer and a 5-2 MHz 2-D broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA) or an Aloka SSD 900 ultrasound unit (Aloka Co. Ltd, Mitakasho, Tokyo) in conjunction with a 5 MHz curvilinear transducer. All images were recorded with either direct digital capture or with a thermal printer. Because of the nature of the dolphin's skin, there is no air layer between the surface and the transducer, so no coupling gel was required.

6.2.3 Scanning procedures

Sonographic examination of the thyroid gland was performed once a week for a period of over 2 years (August 2006 to January 2009). During the sonographic examination of the thyroid gland, the dolphin was positioned in dorsal recumbence close to the poolside, with its tail supported by a trainer. The transducer was initially placed in a transverse orientation at the thoracic inlet, midway between the insertions of the pectoral flippers. The transducer was then moved cranially until the left and right lobes of the thyroid gland were identified. The thyroid volume was measured using the same protocol of Method A as used in Chapter 4 (see Section 4.2.1.1).

Echogenicity and homogeneity of the thyroid gland were also evaluated. The echogenicity of the thyroid gland was compared with sternocephalicus muscle and classified into hypoechoic, isoechoic or hyperechoic. The homogeneity of the thyroid parenchyma was categorized into homogeneous or heterogeneous on the basis of the presence or absence of any nodules or space-occupying lesions.

6.2.4 Age

Age assessment of 8 animals collected from the wild (Jessie, Angel, Gina, Ester, Ada, Hicky, Molly, Mini) was based on their body size, growth patterns and length of time in captivity, which have been performed by the resident veterinarians in the Ocean Park, Hong Kong. The remaining 9 subjects (Pinky, Maya, Hoi Kei, Ginsan, Anson, Nona, Toto, Leo, Perky) were born into captivity and are therefore of known ages. On the basis of the age of each dolphin at the time of the ultrasound examinations, collected data was categorized into 3 age groups: < 5 years (calf), > 5-10 years (juvenile) or > 10 years (adult). At the end of the study, 3 subjects were calves, 5 subjects were juveniles and 9 subjects were adults, and the mean age of the population was 16 years (range, 3 to 37 years).

6.2.5 Season

The captive animals in the present study provided the opportunity for samples to be collected throughout the year from known aged animals of varied age classes. Seasonal variability of the thyroid volume and the sonographic features of the thyroid gland were evaluated by longitudinal ultrasound examinations of the dolphins throughout the 4 seasons. Hong Kong is located near 22° N latitude, on the south coast of China. The climate of Hong Kong is governed by the Asian monsoon circulation and is thus more varied than is typical for other tropical areas. According to a recent publication on climate change in Hong Kong by the Hong Kong Observatory, spring refers to the period from March to May, summer from June to August, autumn from September to November and winter from December to February (Leung et al., 2004). On the basis of the time of sample collection, data was categorized into the 4 seasons accordingly.

6.2.6 Sexual maturity

Weekly ovarian ultrasound was performed on all the female dolphins using the established method (Brook, 2001), and reported by veterinarians in Ocean Park,

Hong Kong, who are experienced in dolphin ovarian ultrasound examination. Archived ultrasound images and examination reports were further evaluated by an experienced sonographer. Both ovaries were assessed in the scan and the ovarian cortex was examined and reported during different stages of the reproductive cycle. In line with a male dolphin reproductive study, weekly ejaculate trials were conducted from the beginning of the data collection. Sexual maturation of the female dolphins was determined by the first observation of ovarian activity, while sexual maturation of male dolphins was determined by the first presence of spermatozoa in the semen, indicating the onset of spermatogenesis. At the end of the study, 14 subjects were sexually mature and 3 were sexually immature. Two subjects had measurements taken for both categories as they became sexually mature during the study period.

6.2.7 Weighing

The dolphin was coaxed to slide out of the water and lie down on the scale platform. Weighing was conducted in the morning during the first feed. Each animal was weighed once for each month since the beginning of the study. At the end of the study, the mean body weight of the studied population (n = 16) was 132.3 kg (range, 106.1 to 185.1 kg).

6.2.8 Body length measurement

Body length measurements were obtained with the dolphin lying flat and straight at the bottom of the pool, in the shallow water against the wall of the poolside. Two targets, 1 at the tip of the rostrum and 1 at notch between the flukes, were placed by 2 trainers to ensure that the dolphin was kept in a straight posture. A flexible measuring tape was placed along the 2 targets, and the dolphin body length measurement was recorded once for each month. At the end of the study, the mean body length of the studied population (n = 16) was 220.1 cm (range, 196 to 244 cm).

6.2.9 Statistical analysis

Analysis of the data was categorized into 2 life history developmental characteristics - somatic growth and reproductive development analysis of thyroid morphology. A three factorial analysis of variance (ANOVA) (SPSS for windows 16.0, SPSS Inc., Chicago, Illinois) was conducted to determine significant differences in thyroid volume among major factors for each category. For somatic growth analysis of thyroid morphology, the factors of animal age, sex and sampling season were evaluated. For reproductive development analysis of thyroid morphology, the factors of animal sex, sexual maturity and sampling season were assessed. Post-hoc tests using Tukey's test of pairwise multiple comparisons (Graphpad InStat 3.05, GraphPad Software San Diego, California) were used to isolate significant differences for age and sampling season as individual effects, whereas post-hoc tests using simple effects analyses were used to examine the simple main effects with contrast test on statistically significant interaction. For the analysis, all thyroid volumes were determined after a log-logit transformation of the standard curve (Robard, 1974) and expressed as cm^3 ± standard error of the mean to meet assumptions of normality and equal variance. Pearson product-moment correlation (SPSS for windows 16.0, SPSS Inc., Chicago, Illinois) was used to investigate any statistically significant association between thyroid volume and body weight or body length. Differences in thyroid gland border sharpness, homogeneity and echogenicity were assessed with Fisher's Exact test (Graphpad InStat 3.05, GraphPad Software San Diego, California) between age groups, sex and sexual maturity separately. For all tests completed, a p-value of less than 0.05 was considered to be statistically significant.

6.3 Results

In somatic growth analysis, significant effects in thyroid volume were detected among the factors of age and sex, as well as in the combined interaction of age and sex (Table 6.2 and Figure 6.1). However, no significant effect on thyroid volume

was detected for sampling season, nor any other combination of interactions ($p > 0.05$).

Table 6.2: Results of the factorial ANOVA for season, sex and age of a group of captive Indo-Pacific bottlenose dolphins in Hong Kong from August 2006 to January 2009.

Effect/Interaction	df	SS	MS	F	*P*
Season	3	0.062	0.021	1.058	0.366
Sex	1	1.230	1.230	63.020	0.001
Age	2	7.354	3.677	188.416	0.001
Season * Sex	3	0.069	0.023	1.185	0.314
Season * Age	6	0.125	0.021	1.071	0.378
Sex * Age	2	2.113	1.057	54.147	0.001
Season * Sex * Age	6	0.044	0.007	0.373	0.896

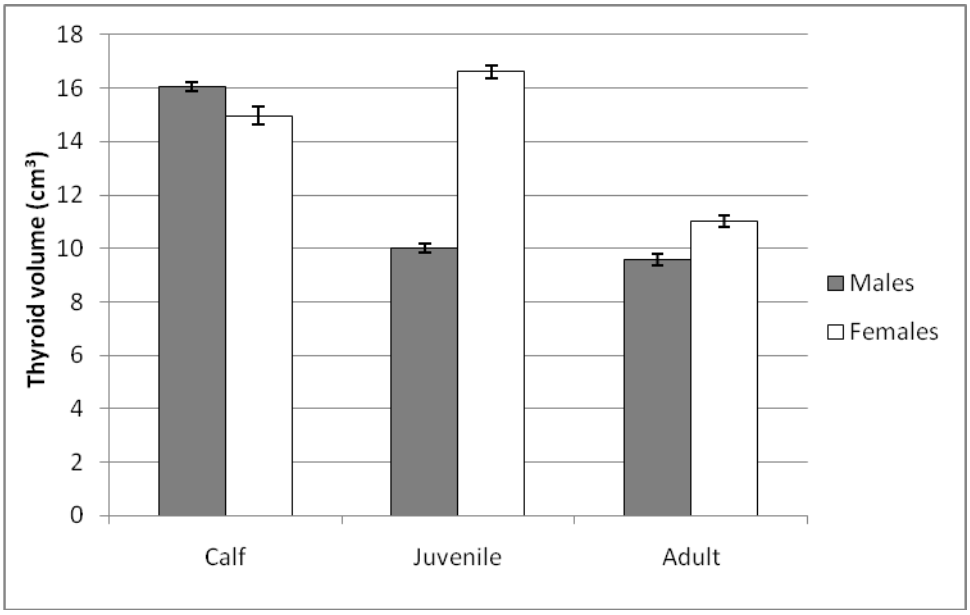

Figure 6.1: Mean and standard error of the mean (SEM) of thyroid volume (cm^3) in male and female captive Indo-Pacific bottlenose dolphins (n = 17) categorized according to different age classes.

Subsequent p-values stated are from Tukey's post-hoc comparisons. The calf thyroid volume (15.65 ± 0.16 cm^3) was significantly greater than that of adolescent (14.62 ± 0.24 cm^3, $p < 0.05$) and adult (10.65 ± 0.16 cm^3, $p < 0.001$). Moreover, adolescent thyroid volume was significantly greater than adult thyroid volume ($p < 0.001$). Thyroid volume measured in females (12.81 ± 0.17 cm^3) was significantly greater than that of males (11.98 ± 0.18 cm^3, $p < 0.001$).

Table 6.2 shows that there was a significant interaction between the factors of age and sex on thyroid volume, $F(2,1360) = 54.15$, $p < 0.001$. Table 6.3 shows the number of subjects, the mean and standard error of the mean of thyroid volume for each variable. Simple effects analyses revealed that for the juvenile and adult subjects, there was a significant difference in the thyroid volume between male and female dolphins ($p < 0.05$). However, the thyroid volume of male and female calves was not significantly different ($p > 0.05$).

Table 6.3: The sampled number of thyroid volume measurements (n = 1384) categorized according to season, sex and age with the mean and SEM given in cm^3.

Males	Calf			Juvenile			Adult		
Season	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**
Spring	36	16.42	0.46	14	9.44	0.21	36	9.54	0.64
Summer	31	16.82	0.28	26	11.21	0.47	48	9.86	0.36
Autumn	57	15.74	0.17	31	9.66	0.20	74	9.21	0.34
Winter	45	15.63	0.27	24	9.45	0.25	52	9.95	0.47
Total	169	16.05	0.15	95	10.00	0.18	210	9.60	0.22

Females	Calf			Juvenile			Adult		
Season	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**
Spring	27	15.41	0.69	31	15.65	0.54	98	11.54	0.53
Summer	17	15.29	1.01	60	16.61	0.45	124	10.83	0.41
Autumn	19	15.09	0.64	74	16.76	0.37	207	10.53	0.34
Winter	36	14.42	0.58	55	16.96	0.45	162	11.50	0.42
Total	99	14.97	0.35	220	16.61	0.22	591	11.03	0.21

Interactions of age and sex indicated that the juvenile (16.61 ± 0.22 cm^3) and adult (11.03 ± 0.21 cm^3) females had significantly greater thyroid volumes than the males in the same age group (10.00 ± 0.18 cm^3, $p < 0.001$ and 9.60 ± 0.22 cm^3, $p < 0.001$, respectively). However, for the calves, there was no significant difference in thyroid volume between females and males ($p > 0.05$).

In the reproductive development analysis, significant effects in thyroid volume were detected in the factor of sexual maturity, as well as in the combined interaction of sex and sexual maturity (Table 6.4 and Figure 6.2). However, no significant effect on thyroid volume was detected for sampling season, nor any other combination of interactions ($p > 0.05$).

Table 6.4: Results of the factorial ANOVA for season, sex and sexual maturity of a group of Indo-Pacific bottlenose dolphins in Hong Kong from August 2006 to January 2009.

Effect/Interaction	Df	SS	MS	F	*P*
Season	3	0.084	0.028	1.124	0.338
Sex	1	0.092	0.092	3.667	0.056
Sexual Maturity	1	3.903	3.903	156.026	0.001
Season * Sex	3	0.039	0.013	0.522	0.667
Season * Sexual Maturity	3	0.101	0.034	1.339	0.260
Sex * Sexual Maturity	1	0.756	0.756	30.214	0.001
Season * Sex * Sexual Maturity	3	0.039	0.013	0.525	0.665

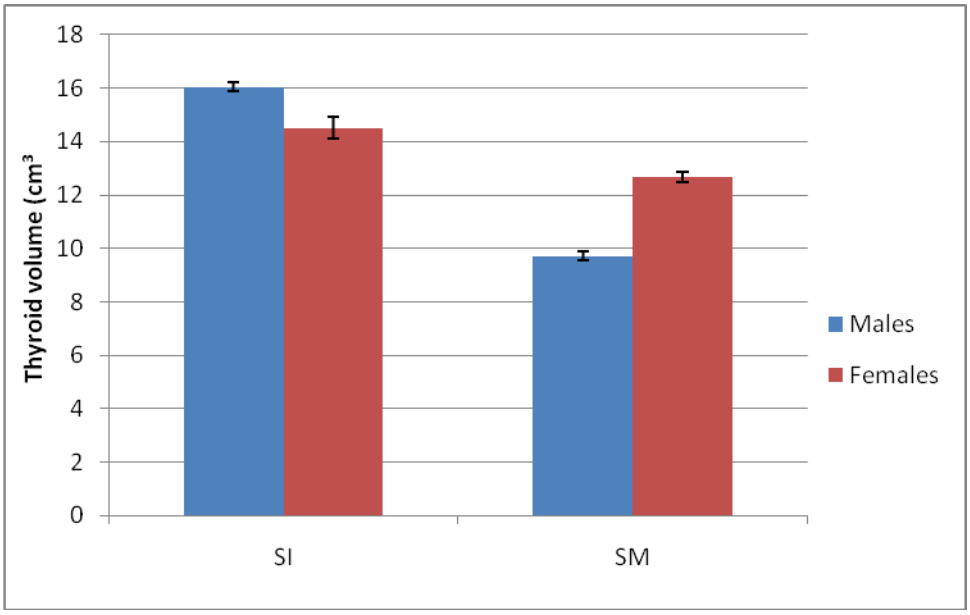

Figure 6.2: Mean and standard error of the mean (SEM) of thyroid volume (cm^3) in male and female captive Indo-Pacific bottlenose dolphins (n = 17) categorized according to sexual maturity (SI = sexually immature; SM = sexually mature).

Table 6.4 shows that there was a significant interaction between the factors of sex and sexual maturity on thyroid volume, [$F(1,1368) = 30.21$, $p < 0.001$]. Table 6.5

shows the number of subjects, the mean and standard deviation of thyroid volume for each variable. Simple effects analyses revealed that in both sexually mature and sexually immature dolphins, there was a significant difference in the thyroid volume between male and female subjects ($p < 0.05$).

Table 6.5: The sampled number of thyroid volume measurements (n=1384) categorized according to season, sex and sexual maturity with the mean and SEM given in cm^3.

SI		Males			Females	
Season	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**
Spring	36	16.42	0.46	10	15.30	0.34
Summer	31	16.82	0.28	17	15.29	1.01
Autumn	57	15.74	0.17	17	15.18	0.70
Winter	45	15.63	0.28	26	13.26	0.64
Total	169	16.05	0.15	70	14.51	0.40

SM		Males			Females	
Season	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**
Spring	50	9.51	0.46	146	12.87	0.42
Summer	74	10.34	0.29	184	12.71	0.37
Autumn	105	9.34	0.24	283	12.19	0.31
Winter	76	9.79	0.33	227	13.08	0.36
Total	305	9.72	0.16	840	12.66	0.18

Interactions of sex and sexual maturity indicated that sexually immature males (16.05 ± 0.15 cm^3) had significantly greater thyroid volumes than sexually immature females (14.51 ± 0.40 cm^3, $p < 0.001$). However, sexually mature males (9.72 ± 0.16 cm^3) had significantly smaller thyroid volumes than sexually mature females (12.66 ± 0.18 cm^3, $p < 0.001$).

Significant associations between thyroid volume and body length [r(280) = 0.565, p < 0.001] as well as body weight [r(311) = 0.407, p < 0.001] were found, and the body size of dolphins is positively correlated with their thyroid volume (Figures 6.3 and 6.4). Using Cohen's (1988) guidelines, the effect size is large for body length and medium-to-large for body weight. The r squared indicates that approximately 32% and 17% of the variance in thyroid volume can be predicted from body length and body weight respectively (Figures 6.3 and 6.4).

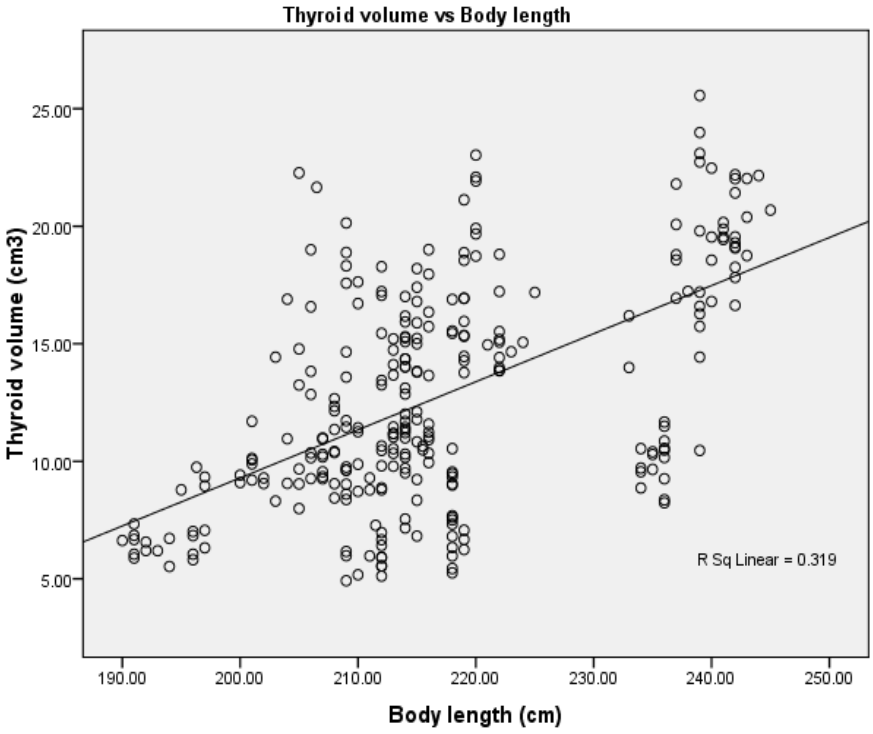

Figure 6.3: Relationship between thyroid volume and body length in a group of captive Indo-Pacific bottlenose dolphins (n = 17) sampled in 2007 and 2008. R^2 indicates that 32% of the variance in thyroid volume can be explained by body length (r = 0.565).

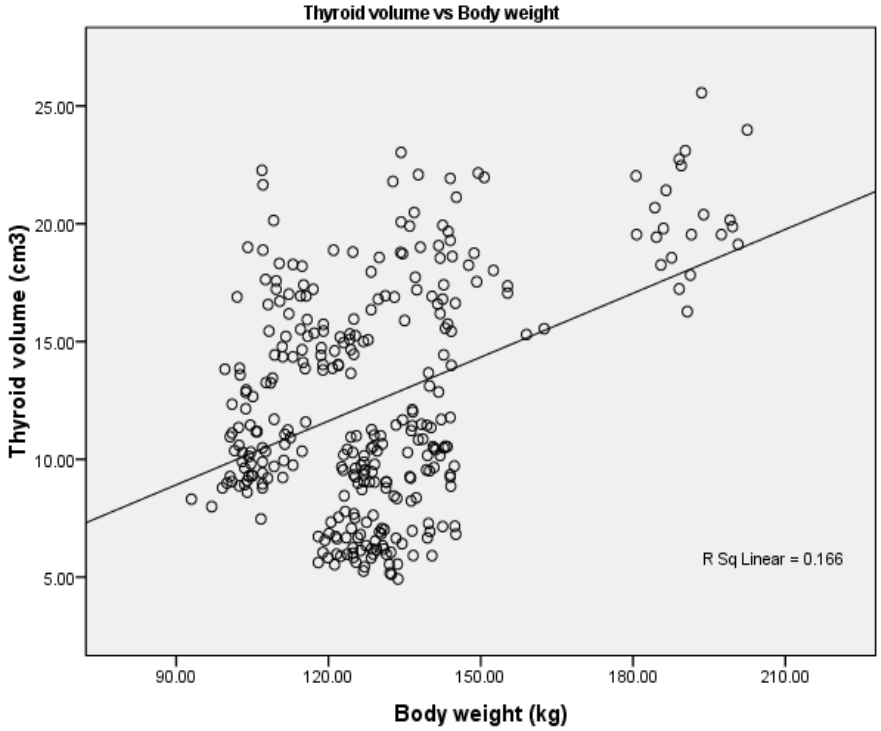

Figure 6.4: Relationship between thyroid volume and body weight in a group of captive Indo-Pacific bottlenose dolphins (n = 17) sampled in 2007 and 2008. R^2 indicates that 17% of the variance in thyroid volume can be explained by body weight (r = 0.407).

When age was considered, the borders of the thyroid gland were usually well-defined (82%) in 14 dolphins, whereas ill-defined borders were observed in 2 adults and 1 calf. The differences in border sharpness among the 3 age groups were not statistically significant ($p > 0.05$). The echopattern of the thyroid gland was generally homogeneous (76%), with echogenic reticulations. The differences in homogeneity among the 3 age groups were not statistically significant ($p > 0.05$). For the dolphins with a heterogeneous thyroid gland, 1 showed hyperechoic foci, 1 showed isoechoic foci with echolucent rim, 1 had hypoechoic foci, and 1 demonstrated a mottled appearance of thyroid gland. The relative echogenicity of the thyroid gland varied among the subjects. The echogenicity of the thyroid gland was usually hypoechoic or isoechoic when compared to the adjacent sternocephalicus muscle [4 (80%) and 1 (20%) in calf; 1 (17%) and 5 (83%) in

juvenile; and 8 (89%) and 1 (11%) in adult]. The differences in thyroid echogenicity were statistically significant ($p < 0.05$) between juveniles and adults, but not for other comparisons ($p > 0.05$).

When sex and sexual maturity were considered as the factors in assessing differences in border sharpness, homogeneity and echogenicity, their differences were not statistically significant ($p > 0.05$).

Echogenicity of the right, left thyroid lobe and isthmus was different in over half of the population (65%).

6.4 Discussion

This study established baseline thyroid morphology, with respect to 2 important life history developmental characteristics: somatic growth and reproductive development, in an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) population under human care using sonography. Ultrasound is a useful imaging tool in the assessment of thyroid morphology and physiology in humans (Hegedüs, 2001; AIUM, 2003; Khati et al., 2003) and companion animals (Cartee et al., 1993; Wisner et al., 2002; Reese et al., 2005; Brömel et al., 2006). Essential sonographic features such as the border sharpness, size, echogenicity and homogeneity of the normal dolphin thyroid gland, together with the understanding of any potential influence on thyroid morphology with different physiological determinants, are needed to offer a basis for the diagnosis of pathology. Information on ultrasound thyroid morphology in relation to animal age, sex, body size, must be identified to avoid misinterpretation of clinical findings altered by the various physiological parameters mentioned.

Our results indicated that under the somatic growth analysis, effects and interactions of animal age and sex should be taken into account while examining thyroid morphology in captive Indo-Pacific bottlenose dolphins. In the studied population,

thyroid volume significantly decreased with advancing age. There are a number of possible explanations for this observation. From an energetic perspective, as the body size increases with advancing age, the surface area to volume ratio of the animal will decrease, thereby reducing the overall heat loss per unit of body weight (Slijper, 1962; Lavigne et al., 1986; Boyd, 2002). Consequently, a decrease in metabolism and thyroid volume may be observed.

Cetaceans possess blubber that helps in thermoregulation, buoyancy control, streamlining, metabolic energy storage and locomotion (Rommel and Lowenstine, 2001). Struntz et al. (2004) described that in neonatal and juvenile dolphins, the blubber mass, depth and lipid content increase with advancing age. The calf tends to have a thinner layer of blubber with lower lipid content, which may not be adequate to provide sufficient insulation when compared to the juvenile and adult. Changes in thermal conductance can be achieved through altering the blubber (Meagher et al., 2008). When the animals grow, the thickness and lipid content of the blubber increase, which helps to enhance the animal's insulation and decrease its conductance (Worthy and Edwards, 1990; Kvadsheim et al., 1996; Dunkin et al., 2005). The lipid content of the blubber reaches the maximum value during the juvenile stage (Struntz et al., 2004). Heat is a product of energy metabolism and, to retain a constant body temperature that exceeds the ambient environmental temperature, marine mammals must balance heat input from metabolism with heat lost to the environment. Poor heat management due to lack of insulation would lead to an increased requirement for heat production through metabolism (Boyd, 2002). The thinner blubber of the calf and its higher metabolism to compensate the heat loss may explain the larger thyroid volume, at this stage of life in dolphins.

Studies indicated that bottlenose dolphins may have different metabolic rates depending on their age and reproductive status, which could, in turn, affect their rate of heat loss to the environment (Lavigne et al., 1986; Scott et al., 1989; Reddy et al., 1994; Waples, 1995; Costa and Williams, 1999; Wells and Scott, 1999). Immature mammals, which are actively growing, have metabolic requirements approximately

twice of that for adult mammals of similar size (Worthy, 2001). Due to their smaller body size, which leads to a larger surface area to volume ratio, dolphin calves may encounter greater heat loss or be more susceptible to cold than older dolphins. Younger animals were more susceptible to cold due to an apparent inability to increase their metabolic rate at low temperatures (Worthy, 2001). Cold, may lead to an adjustment that is mediated via thyroid releasing hormone to increase the secretion of thyroid hormones (Boyd, 2002), which in turn, increased the thyroid volume. Furthermore, the homeostatic heat exchange system may not be fully developed in the dolphin calf. As the thyroid volume of the calf was observed to be the largest among all age groups, this may indicate that the dolphin calf proceeds with a hyperthyroidal status to maintain heat production, which is suspected to play a role in the thermoregulation.

The dolphin's diet may also shed a light on the discrepancy in thyroid volume among different age groups. During the early life history stages, the dolphin is mainly relied on its mother's milk for all or a vast majority of its nutrition source (Wells and Scott, 1999). The thyroid gland of human infants is vulnerable to maternal iodine, maternal thyroid status and milk iodine concentration. Previous literature has documented the presence of T4 and iodine in mammalian milk (Dorea, 2002). Therefore, thyroid activity as well as morphology of the dolphin calf may be influenced by the nutritional status of the mother. In general, dolphins lactate for 1-3 years while nursing a healthy calf in captivity and studies confirmed that dolphin could proceed cyclic estrous pattern, or even conception while lactation (Yoshioka et al., 1989; West et al., 2000). It has been proven that dolphin's milk contains progesterone (West et al., 2000), which may affect the thyroid morphology of dolphin calves. It has been suspected that progesterone increases thyroid metabolism by positively stimulating the binding affinity of plasma proteins for thyroid hormones, thereby increasing thyroid volume.

As the calf transits into the juvenile stage, several major shifts in its life history occur. As the dolphin's surface area to volume ratio decreases with growth, there is

a lesser demand of energy for compensating heat loss, whereas a greater demand for energy to support growth in the body compartments, such as muscle and reproductive organs (Cockcroft and Ross, 1990; Worthy, 2001; Boyd, 2002; McLellan et al., 2002). Sub-adult bottlenose dolphins in the wild forage independently (Cockcroft and Ross, 1990), and when these animals reach puberty they experience a growth acceleration that would greatly increase their energetic demands (Read et al., 1993). In its diet, maternal milk is no longer a source of nutrition, although iodine intake should remain sufficient owing to the primary consumption of fish and other seafood. In humans, thyroid growth in adolescents with a normal iodine supply is mainly influenced by the growth factors during somatic development, and is modulated by the sex steroids for reproductive development. The effect of estrogen and progesterone may account for the higher incidence of goitre in girls with mild iodine deficiency, leading to variations in thyroid volume (Poppe et al., 2007). The shift of energetic demand from somatic growth to reproductive development may also account for the decrease in thyroid volume when dolphins aged.

Captive duration of the dolphin may also explain the variation in thyroid volume among different age groups. Adult dolphins in this population presumably stayed longer in the captive environments than the younger dolphins, where a constant food supply were provided mandatory and other captive conditions such as water temperature were fixed throughout the year. It is suggested that there may have a degenerative progress of thyroid gland, leading to a decrease in thyroid volume in dolphins when aged in captivity (Arvy, 1970). Studies revealed that the average follicle diameter of the thyroid gland in wild dolphin populations may appear to be larger than that of captive dolphin populations (200 μm vs 50 μm) (Harrison, 1969; Arvy, 1970; Cowan and Tajima, 2006). Necropsy reports of the captive adult dolphins found that thyroid follicles were uniformly small, containing only remnants of the condensed and densely stained colloid, suggesting severe thyroid depletion. The thyroid depletion in adult dolphins seems to indicate impairment in iodine absorption, a dietary iodine deficiency or a more rapid utilization and

turnover rate of iodine when dolphin aged. Further studies are needed to investigate the implications of possible progressive thyroid depletion in captive aged dolphin populations.

Overall, female dolphins had a significantly larger thyroid gland than male dolphins. This is contrary to previous studies in humans demonstrating that thyroid volume in females tends to be smaller than that in males (Hegedüs et al., 1983; Berghout et al., 1987; Hintze et al., 1991; Hsiao and Chang, 1994). Although some reports on humans also found a sex difference in the thyroid size, it has been accepted that the sex difference is resulted from differences in body weight rather than the influence of sex itself on the thyroid size (Berghout et al., 1987; Hsiao and Chang, 1994; Fleury et al., 2001; Eugene et al., 2005; Lee et al., 2006; Duan et al., 2009). The difference in the thyroid volume is agreed to be related to the difference in physique between sexes, rather than a difference in the hormonal environment (Hegedüs et al., 1983; Berghout et al., 1987; Hintze et al., 1991; Hsiao and Chang, 1994). The thyroid volume difference between Asians and Caucasians can also be explained by differences in physique. However, the result of the present study was different from that in the human studies. There was no significant sex difference in the body length and body weight of dolphins, despite this study demonstrating a significant difference between the thyroid volumes of males and females. It is suggested that there may have other physiological factors influencing the thyroid volume in association with sex. Previous literature regarding the influence of sex in the marine mammal thyroid was scarce. St. Aubin et al. (1996) investigated the effect of sex, as well as other demographic parameters on the thyroid function of the Atlantic bottlenose dolphin (*Tursiops truncatus*). Sex was found to have the most consistent influence on thyroid hormones, with higher hormone levels observed in females. Sex-related differences were found in total T4, free T4 and free T3 in wild *Tursiops*, while only total T3 differed according to sex in semidomesticated *Tursiops*. The author explained that hormonal changes associated with reproduction in females can indirectly lead to elevations in total T4 and total T3 by increasing hormone binding capacity in plasma (St. Aubin et al., 1996). This contrasts with the action of

testosterone in males, as it is demonstrated to cause a decrease in T4-binding globulin and reduces the binding of T4, thereby reducing total T4 levels (Bahrami et al., 2009). Therefore, the thyroid volume differences in sex in the present study may possibly be related to the innate differences in the hormonal environment. In humans, prevalence of goiter between females and males are different (Ahuja, 2000; Bruneton et al., 2002; Farahati et al., 2006), suggesting sex-specific determinants and mechanisms that promote and/or prevent thyroid enlargement disorders. In females, parity and iodine status were associated with goiter frequency, and the number of pregnancies associated with goiter prevalence. Thus, it may be possible that the significantly larger thyroid gland in female dolphins in the present study may be attributed to the presence of mild/moderate level of goiter in females, which may not be noticeable in our ultrasound examinations. Garner et al. (2002) reported the presence of diffused hyperplastic goiter in 11 perinatal Atlantic bottlenose dolphins (*Tursiops truncatus*) and claimed that the goiter might have been attributed to an imbalance of maternal dietary iodine levels and an inherited dyshormonogenetic inability to synthesize or secrete adequate amounts of thyroid hormones leading up to the time of birth. Further studies are suggested to clarify whether the greater thyroid volume in female dolphins are associated with the difference in iodine status with respect to the opposite sex and parity, leading to different extent of goiter.

Interactions of age and sex indicated that juvenile and adult females also had significantly greater thyroid volume compared to males of the same age group. There was no significant difference in thyroid volume between male and female calves. In calves, energy expenditure is primarily directed towards somatic growth, which includes theromoregulation, development of major organs and muscle tissue for sustained locomotion. Thus, sex differences on thyroid growth remain indiscrete at this life stage. At the juvenile and adult stages, thyroid growth may no longer be primarily influenced by factors involved with somatic growth, but could be further modulated by sex steroids associated with reproductive development. Similar observations have been reported in humans. Chanoine et al. (1991) demonstrated

that the growth of the thyroid gland in residents of iodine-sufficient areas is consistent with the body growth, and thyroid volume increases until the age of 8, without being influenced by the sex. Garel and Léger (2007) found that from 7 days to 8 years of age, the volume of the thyroid gland increases slowly, with no significant difference between girls and boys. At puberty, thyroid volume is greater in girls than in boys (Müller-Leisse et al., 1988, Bruneton et al., 2002). Variability of thyroid volume throughout the normal estrous cycle in females has also been observed, in contrast to males (Hegedüs, 2001). The hormone profile in male bottlenose dolphins remains relatively consistent throughout life after pubertal development. However, female bottlenose dolphins are subject to polyestrous cycling, a process that is intimately related to the endocrine system. Sex steroid receptors were identified in normal and pathological mammalian thyroid tissues, and it is reported that estrogens might precede a promoting effect and androgen a rather inhibitory effect on thyroid tissues (Kuhl et al., 1985; Métayé et al., 1993; Rossi et al., 1996; Bahrami et al., 2009). This may account for the significantly greater thyroid volume observed in female dolphins at the juvenile and adult stages.

For reproductive development analysis, a significant effect in thyroid volume was found in the factor of sexual maturity, as well as a significant interaction in sexual maturity and sex. Sexually immature subjects had significantly greater thyroid volumes than sexually mature subjects. This may be accounted for the dominancy of somatic growth instead of reproductive development at the early stages of life. Sexually immature subjects have higher metabolic demands for thermoregulation as well as organ growth and development, thus having a greater thyroid volume. The emphasis on somatic growth in sexually immature subjects is intended to achieve a larger body size for optimal reproductive fitness, as they only invest energy in their own development, differentiation, and maintenance (Berta et al., 2006a). When the subject attains sexual maturity, the thyroid physiology becomes further complicated by cyclic influences of sex steroids in females, while in males, these influences are not as pronounced. Different reproductive events with correspondently different energy expenditures may lead to various changes in thyroid physiology (Reddy et

al., 1994). Previous studies in humans have reported that the thyroid volume varied significantly during the normal menstrual cycle in females of reproductive age, and thyroid function/physiologic characteristics altered during different reproductive phases of a woman's life (Hegedüs, 2001; Redmond, 2004; Poppe et al., 2007). Rasmussen et al. (1989a) found that thyroid volume increased with a mean variation of 30% during pregnancy. At 12 months after delivery, thyroid volume had not resumed to the values found in early gestation, which indicated that pregnancy may possibly be a prolonged stimulus for the thyroid gland and alterations persist well past late postpartum (Glinoer et al., 1992).

In domestic mammals, Reimers et al. (1984) found that the serum T4 levels in pregnant adult Beagle subjects were similar to those in diestrous subjects but were greater than the levels found in subjects at other reproductive states. In cetaceans, St. Aubin et al. (1996) found that wild female bottlenose dolphins had significantly higher levels of total T4, free T4, and free T3, which were possibly related to the reproduction and lactation of the animals. West et al. (2003) observed a different pattern of T4 concentrations in female bottlenose dolphins, and found that both total T4 and free T4 dropped considerably as pregnancy progressed. Similar observation was also found in pregnant or lactating baleen whales, which had significantly lower thyroid hormone concentrations when compared to other sex and reproductive groups investigated (Rosa et al., 2007). Lactation itself appears to have an effect on thyroid function. Cowan and Tajima (2006) reported that thyroid glands in pregnant and lactating females were larger than non-pregnant animals of comparable size.

Interactions of sexual maturity and sex revealed that sexually immature males were greater in thyroid volume than sexually immature females, while sexually mature females had greater thyroid volumes than sexually mature males. Marine mammals typically show sexual bimaturism (Evans and Stirling, 2001). As part of a polygynous species, male bottlenose dolphins attain sexual maturity later than females (Scott et al., 1989; Evans and Stirling, 2001; Berta et al., 2006b). This allows additional time for somatic growth, giving the males a larger body size

before competing for females upon reaching sexual maturity. The larger thyroid volume observed in sexually immature males compared to sexually immature females in the present study may be due to increased metabolic demand from higher energy expenditure during somatic growth. The increased metabolic demand leads to an increase in thyroid function and physiology, resulting in a larger thyroid volume for sexually immature males. Since female bottlenose dolphins attain sexual maturity earlier than males (Scott et al., 1989; Evans and Stirling, 2001; Berta et al., 2006b), they start reproducing earlier and are subjected to a variety of hormonal influences from different reproductive events, which may have profound effects on their thyroid physiology. Compared with sexually mature males, sexually mature females direct more of their energy expenditure on reproduction, particularly during pregnancy and lactation (Reddy et al., 1994). As a result of these high energy demanding reproductive events, the thyroid gland increases in volume to augment the metabolic processes in sexually mature females. Since observations in the reproductive development analysis were not specifically categorized according to reproductive events, further study could be useful to understand the underlying associations between thyroid morphology and reproductive events.

Both body length and body weight were found to be positively correlated with thyroid volume, but in different extents. Positive significant correlations have been demonstrated between dolphin thyroid weight, standard body length and body mass (Turner et al., 2006). In the present study, body weight correlated weakly with thyroid volume, which may be attributed to the relative decrease in the thickness of the blubber layer as the animal increases in size. Cowan and Tajima (2006) suggested that the thyroid weight lags behind when the animal increases in size. In the present study, body length was found to have a comparatively stronger correlation with thyroid volume, in similarity to previous findings of the thyroid weight to body length correlation in bottlenose dolphins (Cowan and Tajima, 2006). This is because body weight is more strongly influenced by health and nutritional status, whereas body length remains consistent throughout life. During the exponential phase of the growth curve, it appears that body length can be used as a

relatively accurate measurement of age for calves and juveniles (Read et al., 1993). However, Turner et al. (2006) stated that body length is not a reliable indicator of age estimation in sexually mature animals.

In the present study, the ultrasound features of bottlenose dolphin thyroid glands varied mostly when age was considered as a factor in the assessment. The borders of the thyroid gland were usually well-defined, since the dolphin thyroid gland was encapsulated, which provided a uniform surface for ultrasound beam reflection. However, ill-defined thyroid gland was also observed in 2 adults and 1 calf. This could be attributed to the possible adipose tissue deposition and connective tissue proliferation surrounding their thyroid glands, leading to a decrease in the acoustic impedance difference between the thyroid parenchyma and the adjacent soft tissues (Burroughs and Shenkman, 1982; Das et al., 2006). In the studied population, the differences in border sharpness among the 3 age groups were not statistically significant.

The gross morphology demonstrated that dolphin thyroid gland usually appeared compact and relatively homogeneous starting from the period of infancy (Cowan and Tajima, 2006), which may be attributed to the generally uniform and homogeneous echopattern of the thyroid gland in the ultrasound examinations. Additionally, the presence of echogenic reticulations within the thyroid gland may be owed to the presence of fibrous bands, which are considered to be normal and increasing in frequency with advancing age (Cowan and Tajima, 2006). Although the variability of follicle size and colloid density tended to increase with age (Arvy, 1970; Ridgway, 1972), the differences in homogeneity of the thyroid gland among the 3 age groups were not significant, and the thyroid gland tended to be homogeneous. Various thyroid disorders were identified and reported histologically (Cowan, 1966; Harrison, 1969; Arvy, 1970; Garner et al., 2002; Cowan and Tajima, 2006). The underlying thyroid disorders may explain the heterogeneous ultrasound appearance of the thyroid gland in the present study. Further studies are suggested to

correlate the histological findings with the observed ultrasound findings in subjects with heterogeneous echopattern of the thyroid gland.

In the present study, the relative echogenicity of the thyroid gland mostly varied in the subjects. The thyroid gland tended to be hypoechoic when compared to the adjacent sternocephalicus muscle in calves (80%) and adults (89%), while the thyroid gland was usually isoechoic in juveniles (83%). The echogenicity of the thyroid gland coincides with the architecture of the organ (follicular size and colloid density) (Müller et al., 1985). Changes in the histological appearance thus may reflect changes in functional status, as well as presence of different thyroid abnormalities (St. Aubin and Geraci, 1989). In cetaceans, a study reported that the thyroid gland of Long-finned Pilot whales exhibited noticeable variation in size and appearance when age advanced, although active formation of follicles were reported in all stages of development in Pacific White-sided dolphins (Harrison, 1969). Generally speaking, the thyroid follicles are more regular in young animals. The colloid stains readily (slightly chromophile), its appearance is more fluid and the epithelial cells are tall. In older animals, the colloid is less hydrated in appearance and a large number of lamellar basophile masses are present in the thyroid gland (Arvy, 1970). Cowan and Tajima (2006) studied the thyroid morphology of 60 stranded Atlantic bottlenose dolphins and reported that in young dolphins, areas predominantly occupied by small follicles were scarcely recognizable as thyroid tissue, the colloid-free follicles appearing as cell clusters rather than follicles.

In humans, the interface between thyroid cells and the colloid exhibits high acoustic impedance, causing more ultrasound waves to be reflected back to the transducer, giving a hyperechoic thyroid gland compared to the sternocleidomastoid muscle. Previous studies demonstrated that the dolphin thyroid cells were smaller than the normal human thyroid cells, resulting in a lower echogenicity (Ridgway, 1972). Therefore, in the calf thyroid gland, the hypoechoic appearance may indicate small active-growing follicles evidencing the enhanced thyroid function/activity to fulfill higher metabolic need for somatic growth. The thyroid development is followed by

the maturation of larger follicles at the juvenile stage, in which the thyroid gland has an isoechoic appearance due to the increased interface between thyroid cells and the colloid exhibits high acoustic impedance. At the adult stage, the thyroid gland may develop various architectures that are influenced by the alternation of sex steroids as well as possibly developing signs of degeneration or subclinical thyroid abnormalities, giving it a hypoechoic appearance again. Since there were no clinical signs of thyroid function disorders observed in the investigated dolphin population, a dolphin thyroid gland with hypoechoic and isoechoic appearances may be considered normal.

In addition, varied echogenicity at different lobes or portions of a single thyroid gland could be possibly explained by uneven distribution of various sizes of follicles within the dolphin's thyroid gland. Shimokawa et al. (2002) reported that irregular or oval follicular lumens were seen in the parenchyma of the thyroid gland, with the size of the follicular lumen appearing larger in the central regions than in the peripheral regions in Risso's dolphins.

6.5 Conclusions

This study was undertaken to gain a better understanding of thyroid morphology in Indo-Pacific bottlenose dolphins by investigating potential determinants such as age, sex, sexual maturity, season and body size. Identification and greater knowledge of these determinants will provide a normative reference to clinically recognize and treat thyroid gland abnormalities in living dolphins, which allows better monitoring of the thyroid gland during the course of treatment. This information would also be helpful for facilities in attaining self-sustainability and allowing optimal captive management.

Chapter Seven

Study Five

Sonographic evaluation of thyroid morphology during different reproductive events in female Indo-Pacific bottlenose dolphins, *Tursiops aduncus*

7.1 Introduction

Interpretation of thyroid morphology in marine mammals should account for dynamic changes associated with life-history events (St. Aubin 2001). In particular, thyroid function and morphology in females are likely affected by the cyclic change of hormonal variation during the estrous cycle and different reproductive events (Chan et al. 1998, Krejza et al. 2004). Abnormal thyroid function is potentially problematic to healthy pregnancies in dolphins and affects calf survivorship (Garner et al. 2002). As dolphins are primarily diagnosed post-mortem, there is substantial autolysis on the thyroid gland tissue, placing limitations on histological investigation and making the findings difficult to serve as a reference for live dolphins. Before thyroid abnormalities can be accurately diagnosed and assessed in living subjects, reliable methods for assessing the normal thyroid morphology must be developed.

In patients with clinical signs of thyroid abnormalities, thyroid function disorders are usually diagnosed by evaluating the serum concentration of total or free thyroxine (T4), as well as thyroid stimulating hormone (TSH) (Foktin et al., 2010). Free hormonal concentration is distinguished from total hormonal concentration by its unbound state and thus greater functional effect on target tissues. Thyroid hormones can be suppressed by non-thyroidal illness, such as euthyroid sick syndrome, and can also be suppressed by medications. Therefore, measuring basal concentrations of total or free T4 alone is inadequate for thyroid disorder diagnoses. Prior to the readily availability of an assay for dolphin TSH (St. Aubin, 2001),

animals with thyroid abnormalities can be morphologically investigated by using ultrasound to determine the thyroid volume and sonographic features.. Previous literature has also suggested that ultrasound may be more sensitive than serum TSH concentration in assessing thyroid disturbance (Stewart et al., 1989).

The application of ultrasound in the differential diagnosis of thyroid disorders in humans and companion animals has been reported (Cartee et al., 1993; Ahuja, 2000; Bruneton et al., 2002; Wisner et al., 2002; AIUM, 2003; Reese et al., 2005; Brömel et al., 2006). Apart from the pathological changes, physiological alternations of thyroid morphology with changes of female hormonal environments and dietary iodine intake have been observed. Previous studies in humans have reported that the thyroid volume varied significantly during the normal menstrual cycle in females of reproductive age, and thyroid physiological characteristics altered during different reproductive phases of women (Hegedüs et al., 1986; Nelson et al., 1987; Brander and Kivisaari, 1989; Hegedüs, 1990; O'Leary et al., 1992; Berghout and Wiersinga, 1998; Chan et al., 1998,1999; Tajtáková et al., 1999; Doufas and Mastorakos, 2000; Krejza et al., 2004; Fister et al., 2006; Soares et al., 2008; Fister et al., 2009). In a companion animal study, serum thyroid hormone concentrations were found to be greater in diestrous females than in anestrous, proestrous and lactating females (Reimers et al., 1984).

Maternal thyroid physiology affects fetal well-being. In humans, thyroid parameters during serum investigation are used as determinants of thyroid disease in pregnant women. During a normal human pregnancy, T4 varies according to trimesters, with an increase in total T4 and a decrease in free T4 (O'Leary et al., 1992; Berghout and Wiersinga, 1998; Doufas and Mastorakos, 2000; Glinoer, 2005). However, West et al. (2003) observed that in bottlenose dolphins, both the total and free T4 dropped considerably as pregnancy progressed. Lactation itself appears to have an effect on thyroid function. St. Aubin et al. (1996) observed a higher level of total T4 in wild female bottlenose dolphins undergoing lactation. When these lactating females were excluded from the analysis, there was no significant difference in thyroid hormone

levels found between semi-domesticated and wild dolphins. Therefore, lactation may have a profound effect on thyroid hormone levels.

Thyroid morphology in dolphins during reproductive events was demonstrated to be different from that in humans, but due to the unclear methodology reported by West et al. (2003), further investigation is needed with a more reliable and accurate measurement tool to monitor the dolphin thyroid morphology in different reproductive stages. In line with the present study, an ovarian imaging project was conducted by experienced in-house veterinarians in a female bottlenose dolphin population in Ocean Park Hong Kong for a length of over 4 years, with a regularly updated storage of their reproductive status reports and known demographic parameters, a unique opportunity was provided to investigate the possible influence of different reproductive states on thyroid morphology in a captive environment. To the best of our knowledge, previous literature is devoid of information on the association between thyroid morphology and reproductive status in normal female bottlenose dolphins. For accurate assessment of the thyroid physiology in adult female bottlenose dolphins, understanding the baseline thyroid morphology during different reproductive events is needed. This study was undertaken to investigate the possible variations of thyroid morphology in female Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) during different reproductive events using sonography.

7.2 Materials and Methods

7.2.1 Subjects

Nine sexually mature female *Tursiops aduncus*, at Ocean Park, Hong Kong were included in the study. At the end of the study, the mean age of the subjects was 18 years (range, 7 - 37 years). All dolphins involved in the study were being trained to cooperate for neck and ovarian sonographic examinations. Characteristics of the subjects in this study were tabulated (Table 7.1).

Table 7.1: Characteristics of the captive female bottlenose dolphins in this study.

Animal	Date of Birth (DD/MM/YYYY)	Reproductive Event	Data Collection Period	Body Weight – Initial to Final (kg)	Body Length – Initial to Final (cm)
Ada	01/01/1983	Lactating, Estrus, Anestrus	Aug 2007 to Jan 2009	115.0 to 106.1	209 to 201
Angel	01/01/1984	Estrus, Anestrus	Aug 2006 to Jan 2009	128.4 to 136.5	212 to 215
Ester	01/01/1988	Pregnancy, Estrus, Anestrus	Jul 2006 to Jan 2009	130.0 to 138.0	212 to 212
Gina	01/01/1983	Estrus, Anestrus	Jul 2006 to Jan 2009	122.6 to 132.8	205 to 208
Hicky	01/01/1988	Estrus, Anestrus	Jul 2006 to Jan 2009	118.0 to 124.0	191 to 196
Hoi Kei	14/05/2001	Estrus, Anestrus	Jul 2006 to Jan 2009	101.0 to 108.3	204 to 214
Jessie	01/01/1972	Lactating, Estrus, Anestrus	Aug 2006 to Jan 2009	193.5 to 185.1	239 to 242
Maya	04/06/2001	Estrus, Anestrus	Jul 2006 to Jan 2009	137.1 to 147.1	239 to 244
Pinky	15/09/1998	Pregnancy, Estrus, Anestrus	Jul 2006 to Jan 2009	121.2 to 140.3	216 to 220

7.2.2 Equipment

All sonographic examinations were performed with either a Philips HD-11 ultrasound unit or a Philips HD-11 XE ultrasound unit, in conjunction with a 6-2 MHz curvilinear 3-D broadband curved array transducer and a 5-2 MHz 2-D broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA) or an Aloka SSD 900 ultrasound unit (Aloka Co. Ltd, Mitakasho, Tokyo) in conjunction with a 5 MHz curvilinear transducer. All images were recorded with either direct digital capture or with a thermal printer. Because of the nature of the dolphin's skin, there is no air layer between the surface and the transducer, so no coupling gel was required.

7.2.3 Scanning procedures

Sonographic examination of the thyroid gland and ovaries was performed once a week for 2.5 years (August 2006 to January 2009). For the ovarian scans, both ovaries were assessed at each examination and the ovarian cortex was examined using the established method (Brook, 2001), and reported during different stages of the reproductive cycle. When folliculogenesis was observed, ovarian scans were performed more frequently to document follicle growth rates and to predict time of ovulation. The size of the dominant follicle was noted and monitored until ovulation. The size and appearance of the corpus luteum was also monitored. Ovarian ultrasound was performed and reported by experienced veterinarians in Ocean Park, Hong Kong and recorded images and reports were further evaluated by an experienced sonographer. The anestruswas defined as the period in which ovulation normally stops, and it was reported that the duration could be up to 2.5 years (Brook, 1997). The lactating state was defined as the period which immediately follows a gestational period of a subject, lasting for up to 2 years or more in captivity (Robeck et al., 2001). Pregnancy was defined as the state in which the subject is fertilized and nurtures the fetus up until the point of delivery, lasting approximately 12 months (Lacave et al., 2004). The estrus was defined as the period in which a female is sexually receptive to males for the purposes of conception, lasting from 27 to 33 days (Brook, 1997).

For the thyroid scans, the dolphin was positioned in dorsal recumbence close to the poolside, with its tail supported by a trainer. The transducer was initially placed in a transverse orientation at the thoracic inlet, midway between the insertions of the pectoral flippers. The transducer was moved cranially until the brachiocephalic vein was identified. The transducer was then moved further cranially until the left and right lobes of the thyroid gland were identified. The thyroid volume was measured using the same protocol of Method A as used in Chapter 4 (see Section 4.2.1.1).

Sonographic features such as echogenicity and homogeneity of the thyroid gland were also evaluated. The echogenicity of the thyroid gland was compared to sternocephalicus muscle and classified into hypoechoic, isoechoic or hyperechoic. The thyroid parenchyma was categorized into homogeneous or heterogeneous on the basis of the presence or absence of any nodules or space-occupying lesions.

7.2.4 Statistical analysis

The ultrasound findings of each dolphin were summarized and tabulated individually according to the reproductive status of the subject. The mean and standard deviation of thyroid volume of all the subjects at different reproductive events were also calculated. This allows for within-subject comparison as well as comparisons across different subjects.

To estimate the effects of the reproductive events for thyroid volume, a generalized linear mixed model was applied by using a statistical package SPSS (SPSS for windows 16.0, SPSS Inc., Chicago, Illinois). The classical regression model was given as follows,

$$Y = X\beta + \varepsilon$$

Y is the vector containing the depending variable, thyroid volume.

X is the known matrix containing the vector of the independent variable, reproductive event.
β is the vector containing the overall mean and all mixed effect parameters.
ε is the vector containing the random effects.

The modelled relationship was determined as follows,

Thyroid Volume = Reproductive Event + Name + Date*Name

Name was defined as the individual dolphin subject. Date was defined as the date of the neck ultrasound examination. Reproductive Event was defined as the reproductive status of the individual dolphin subject.

When the final model was run, 1 of the reproductive events was fixed as the Intercept, and multiple t-tests were performed for direct comparison against the other events. The analyses provided the mean modelled thyroid volume, the standard deviation, the degrees of freedom, and the p value for significance.

7.3 Results

7.3.1 Statistical Model

Statistical validity of the final model was confirmed with information criteria such as Deviance, Akaike's information criterion (AIC), Akaike's information criterion with small sample correction (AICc), consistent Akaike's information criterion (CAIC) and Bayesian information criterion (BIC) when compared with the following modeled relationships:

Thyroid Volume = Name

Thyroid Volume = Name + Date*Name

The information criterion value showed a decrease when compared to the aforementioned models, indicating that the final model was a better fit. The final model with reproductive event produces a statistically significant ($p < 0.05$) better fit. Therefore reproductive event is a significant predictor for thyroid volume measurement in bottlenose dolphins.

The Estimates of Fixed Effects from the linear mixed model are shown in Table 7.2.

Table 7.2: Information criteria for model validation.

Model	Deviance	AIC	AICC	CAIC	BIC
(Name)	3172	3178	3178	3195	3192
(Name + Date*Name)	3169	3175	3175	3192	3189
(Reproductive Event + Name + Date*Name)	3161	3167	3167	3184	3181

Statistical validity of the model was confirmed with information criteria such as Deviance, Akaike's information criterion (AIC), Akaike's information criterion with small sample correction (AICc), consistent Akaike's information criterion (CAIC) and Bayesian information criterion (BIC).

This study demonstrated that reproductive event is a significant predictor for thyroid volume measurement, and significant variability of thyroid volume was found among different reproductive events in female bottlenose dolphins. Among the 4 reproductive events investigated, there was a significant difference ($p < 0.05$) found between the fixed effects lactation and estrus, as well as between the fixed effects lactation and anestrus (Table 7.3). The modelled thyroid volume had a mean of 12.51 mL, when all predictors had null effect. When anestrus was chosen as the reference parameter, effects of the other reproductive events, i.e. estrus, lactation, and pregnancy were observed. When compared to anestrus, estrus led to a decrease in the modelled thyroid volume by 0.26 units. Lactation and pregnancy both increased the modelled thyroid volume by 0.91 and 0.26 units respectively, when compared to anestrus. However, no observable change in thyroid sonographic features was found during different reproductive events.

Table 7.3: The Estimates of Fixed Effects from the linear mixed model. showing the relative effect of each reproductive event on thyroid volume when compared to the Intercept (baseline reproductive event). A p-value of less than 0.05 was considered to be statistically significant.

Parameter	Estimate	Std. Error	df	T	Sig.
Intercept/Pregnancy	12.54	1.74	8.32	7.17	<0.05
[Anestrus]	-0.26	0.26	768.61	-0.98	0.33
[Estrus]	-0.52	0.31	716.80	-1.66	0.10
[Lactating]	0.65	0.46	772.24	1.42	0.16

Parameter	Estimate	Std. Error	df	T	Sig.
Intercept/Anestrus	12.28	1.73	8.02	7.09	<0.05
[Estrus]	-0.26	0.18	383.42	-1.41	0.16
[Lactating]	0.91	0.37	772.97	2.41	<0.05
[Pregnancy]	0.26	0.26	768.61	0.98	0.329

Parameter	Estimate	Std. Error	df	T	Sig.
Intercept/Estrus	12.02	1.74	8.14	6.91	<0.05
[Anestrus]	0.26	0.19	383.42	1.41	0.16
[Lactating]	1.18	0.39	769.90	3.03	<0.05
[Pregnancy]	0.52	0.32	716.80	1.66	0.10

The ultrasound findings of each subject were summarized and tabulated according to different reproductive events (Table 7.4). A plot of thyroid volume measurements obtained during the sampling period was produced for each of the 9 studied subjects (Figure 7.1).

Table 7.4: Thyroid volume measurements and the corresponding reproductive characteristics of individual *T. aduncus* during a) estrus, b) pregnancy, and c) lactation.

a

ID	Data Collection Period	Number of Estrous Cycle	Number of Estrous Cycle with Decreased Thyroid Volume	Number of Estrous Cycle with Increased Thyroid Volume	Lowest Mean Thyroid Volume (mL)	Highest Mean Thyroid Volume (mL)	Treatment Period with Regu-Mate®
F2	Aug 2006 - Jan 2009	6	5	1	9.66	12.49	N/A
F4	Jul 2006 - Jan 2009	4	3	1	8.45	10.83	N/A
F5	Jul 2006 - Jan 2009	8	4	3	5.82	6.98	Dec 2007 - Mar 2008 Sept 2008 - Jan 2009
F6	Jul 2006 - Jan 2009	2	0	1	10.14	12.65	Dec 2007 - Dec 2008
F8	Jul 2006 - Jan 2009	4	3	1	14.32	19.84	Dec 2007- Jul 2008 Aug 2008 - Oct 2008

b

ID	Data Collection Period	Pregnancy Period	Number of Estrous Cycle	Number of Estrous Cycle with Decreased Thyroid Volume	Number of Estrous Cycle with Increased Thyroid Volume	Lowest Mean Thyroid Volume (mL)	Highest Mean Thyroid Volume (mL)	Average Thyroid Volume During Pregnancy (mL)	Treatment Period with Regu-Mate®
F3	Jul 2006 - Jan 2009	Aug 2006 - Aug 2007	1	1	0	5.96	6.93	6.93	Dec 2007 - Dec 2008
F9	Jul 2006 - Jan 2009	Mar 2007 - Feb 2008	3	1	2	15.73	20.53	17.85	Apr 2008 - Dec 2008

c

ID	Data Collection Period	Number of Estrous Cycle	Number of Estrous Cycle with Decreased Thyroid Volume	Number of Estrous Cycle with Increased Thyroid Volume	Lowest Mean Thyroid Volume (mL)	Highest Mean Thyroid Volume (mL)
F1	Aug 2007 - Jan 2009	1	1	0	8.53	9.28
F7	Aug 2006 - Jan 2009	4	2	2	17.68	21.98

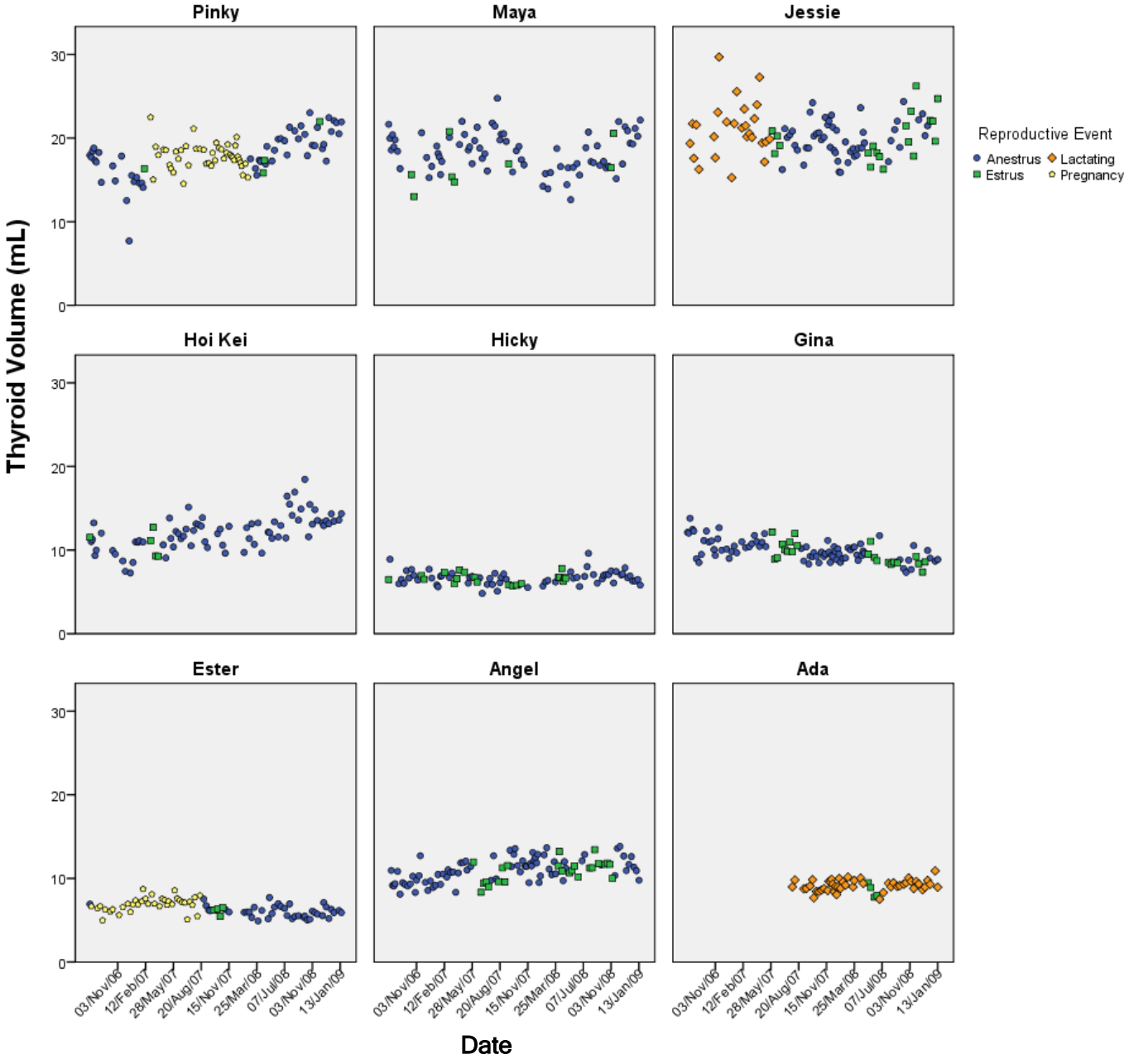

Figure 7.1: Individual plots showing the thyroid volume along with the corresponding reproductive event during the time of the measurement.

7.3.2 Estrus

Angel was monitored from August 2006 to January 2009. During that period of time, 6 estrous cycles were observed in alternation with the anestrus. The 1st, 2nd, 4th, 5th and 6th estrous cycle was accompanied by a drop in thyroid volume when compared with the previous anestrus. The lowest mean thyroid volume observed during the sampling period was 9.66 mL during estrus, and the highest mean thyroid volume was 12.49 mL during anestrus.

Gina was monitored from July 2006 to January 2009. During that period of time, 4 estrous cycles were observed in alternation with the anestrus. The 1st, 3rd and 4th estrous cycle was accompanied by a drop in thyroid volume when compared with the previous anestrus. The anestrus immediately following the 2nd estrous cycle had a higher increase in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 8.45 mL during estrus, and the highest mean thyroid volume was 10.83 mL during anestrus.

Hicky was monitored from July 2006 to January 2009. During that period of time, 8 estrous cycles were observed in alternation with the anestrus. The 2nd, 4th, 5th and 6th estrous cycle was accompanied by a drop in thyroid volume when compared with the previous anestrus. The anestrusimmediately following the 1st, 7th and 8th estrous cycle had a higher increase in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 5.82 mL during estrus, and the highest mean thyroid volume was 6.98 mL during anestrus. Hicky was undergoing treatment with altrenogest (Regu-mate; Intervet) from December 2007 to March 2008, and from September 2008 onwards.

Hoi Kei was monitored from July 2006 to January 2009. During that period of time, 2 estrous cycles were observed in alternation with the anestrus. The anestrus immediately following the 2nd estrous cycle had a higher increase in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 10.14 mL during anestrus, and the highest mean thyroid volume was 12.65 mL also

during anestrus. Hoi Kei was undergoing treatment with altrenogest (Regu-mate; Intervet) from December 2007 to December 2008.

Maya was monitored from July 2006 to January 2009. During that period of time, 4 estrous cycles were observed in alternation with the anestrus. The 1st, 2nd and 3rd estrous cycle was accompanied by a drop in thyroid volume when compared with the previous anestrus. The anestrus immediately following the 4th estrous cycle had a higher increase in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 14.32 mL during estrus, and the highest mean thyroid volume was 19.84 mL during anestrus. Maya was undergoing treatment with altrenogest (Regu-mate; Intervet) from December 2007 to July 2008, and from August 2008 to October 2008.

7.3.3 Pregnancy

Ester was monitored from July 2006 to January 2009. During that period of time, pregnancy was observed from August 2006 to August 2007. The anestrus immediately after pregnancy was accompanied by a drop in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 5.96 mL during estrus, and the highest mean thyroid volume was 6.93 mL during the pregnancy. The average thyroid volume for Ester during pregnancy was 6.93 mL. Ester was undergoing treatment with altrenogest (Regu-mate; Intervet) from December 2007 to December 2008.

Pinky was monitored from July 2006 to January 2009. During that period of time, pregnancy was observed from March 2007 to February 2008. The anestrus immediately after the pregnancy was accompanied by a drop in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 15.73 mL during anestrus, and the highest mean thyroid volume was 20.53 mL also during anestrus. The average thyroid volume for Pinky during pregnancy was 17.85 mL.

Pinky was undergoing treatment with altrenogest (Regu-mate; Intervet) from April 2008 to December 2008.

7.3.4 Lactation

Ada was monitored from August 2007 to January 2009. Lactation was observed for this entire period. During this period of time, 1 estrous cycle was observed during June 2008 and had a smaller thyroid volume observed compared to the previous and the following measurements in lactation. The lowest mean thyroid volume observed during the sampling period was 8.53 mL during estrus, and the highest mean thyroid volume was 9.28 mL during lactation.

Jessie was monitored from August 2006 to January 2009. During that period, lactation was observed from August 2006 to March 2007. The estrus immediately after lactation was accompanied by a drop in thyroid volume. The lowest mean thyroid volume observed during the sampling period was 17.68 mL during estrus, and the highest mean thyroid volume was 21.98 mL also during estrus. 4 estrous cycles were observed in alternation with the anestrus. The 2nd estrous cycle was accompanied by a drop in thyroid volume when compared with the previous anestrus. The anestrus immediately following the 1st, 2nd, and 3rd estrous cycle had a higher increase in thyroid volume.

7.4 Discussion

This study demonstrated that reproductive event is a significant predictor for thyroid volume measurement. Significant variation of thyroid volume was found among different reproductive events in female bottlenose dolphins. Among the 4 reproductive events investigated, there was a significant difference ($p < 0.05$) in thyroid volume found between the fixed effects lactation and estrus, as well as between the fixed effects lactation and anestrus. For the adult female bottlenose dolphins in the study population, the measured thyroid volume was highest during

lactation when compared to other reproductive events. This may be possibly due to the fact that lactation is generally considered the most energetically expensive aspect of reproduction for female mammals (Millar, 1977; Gittleman and Thompson, 1988; Wade and Schneider, 1992; Reddy et al., 1994; Hanwell and Peaker, 1997; Ellison, 2003; Schneider, 2004). During gestation and lactation, a substantial portion of the mother's total metabolism is allocated to support her offspring (Gittleman and Thompson, 1988; Ellison, 2003). A larger thyroid volume, with increased production of thyroid hormones, is perhaps needed in lactating females in order to ensure sufficient metabolic energy production.

The present findings are in accord with the findings of the previous studies that energetic requirements for lactation and pregnancy are far greater than that of the estrus and anestrus, despite the fact that these studies concentrated on the measurement of metabolic needs rather than thyroid physiology (Gittleman and Thompson, 1988; Wade and Schneider, 1992; Ellison, 2003). In a previous study, Cowan and Tajima (2006) performed a thyroid morphological and histological investigation using fresh carcasses and observed that the thyroid glands were larger in pregnant and lactating females compared with non-pregnant bottlenose dolphins of similar body size. Differences in thyroid hormone concentrations of bottlenose dolphins during different reproductive events were also noted in previous studies (St. Aubin et al., 1996; West et al., 2001). However, these studies did not compare their findings across different reproductive events, resulting in discrepancies. St. Aubin et al. (1996) found that wild female dolphins had significantly higher levels of total T4, free T4 and free triiodothyronine (T3) when compared to the semi-domesticated female dolphins. The authors suggested that it may be attributed to reproduction and lactation, since lactating subjects themselves as a group were sufficient to bias the results of the entire wild population.

Comparatively, West et al. (2001) reported that thyroid function in bottlenose dolphins was different between pregnant and non-pregnant animals, with the total T4 and total T3 slightly lower in pregnant animals, but the difference was not

statistically significant. The authors also observed a different pattern of T4 concentrations in female bottlenose dolphins in accordance to the gestational stage, with both total T4 and free T4 dropping considerably as pregnancy progressed. However, only limited serum samples were analyzed in this study (n = 100), and the authors did not differentiate the reproductive events of certain animals. Similar observations were found in baleen whales, with pregnant or lactating females having significantly lower thyroid hormone concentrations compared to other sex and reproductive groups investigated (Rosa et al., 2007). Therefore, lactation appears to have an effect on thyroid function in cetaceans.

Another possible reason accounting for the increased thyroid volume in lactating dolphins may be the milk production for nursery. This could be observed by the ingestion rates of the dam during gestation, as they are generally not as high as during lactation (Wade and Schneider, 1992). Reddy et al. (1994) also reported that a marked increase in food energy intake (kcal) did not occur in the pregnant dolphins until just prior to giving birth. Food energy consumption by bottlenose dolphins varies with age and is usually high during the period of lactation (Reddy et al., 1994). Therefore, the inference of milk production appears to be more costly in terms of energy consumption (Gittleman and Thompson, 1988). In humans, the energy consumption for milk production during lactation is usually around 700 kcal/day (Dewey, 1997), which can represent as much as a third of a woman's entire energy flux (Ellison, 2003). Comparatively, in dolphins, adult females showed an average consumption of 38-67 kcal/kg; subadult females, 56-73 kcal/kg; pregnant females, 36-89 kcal/kg; and lactating females, 88-153 kcal/kg. This demonstrated that milk production in dolphins influences the energy requirement relative to body weight, as the lactating females required the highest food intake to maintain basal metabolic function. As the thyroid gland is an important mediator of energetic processes, the increased thyroid volume in lactating dolphins may serve to maximize the metabolism involved and thereby balances off the energy consumption for milk production.

Female ovarian function is particularly sensitive to energy balance and energy flux, resulting in the synchronization of conception with favorable energetic conditions (Wade and Schneider, 1992; Ellison, 2003). Several authors have noted that the endocrine system is essential in shaping life histories at the physiological level by mediating metabolic allocation to competing domains (Lockyer, 2007; Foktin et al., 2010). Thyroid physiology plays an important role in normal reproductive function both through direct effects on the ovaries and indirectly by interacting with ovarian steroid binding proteins (Doufas and Mastorakos, 2000). During estrus and pregnancy, the ovarian steroids, namely estradiol and progesterone, induce coordinated changes in the procurement, ingestion, metabolism, storage, and expenditure of metabolic fuels (Wade and Schneider, 1992). Ovarian function in adult, non-pregnant, non-lactating women shows a consistent pattern of variation in association with indices of energetic conditions (Ellison, 2003). Total energy investment during pregnancy involves many components including net production of fetal, uterine, placental and mammary tissue, production costs, and increased maintenance costs associated with these new tissues (Gittleman and Thompson, 1988; Wade and Schneider, 1992; Schneider, 2004).

In the present study, no significant differences in thyroid volume were found between estrus and anestrus as well as pregnancy and anestrus. The effect of estradiol and progesterone on thyroid morphology and function has been previously established (Kuhl et al., 1985; Dalla Valle et al., 1998; Krejza et al., 2004; Sosić-Jurjević et al., 2005; Sekulić et al., 2007). Estrogens have been shown to conflictingly exert stimulatory (Kuhl et al., 1985; Furlanetto et al., 1999), inhibitory (Sosić-Jurjević et al., 2005; Sekulić et al., 2007), or no effects (Ceresini et al., 2008) on thyroid activity, while progesterone was found to be not in synchronization with any oscillations of human thyroid flow velocity (Krejza et al., 2004), although a vasoconstrictor effect on the blood vessels was suggested (Miyamoto et al., 2005). In diestrous dogs, it has also been postulated that progesterone may also enhance the binding affinity of plasma proteins for thyroid hormones, resulting in an increase in serum concentrations of total T4 and T3 (Reimers et al., 1984). The levels of

estrogens and progesterone fluctuate markedly on a day-to-day basis during estrus (Sawyer-Steffan et al., 1983; Robeck et al., 2001). As such, there are no observable long term changes in hormonal levels as seen in pregnancy and lactation. The effect of estrus on thyroid volume may have been diminished due to the sex hormonal variability of the 3 phases within the estrous cycle. Further study in investigating the possible variations of thyroid morphology in female Indo-Pacific bottlenose dolphins during the estrous cycle would provide a better insight on its effect.

In humans, increase in thyroid volume during pregnancy could be caused either by the demands of the growing fetus or by the hyperemia of the thyroid gland caused by the increased blood volume of the mother (Brander and Kivisaari, 1989; Berghout and Wiersinga, 1998; Glinoer, 2005). In dolphins, Lacave et al. (2004) proposed to divide the gestational period into 3 phases of 4 months each. However, the lack of statistical significant difference in thyroid volume between anestrus and pregnancy in the present study is possibly due to the absence of an observable "trimester-like" gestational period in bottlenose dolphins. In humans, at the beginning of gestation, there is an increased iodine clearance due to increased renal blood flow and glomerular filtration (Glinoer, 2005). In addition, thyroid hormones may increase throughout the first trimester of gestation because of the thyrotrophic effects of elevated serum human chorionic gonadotropin levels (O'Leary et al., 1992; Berghout and Wiersinga, 1998; Poppe et al., 2007). Studies of pregnant women at post-mortem have demonstrated increased vascularity and cellular hypertrophy of the thyroid gland (Stoffer et al., 1957). Plasma volume and total blood volume increase progressively during pregnancy, and reach their peak in the third trimester (Frederiksen, 2001; Glinoer, 2005). Further study with a larger sample size of pregnant subjects is needed to discern the effects of pregnancy over thyroid morphology, particularly with consideration to a possible "trimester-like" gestational period.

Several individual subjects had specific circumstances that were observable in thyroid volume measurements and monitoring of reproductive events. Ada and

Jessie were both lactating females. Jessie's thyroid volume fluctuated markedly whilst Ada's thyroid volume remained relatively constant during the lactating period. This could be explained by the difference in the ability to meet adequate energy demands for the resumption of ovarian function as Jessie appeared to be slower than Ada in meeting the energy demands. During pregnancy and lactation, Jessie's body may react several times as an attempt to meet the required energy demands; this may cause fluctuation of the thyroid function as well as the thyroid volume. During unsupplemented lactation, the high metabolic investment in a nursing offspring represents a significant constraint on the availability of metabolic energy to support a new pregnancy (Ellison, 2003). This high metabolic load generated by milk production during lactation is usually accompanied by ovarian suppression and amenorrhea, precluding any risk of a new conception (Gittleman and Thompson, 1988; Wade and Schneider, 1992; Ellison, 2003; Schneider, 2004). As the metabolic burden of supporting the infant is shifted away from milk production, the potential for meeting the energetic cost of a new pregnancy is restored and ovarian function resumes (Valeggia and Ellison, 2001; Ellison and Valeggia, 2002). The difference in adequate energy demands was observed by the fact that Jessie and Ada resumed their estrus at 24 months and 15 months after parturition respectively. Therefore, when compared to Ada, Jessie's thyroid volume appeared to be more variable.

Pinky and Ester were the 2 pregnant females in the study period, both having problems with either producing healthy offspring or nursing the calf. Pinky gave birth to a stillborn possibly due to her inexperience in parturition, whereas Ester had inadequate milk production resulting in the malnutrition and death of her calf. It was noted that Ester had a small thyroid gland similar to Hicky's, an impotent female, and it was suspected that both Ester and Hicky may be approaching the state of subclinical hypothyroidism. Their thyroid volumes were the smallest among all subjects measured during the sampling period and the average serum free T4, total T3 and total T4 levels (1.45 ng/dL, 78.09 ng/dL and 6.65 μg/dL for Ester; 1.50 ng/dL, 62.64 ng/dL and 7.06 μg/dL for Hicky) were at the lower bound of the reference ranges previously reported (St. Aubin et al., 1996; St. Aubin, 2001).

Although commercially available reagents for measuring human TSH appear to be ineffective in bottlenose dolphins (St. Aubin, 2001) and no serum TSH level could be therefore deduced from the present study, there was a higher chance that Ester and Hicky were in state of subclinical hypothyroidism due to the lower value in the available parameters of thyroid function reported (Foktin et al., 2010). Thyroid status plays a role in the secretion and action of ovarian steroids (Johnson, 2002; Glinoer, 2005; Poppe et al., 2007). Successful implantation and development of an embryo is determined by progesterone and estrogen on one hand, and adhesion molecules, growth factors and cytokines on the other (Redmond, 2004; Foktin et al., 2010). Although many pregnancies are successfully carried to term in women with hypothyroidism, abortion may result from impaired progesterone production due to the diminished trophic and stimulatory effect of thyroid hormones on chorion and corpus luteum function (Maruo et al., 1992; Redmond, 2004; Glinoer, 2005; Poppe et al., 2007). A similar association between hypothyroidism and fetal resorption, abortion and stillbirth has been suggested in female dogs, although no published documentation of this association is available for dolphins (Johnson, 2002; Feldman and Nelson, 2004). Therefore, due to their small thyroid volume, there may have been a failure in achieving the required metabolic energy to support the pregnancy and milk production respectively.

Gina and Angel were both non-pregnant, non-lactating females with normal cycling, and both had the largest thyroid volume during anestrus and the smallest thyroid volume during estrus. Their similar ages may be the reason for their similar thyroid volume changes across different reproductive events.

Hoi Kei and Maya were also non-pregnant, non-lactating females with normal cycling. Compared to the other females, they were the youngest and first initiated cycling during the sampling period. They received oral altrenogest (Regu-mate; Intervet) since December 2007, with a pause in the treatment during the summer of 2008. Regu-mate® has been used in a variety of cetacean species for both estrous synchronization and reversible contraception without adverse effects reported

(Robeck et al. 2001, AZA CAG 2004). It promotes suppression of the release of gonadotropin and the blocking of ovarian follicular development and estrus (Biancani et al. 2009). Hoi Kei and Maya were observed to have an increasing thyroid volume since the treatment of oral altrenogest. The increased thyroid volume may be due to the effect of oral altrenogest, which mimics as the action of endogenous progesterone.Progesterone itself has been reported to enhance the binding affinity of plasma proteins for thyroid hormones, resulting in an increase in serum concentrations of total T4 and T3 (Doufas and Mastorakos, 2000; Krejza et al., 2004). With the increase in thyroid hormones, the thyroid metabolism is augmented, possibly resulting in the increasing thyroid volume observed.

7.5 Conclusions

In conclusion, results of the present study demonstrated that reproductive event is a significant predictor for thyroid volume measurement and significant variability of thyroid volume was found among different reproductive events in female bottlenose dolphins. A significantly larger thyroid volume in lactating females was presented compared to estrous and anestrous females which may be due to the high energy requirements and milk production during lactation. The thyroid volume observed in estrous and pregnant females was comparable to anestrous females. Individual subjects with specific circumstances regarding the effect of different reproductive events on thyroid volume measurements were addressed. Thyroid volume variability during different reproductive events should be taken into account when examining female dolphins so as to obtain a diagnostically meaningful assessment.

Chapter Eight

Study Six

Sonographic evaluation of thyroid morphology during the normal estrous cycle in the Indo-Pacific bottlenose dolphin, *Tursiops aduncus*

8.1 Introduction

Interpretation of thyroid morphology in marine mammals should take into account the dynamic changes that occur in association with life-history events (St. Aubin, 2001). Thyroid function and morphology in females are likely to be affected by the cyclic change of the hormonal variation during their estrous cycle and different reproductive events (Chan et al., 1998; Krejza et al., 2004). In humans, although most of the thyroid hormones do not have significant variation during the normal menstrual cycle (Hegedüs, 1990), there is a positive correlation between the serum thyroglobulin (Tg) level and the thyroid volume (Rasmussen et al., 1989b). Significant thyroid volume changes have been detected in women during the normal menstrual cycle, although the pattern of change varied among individuals. With the use of Doppler ultrasound, it has been found that the vascular resistance of the superior thyroid artery decreased whereas its blood flow velocity increased, during the ovulatory and luteal phases (Chan et al., 1998; Krejza et al., 2004). This finding may reflect the increased metabolism and functional activity of the thyroid gland during these phases of the estrous cycle (Chan et al., 1998; Krejza et al., 2004). Increase in blood flow velocity of the thyroid gland during the estrous cycle causes hypertrophy of thyroid cellular tissues, leading to the increase in thyroid volume (Hegedüs et al., 1986; Hegedüs, 1990). For the companion animals, studies reported that there was an increase in serum thyroid hormones concentration in diestrous female dogs when compared to the females of different reproductive physiological states and males; this may be due to the elevated progesterone levels during diestrus (Wenzel, 1981; Reimers et al., 1984).

Assessment of the thyroid morphology is important in the diagnosis and management of different thyroid diseases. Ultrasound is a useful, real-time, safe and relatively low-cost imaging tool in the assessment of thyroid morphology and physiology in humans (Ahuja, 2000; Hegedüs, 2001; AIUM, 2003) and companion animals (Cartee et al., 1993; Kaptein et al., 1994; Wisner et al., 2002; Brömel et al., 2006). Studies suggested that ultrasound may be a more sensitive index of thyroid disturbance than serum thyroid stimulating hormone (TSH) concentration (Stewart et al., 1989). To the best of our knowledge, previous literature is devoid of information on thyroid morphology in neither female cetaceans nor any species of marine mammals with different reproductive physiological states. Recognizing any changes in the thyroid gland during the estrous cycle and in different reproductive events in female bottlenose dolphins may help the diagnosis of pathological conditions and the monitoring of treatment responses. Thus, this study was undertaken to investigate the possible variations of thyroid morphology in female Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) during the estrous cycle using ultrasound.

8.2 Materials and Methods

8.2.1 Subjects

Thirteen sexually mature *Tursiops aduncus*, at Ocean Park, Hong Kong (4 males and 9 females) were included in the study. At the beginning of the study, the mean age of the subjects was 17 years (range, 5-34 years). All dolphins involved in the study were being trained to cooperate for neck and ovarian sonographic examinations.

8.2.2 Equipment

All sonographic examinations were performed with either a Philips HD-11 ultrasound unit or a Philips HD-11 XE ultrasound unit, in conjunction with the same

6-2 MHz curvilinear 3-D broadband curved array transducer and 5-2 MHz 2-D broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA) or an Aloka SSD 900 ultrasound unit (Aloka Co. Ltd, Mitakasho, Tokyo) in conjunction with a 5 MHz curvilinear transducer. All images were recorded with either direct digital capture or with a thermal printer. Because of the nature of the dolphin's skin, there is no air layer between the surface and the transducer, so no coupling gel was required.

8.2.3 Scanning procedures

Sonographic examination of the thyroid gland was performed once a week for 2 years (August 2006 to August 2008) for females and once a week for 2 months (in between August 2006 to August 2008) for males. Four sexually mature male dolphins were also included in the present study as the control group. All ultrasound examinations of the thyroid gland were performed with the dolphin positioned in dorsal recumbence close to the poolside, with its tail supported by a trainer. The transducer was initially placed in a transverse orientation at the thoracic inlet, midway between the insertions of the pectoral flippers. The transducer was moved cranially until the brachiocephalic vein was identified. The transducer was then moved further cranially until the left and right lobes of the thyroid gland were identified. The thyroid volume was measured using the same protocol of Method A as used in Chapter 4 (see Section 4.2.1.1).

For the 9 female dolphins, sonographic examination of the ovaries was performed once a week for 2 years (August 2006 to August 2008) using the established method (Brook, 2001), to monitor their reproductive status. Both ovaries were assessed at each examination and the ovarian cortex was examined and reported during different phases of the reproductive cycle. When folliculogenesis was observed, ovarian scans were performed more frequently to document follicle growth rates and to predict time of ovulation. The size of the dominant follicle was noted and monitored until ovulation. The size and appearance of corpus luteum (CL) was also

monitored. Ovarian ultrasound was performed and reported by experienced veterinarians in Ocean Park, Hong Kong and recorded images and reports were further evaluated by an experienced sonographer in dolphin ovarian sonography.

8.2.4 Statistical analysis

Evaluation of the thyroid morphology in an estrous cycle for each female was divided into 3 phases: the follicular, ovulatory and luteal phases. Data measured from 3 days prior to and 3 days after the predicted day of ovulation were included in the ovulatory phase. Data measured prior to the ovulatory phase from on the onset of folliculogenesis were considered to be in the follicular phase, and data obtained after the ovulatory phase until the corpus luteum completely regressed were included in the luteal phase. The thyroid volume in each phase of the cycle was compared and evaluated by the repeated measures analysis of variance (ANOVA) (GraphPad InStat, GraphPad Software, Inc., San Diego, CA, USA). Similarly, the thyroid volume measured once a week for 2 months for each male was categorized into 8 ultrasound examinations and was also subjected to the repeated measures ANOVA. When statistically significant differences were found, the Tukey-Kramer multiple comparisons test was used as the post-hoc test. A p value less than 0.05 was considered significant. The thyroid volumes in different phases during the normal estrous cycle of female dolphins were compared with those measured in the 8 ultrasound examinations in male dolphins using unpaired t-tests (GraphPad InStat, (GraphPad InStat, GraphPad Software, Inc., San Diego, CA, USA)

8.3 Results

Nine normal estrous cycles were observed and studied in 4 female dolphin subjects, between 18 and 34 years of age (mean, 24.3 years). Data from the other 5 females were excluded due to incomplete or abnormal estrous cycles, pregnancy or lactation observed. Four male dolphins, aged between 7 and 24 years (mean, 16.3 years),

were included in the study as a control group. The changes of thyroid volume in female and male dolphins are shown in Figures 8.1 and 8.2.

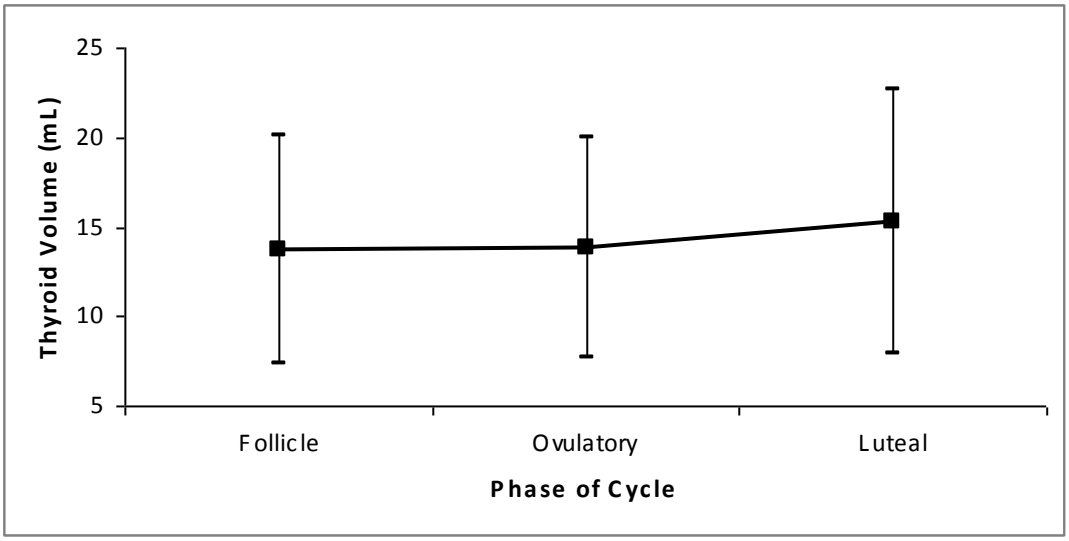

Figure 8.1: Changes of the mean thyroid volume in sexually mature female Indo-Pacific bottlenose dolphins. The mean thyroid volume in the luteal phase was significantly higher than in the follicular phase ($p < 0.05$) or in the ovulatory phase ($p < 0.05$). The difference of the mean thyroid volume between the follicular and the ovulatory phases was not significant ($p > 0.05$).

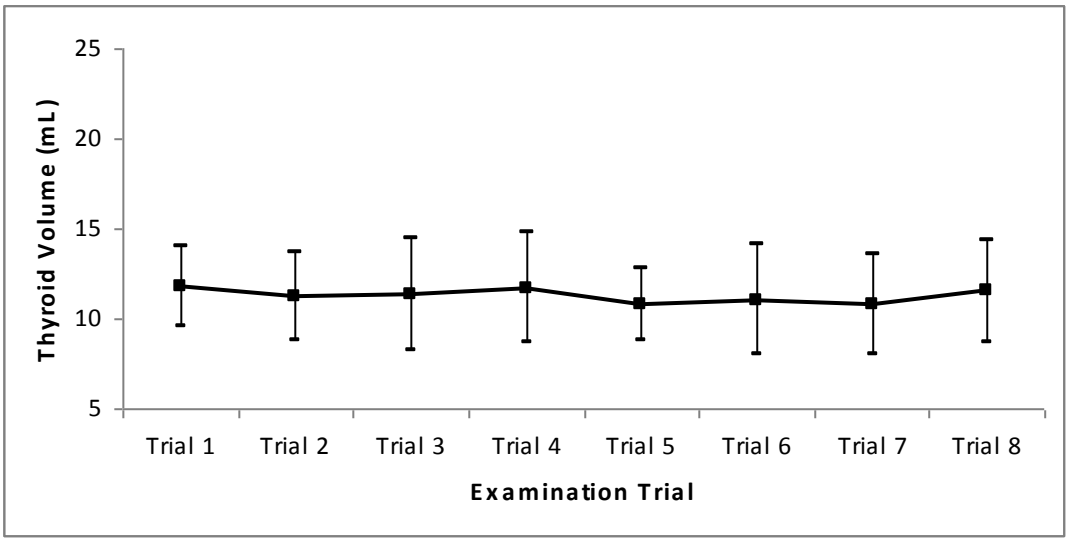

Figure 8.2: Changes of the mean thyroid volume in sexually mature male Indo-Pacific bottlenose dolphins. No significant variation of mean thyroid volume during the 8 weeks of the study ($p > 0.05$). The mean value of the thyroid volume over 8 examinations was 11.27 cm^3 ± 2.41 cm^3.

In the 4 female subjects, the thyroid glands were hypoechoic when compared with sternocephalicus muscle, with border sharpness varied from well-defined (50%) to ill-defined (50%) and homogeneity varied from homogeneous (50%) to heterogeneous (50%) during the estrous cycle. No observable change was found in any thyroid sonographic features of individual subject during the normal complete cycle. However, there was a significant difference in the thyroid volume during the estrous cycle in the female dolphins ($p < 0.05$), and the mean thyroid volume was 13.72 cm^3 ± 6.39 cm^3, 13.85 cm^3 ± 6.12 cm^3, 15.31 cm^3 ± 7.37 cm^3 for the follicular, ovulatory and luteal phases respectively. Tukey-Kramer multiple comparisons tests revealed that the mean thyroid volume in the luteal phase was significantly higher than in the follicular phase ($p < 0.05$) or in the ovulatory phase ($p < 0.05$). The difference in the mean thyroid volume between the follicular and the ovulatory phases was not statistically significant ($p > 0.05$). In male dolphins, there was no significant variation of the mean thyroid volume during the 8 weeks of the study ($p > 0.05$). The mean thyroid volume of the 4 male subjects over 8 examinations was 11.27 cm^3 ± 2.41 cm^3. The mean thyroid volume measured in the all phases of the

female dolphins (14.29 cm^3 ± 6.43 cm^3) was significantly higher than that in the 8 examinations of the male dolphins (11.27 cm^3 ± 2.41 cm^3) ($p < 0.05$).

8.4 Discussion

This study showed significant variation of thyroid volume throughout the estrous cycle in female bottlenose dolphins. During the normal estrous cycle, the thyroid volume increased from the follicular phase to the luteal phase with the mean thyroid volume in the luteal phase being significantly higher than in the follicular and ovulatory phases. Increase in thyroid volume during the estrous cycle may be due to the hypertrophy of thyroid cellular tissues, which is affected by an increase in blood flow velocity to the thyroid gland (Rasmussen et al., 1989a). Chan et al. (1998) reported that thyroid perfusion was found at a minimal in the follicular phase, whereas there was an increase of blood flow to the thyroid gland in the ovulatory and luteal phases, thereby implying an increase in thyroid metabolism and functional activity. The thyroid volume alternation would possibly reflect the amount of hormone released into circulation (Harrison and Young, 1970), or inevitably, its function and activity.

The thyroid volume in different phases during an estrous cycle in female dolphins was compared with those measured in the 8 ultrasound examinations for the male dolphins. No significant variation of the thyroid volume was found in the males over 8 weeks. The sex difference in thyroid volume suggested that a "cyclic" effect on thyroid metabolism in female dolphins may exist, indicating a possible relationship between the thyroid physiology and female reproductive activity, in which there may have substantial influence by the sex steroids involved during the estrous cycle. A close relationship between gonadal and thyroid function has been reported in humans and other experimental animals (Miki et al., 1990; Gerhard et al., 1991; Sosić-Jurjević et al., 2005; Sekulić et al., 2007). Immunohistochemical studies, binding assays as well as biochemical studies have demonstrated the presence of estrogen, progesterone and androgen receptors in the thyroid tissues of different

mammals (Money et al., 1989; Miki et al., 1990; Métayé et al., 1993; Bonacci et al., 1996; Rossi et al., 1996; Memon et al., 2005; Zagrodzki et al., 2007), which allows for the influence of female gonadal steroids on thyroid follicular cells. Apart from this effect, gonadal steroids may also affect the regulation of the hypothalamic TRH-pituitary TSH axis, as well as altering the level of serum thyroxine (T4)-binding proteins (Ramey et al., 1975; Huang et al., 1995; Sekulić et al., 2007).

Although several studies have demonstrated the presence of estrogen receptors in both normal and pathological thyroid tissues (Miki et al., 1990), no common consensus has been established on the effect of estrogens toward thyroid activity. From a vast majority of pharmacological studies, estrogens have been shown to conflictingly exert stimulatory (Kuhl et al., 1985; Furlanetto et al., 1999), inhibitory (Sosić-Jurjević et al., 2005; Sekulić et al., 2007), or no effects (Ceresini et al., 2008) on thyroid activity. It is suggested that estrogen positively stimulates thyroid follicular cell growth, and influences its function through the reduction of sodium-iodide symporter gene expression and iodine uptake in either the presence or absence of TSH, resulting in hyperplasia of follicular cells and a significant decrease of serum thyroid hormones (Furlanetto et al., 1999; Furlanetto et al., 2001; Sosić-Jurjević et al., 2005). While other studies reported that the hypothalamic-pituitary-thyroid axis was unaffected by estrogens in women given oral contraceptives, although an estrogen-induced increase in pituitary sensitivity of TSH response to thyrotropin-releasing hormone (TRH) has been suggested, with the rise of T4-binding protein and decrease in T4/TBG (thyroxine-binding globulin) ratio (Ramey et al., 1975; Rey-Stocker et al., 1981). Ceresini et al. (2008) found that a 1-year estrogen administration did not affect the total thyroid volume, or the total volume or number of thyroid nodules (Ceresini et al., 2008). However, estrogens and androgens have been suggested to exert opposing effects, with estrogen positively influencing and androgen negatively influencing the thyroid size. Studies on experimental animals revealed that a neonatal treatment of estradiol alone exerted a protracted inhibitory effect on male and female rat thyroid function (Sekulić, 1986, 1988), which is evidenced by their decreased ability for biosynthesis

and reabsorbtion of Tg. Sosić-Jurjević et al. (2005) also reported the inhibitory effect of estradiol on thyroid gland of middle-aged female rats, which significantly decreased volume density and height of centrally located follicular epithelium, follicular activation index and serum level of total thyroid hormones in the administration of chronic estradiol treatment.

While concentrations of serum estrogens were not measured in this study, previous research analyzing urinary estrogen metabolites in bottlenose dolphins has demonstrated that estrogen concentrations rise in parallel with follicular growth, peaking just prior to ovulation (Robeck et al., 2005). The significant difference in dolphin thyroid volume between estrus and luteal phase may be due to the higher thyroid metabolic rate directly stimulated by progesterone secreted during the in luteal phase. However, the differences may simple be a reflection of sustained lower concentration of TSH and Tg in response to estrogen decrease, stimulating the feedback mechanism and causing an increase in thyroid metabolism.. The present study focused on the thyroid and ovarian morphological evaluation, the effect of estrogen concentrations to the thyroid morphology was not fully evaluated, and further studies with corresponding estrogen concentrations during the weekly thyroid ultrasound examinations are suggested.

Progesterone receptors have also been reported to be present in thyroid cells (Bonacci et al., 1996), although data on the effects of progesterone on thyroid cell physiology are scant. A lack of strong support from any available studies that thyroid tissue contains a significant amount of progesterone receptors which implies that the effect of progesterone on human thyroid seems likely to be indirect (Memon et al., 2005). Progesterone was found to be not in synchronization with any oscillations of human thyroid flow velocity (Krejza et al., 2004), although a vasoconstrictor effect on the blood vessels was suggested (Miyamoto et al., 2005). A positive correlation between progesterone and thyroid volume was noted, and it was believed that the increased thyroid volume was not attributed predominantly by

the onset of menses or raised progesterone levels (Zagrodzki et al., 2007). Effect of progesterone on the thyroid of some experimental and companion animals has been investigated and the results were differed from those of humans. Huang et al. (1995) suggested that progesterone potentiated the stimulatory effects of estrogen at the level of plasma prolactin, hypophyseal plasma TRH, and dopamine in rats. In female canine, progesterone affects serum T4 and 3,5,3'-triiodothyronine (T3) concentrations (Feldman et al., 2004). In another study, serum T4 and T3 concentrations of diestrous females were greater than those from anestrous, proestrous and lactating females or male canine (Reimers et al., 1984). It has been postulated that progesterone may enhance the binding affinity of plasma proteins for thyroid hormones, resulting in an increase in serum concentrations of total T4 and T3.

Progesterone levels have been proven to be useful indicators of ovarian activity in captive *Tursiops* (Robeck et al., 2005). An episodic elevation of progesterone levels during the luteal phase of estrous cycle was reported in bottlenose dolphins, in which the corpus luteum was believed to be the primary source of progesterone secretion. Although there is an individual variation on the degree of progesterone level change during an estrous cycle, progesterone levels generally increased gradually from the follicular phase to luteal phase, with the highest value recorded when the maximum corpus luteum diameter was measured. This may partly account for the significant difference in dolphin thyroid volume due to increased binding affinity of plasma proteins for thyroid hormones in response to progesterone, leading to an increase in thyroid metabolism.

Both of the aforementioned sex steroids were shown to influence the thyroid metabolism, function and morphology; thus attention has been directed towards understanding the possible combined effect of estrogen and progesterone simultaneously on thyroid function. Administration of estrogen and progesterone caused an increased response of TSH cells to hypothalamic TRH in sheep (Wright et al., 1978) and a dramatic effect on the structure of thyroid tissue and the

ultrastructure of follicular epithelial cells in a stimulatory manner in peripubertal male pigs. Huang et al. (1995) suggested that progesterone may have the stimulatory effects of estradiol at the level of plasma prolactin, hypophyseal plasma TRH, and dopamine in rats (Huang et al., 1995). Therefore, this possible increased response of pituitary TSH cells to hypothalamic TRH after treatment with estradiol and progesteron may account for the thyroid volume alternation described under our experimental conditions.

Despite observing cyclic changes in the dolphin thyroid volume and that the possible influence of sex steroids on these changes was suggested, the measurement of the corresponding concentrations of estrogen and progesterone of this population was out of the scope of our study. Further study in evaluating dolphin thyroid morphology using ultrasound in conjunction with the measurement of the corresponding concentrations of sex steroids is suggested. In addition, St. Aubin (2001) proposed that hormonal changes associated with reproduction in female dolphins canindirectly lead to elevations in total T4 and total T3, by increasing hormone binding capacity in plasma. Since the levels of estrogen and progesterone hormones alter differently among individual dolphins and fluctuate markedly on a day to day basis over an estrous cycle, serially longitudinal investigations conducted with a larger sample size over the entire estrous cycle is suggested to distinguish transient changes in sex steroids and also, over the pregnancy and lactation periods to understand the possible effects on thyroid metabolism and function derived from longer-term adjustment associated with these reproductive events.

8.5 Conclusions

To conclude, results of the present study showed no observable change in the dolphin thyroid sonographic features during the estrous cycle. However cyclic changes of thyroid volume in female Indo-Pacific bottlenose dolphinsduring the estrous cycle were found, with the thyroid volume increasing from the follicular phase to the luteal phase. This pattern of thyroid volume variation was not found in

the male dolphins. The sex difference suggests that the change may possibly be associated through the action of several female sex steroids, with their serum concentrations fluctuating naturally during the estrous cycle. Thyroid volume variability during the estrous cycle should be taken into account when examining the thyroid gland of female dolphins so as to obtain a diagnostically meaningful assessment.

Chapter Nine

Summary and Suggestions for Future Research

9.1 Summary of the thesis

This thesis assesses the anatomy and physiology of the thyroid gland of a population of Indo-Pacific bottlenose dolphins with the application of sonography.

Chapter 1 provides a brief introduction of the background and objectives of the present study. Chapter 2 describes the anatomy of the bottlenose dolphin thyroid gland, and includes a review of the function of mammalian thyroid gland, a review of common thyroid abnormalities in marine mammals, a review of the methodology of investigating thyroid physiology, a review of common diagnostic imaging methods to assess the morphology of mammalian thyroid gland, a review of sonography on the mammalian thyroid gland, a review of the effects of common demographic parameters on thyroid physiology, a review of the effects of various reproductive status on thyroid physiology, and a review of the effects of animal illness on thyroid physiology.

Sonography has been proven to be an effective imaging tool in assessing thyroid glands and screening for thyroid pathologies in humans. For accurate diagnosis, the normal sonographic features of the thyroid gland should be established, as they are essential for morphological investigation, pathological evaluation and the follow-up of treatment regimens. To the best of our knowledge, the formal literature is devoid of any reference to the sonographic evaluation of normal thyroid glands and the adjacent neck structures of any marine mammal species. Essential sonographic features of the normal dolphin thyroid gland, which are crucial to offer a basis for the diagnosis of pathology, have not been documented. Description of a simple scanning protocol that enables repeatable visualization of the thyroid gland of bottlenose dolphins, and the sonographic features of normal dolphin thyroid gland

and adjacent neck structures was noted in Chapter 3. In the 18 *T. aduncus* at Ocean Park, Hong Kong, 1,404 neck ultrasound examinations were performed in this captive population during the period of August 2006 and January 2009. Standardised scanning protocol was used for all ultrasound examinations. Thyroid gland was assessed for its shape, echogenicity and homogeneity. Adjacent neck structures such as cervical lymph nodes, musculatures and vasculatures were also assessed. All dolphin thyroid glands were categorized into 4 different morphological configurations by evaluating the contours of the thyroid gland in different 2-D and 3-D ultrasound images. The shape of the thyroid lobes appeared elliptical or fusiform in the transverse scan plane, and round to oval in the longitudinal scan plane. The thyroid capsule was usually appeared echogenic and the borders of the thyroid gland were usually well-defined (82%), whereas ill-defined borders were observed in the thyroid gland of 3 subjects (18%). The echopattern of the thyroid parenchyma was generally uniform and homogeneous (76%), with the presence of echogenic reticulations. The relative echogenicity varied between subjects from hypoechoic (61%) to isoechoic (39%) when compared with the sternocephalicus muscle. Echogenicity of the right, left thyroid lobe and isthmus was different in over half of the subjects (65%). Two morphological configurations of the thyroid gland, Type A: two lobes joined by an isthmus (n = 4); and Type C: a shield-like, single mass, roughly diamond-shaped, placed ventrally on the trachea (n = 14) were identified. Major blood vessels such as brachiocephalic trunk, subclavian arteries, internal and external carotid arteries, omooccipital artery, superior thyroid arteries, brachiocephalic vein, internal jugular veins and superior thyroid veins were visualized. Cervical lymph nodes were also visualised on ultrasound.

In the morphological evaluation of thyroid glands, the assessment of thyroid volume plays an indispensable role in the diagnosis and management of different thyroid diseases. In order to accurately diagnose and monitor thyroid diseases, reliable methods for assessing the thyroid size must be developed to correlate with existing biochemical and clinical data. There is scant information in the literature about ultrasound measurement of thyroid size in dolphins. The only published report of

dolphin thyroid ultrasound focused on determining anatomical landmarks, and the assessment of thyroid size was not comprehensive. An evaluation of the accuracy of dolphin thyroid volume as determined by four 2-D ultrasound measurement methods, with the standard of reference determined by 3-D ultrasound measurements has now been undertaken (Chapter 4), and the inter- and intra-operator variability of the aforementioned ultrasound measurement methods has been evaluated. The accuracy of different 2-D ultrasound methods in measuring thyroid glands with various morphological configurations has also been investigated. A total of 16 *Tursiops aduncus* (6 males and 10 females) were included in the study. Each session of ultrasound examination begun with the 2-D ultrasound measurements (Methods A – D), followed by the 3-D ultrasound measurement (Method E). During the period of August 2006 and December 2008, a total of 856 individual ultrasound scans were conducted in the 16 *T. aduncus* with the use of the 2-D and 3-D ultrasound thyroid volume measurement methods (2 subjects were not included in this study since 1 was just under training for ultrasound examination and the other subject deceased during the mentioned period of time). All 2-D and 3-D ultrasound measurements were performed by the same operator and all images required for measurements of a single subject were collected at the same session. For the reliability test, 3 operators scanned the thyroid gland of the 16 subjects twice in the same session to investigate the measurement reliability of Methods A – E. The present study shows that there is a considerably high reproducibility (77.6% - 86.2%) and repeatability (78.1% - 99.7%) in thyroid volume measurement using both 2-D and 3-D ultrasound. Methods A and B are more accurate and reliable than Methods C and D in 2-D ultrasound dolphin thyroid measurement, regardless of the thyroid morphological configuration. The sample size of the present study is small, and further studies with a larger sample are suggested.

Thyroid hormone test kits were used to evaluate the association between thyroid morphology and blood sampling of thyroid hormones in captive Indo-Pacific bottlenose dolphin subjects (Chapter 5). During the period of August 2006 to July 2008, a concurrent total of 241 blood samples and ultrasound thyroid volume

measurements were obtained from the 17 subjects (1 subject was not included because she was just under training for ultrasound examination during the mentioned period of time). Analyses were stratified by age (calf, juvenile and adult), sex and sexual maturity. For thyroid volume, significant association was found in both free T3 and total T3. When the data were grouped according to the age of the subjects, calves had significant associations in thyroid volume with free T3, free T4 and total T4. In juveniles, a significant association with free T3 and free T4 was found with thyroid volume. In adults, all serum thyroid hormones were found to be significantly associated with thyroid volume. For sex analysis, all serum thyroid hormones were found to be significantly associated with thyroid volume in the males, while no significant associations were observed for the females. For sexual maturity, a significant association was found in free T3, free T4 and total T4 with thyroid volume in sexually immature subjects, but no significant association was found in sexually mature subjects.

Understanding the determinants of the thyroid gland such as age, sex, sexual maturity, body size, and season are vital in the diagnosis of pathologies and monitoring of the thyroid gland during the course of treatment. It is important to recognize the potential influence of these determinants when interpreting physical thyroid changes and serum thyroid hormone levels. The literature is devoid of any reference to possible determinants on thyroid morphology in bottlenose dolphins. Therefore, a study was undertaken to investigate the possible variations in thyroid morphology of a group of Indo-Pacific bottlenose dolphins under human care with different demographic factors using sonography (Chapter 6). In the 17 subjects included in this study during the period of August 2006 to January 2009 (1 subject was not included because she was just under training for ultrasound examination during the mentioned period of time), a total of 1384 sonographic examinations were performed. Data were analysed according to the 2 life history developmental characteristics - somatic growth and reproductive development analysis of thyroid morphology. For somatic growth analysis of thyroid morphology, the factors of animal age, sex and sampling season were evaluated. For reproductive development

analysis of thyroid morphology, the factors of animal sex, sexual maturity and sampling season were assessed. For somatic growth analysis, significant effects in the thyroid volume were found among the factors of age and sex, as well as in the combined interaction of age and sex. For reproductive development analysis, significant effects in thyroid volume were found for the factor of sexual maturity, as well as in the combined interaction of sex and sexual maturity.

In bottlenose dolphins, trends in reproductive events were different from those of humans. However, possible influences of different reproductive states on thyroid morphology are unclear. The present study used sonography to evaluate the possible variations of thyroid morphology at different reproductive states of a group of bottlenose dolphins in a captive environment (Chapter 7). Nine sexually mature female *T. aduncus* were included in this study. To estimate the effects of the reproductive events for thyroid volume, a generalized linear mixed model was used. In the present study, reproductive event was found to be a significant predictor for thyroid volume measurement. A significant difference in the thyroid volume was found between lactation and estrus, as well as between lactation and anestrus.

Based on the findings of Chapter 7, variations of thyroid morphology during the estrous cycle of female Indo-Pacific bottlenose dolphins were investigated (Chapter 8). Thirteen sexually mature subjects, consisting of 9 females and 4 males, were included in the investigation. The estrous cycle was divided into 3 phases for evaluation: the follicular, ovulatory and luteal phases. For sex comparison, thyroid volumes measured in different phases during the normal estrous cycle of female dolphins were compared to those measured in 8 ultrasound examinations of the male dolphins during the same period of time August 2006 to August 2008. Nine normal estrous cycles were observed and evaluated. The thyroid volume during luteal phase was found to be significantly larger than that during the follicular phase or the ovulatory phase. The thyroid volume during follicular phase was not significantly different from that during ovulatory phase. In male dolphins, there was no significant variation of the thyroid volume during the study period. The thyroid

volume measured in all estrous cycle phases of the female dolphins was significantly larger than that measured in the 8 examinations of the male dolphins. Thyroid volume variability during the estrous cycle should therefore be taken into account when examining the thyroid gland of female dolphins so as to obtain a diagnostically meaningful assessment.

9.2 Suggestions for Future Research

Intrinsic areas that could possibly derived from the present study are as follow

- Development of dolphin serum TSH measurement method

- Association between thyroid morphology with male sex steroid can be investigated retrospectively, i.e. T level through out the life cycle

- A longitudinal study on thyroid development from the neonatal stage until adulthood, in conjunction with changes in THs, somatic growth and blubber thickness

Toxicity (carcinogenicity, teratogenicity, immunotoxicity etc.) of several pollutants in some experimental and wild animals are well established (Safe, 1993). Because of the remarkable structural similarity of some organochlorinated pollutants (POPs) such as PCBs, dioxins and pesticides etc. to thyroid hormones, attention has been directed to the possible effects of these toxicants on the thyroid gland as described by Brucker-Davis (1998), Hagmar (2003), Langer et al. (2005, 2007) and Kloas et al. (2009).

One of the possible effects of organochlorines on the thyroid gland is a reduction of plasma thyroxine (T4) level, which is due to the displacement of T4 from protein binding (Kloas et al., 2009) and increased hepatic metabolism of T4 resulting from

the induction of UDP-glucuronosyl-transferase (Schuur et al., 1997). Because of a structural similarity to T4, PCBs may also interfere with the transport of T4 into cells and T4 to T3 conversion. They may also mimic thyroid hormone action and modulate the mechanism of T3 binding to its nuclear receptor, resulting in gene expression (Langer et al., 2007).

In neonates, perinatal exposure to maternal milk containing polychlorinated dioxins and furans resulted in increased TSH levels after 11 weeks (Pluim et al., 1993). In addition, in mother-infant pairs, such exposure resulted in a decreased T4 level in blood (Koopman-Esseboom et al., 1994). Irreversible neurological damage resembling that seen in those with thyroid hormone deficiency *in utero* or in infancy was also observed (Porterfield, 1994). In occupationally exposed workers, an increased prevalence of thyroid disorders and thyroid cancer has been reported (Saracci et al., 1991).

A number of studies have documented the concentrations of pollutants in cetacean species residing in various bodies of water (Ramu et al., 2005; Haraguchi et al., 2009; Houde et al., 2009; Lailson-Brito et al., 2010; Moon et al., 2010; Mwevura et al., 2010; Yordy et al., 2010), and highlighted the potential adverse health effects from pollutant exposure. Resident mammalian species can act as ideal wildlife sentinels for the effects of environmental contaminants on marine ecosystems. For risk assessment on human health, they would be useful animal models for investigating long-term impacts. To the best of my knowledge, the literature is devoid of the effects of PCBs and various organochlorinated substances on thyroid morphology and function of any cetacean species. Therefore, further studies to investigate the possible effects of pollutants on thyroid morphology and physiology in cetaceans are suggested.

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Appendix 1

Captivity Facility

There are 6 interconnected pools at 1 of Ocean Park's cetacean facilities called Ocean Theatre, where the subjects of the study are maintained. The facility is outdoors and semi-covered, except for the main show demonstration pool. Each tank is equipped with solid metal gates. Pool layout is shown in Figure A1.1. Tank dimensions are shown in Table A1.1.

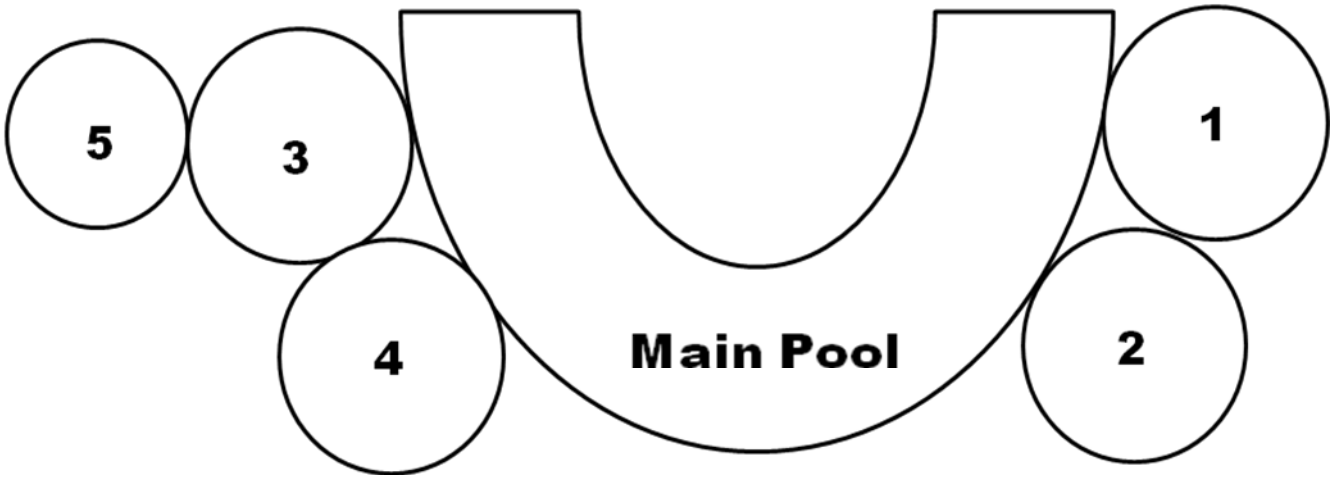

Figure A1.1: Configuration of pool layout at Ocean Theatre.

Table A1.1: Dimensions of the tanks at Ocean Theatre.

Tank	Minimum Horizontal Dimension (m)	Minimum Depth (m)	Surface Area (m^2)	Volume (m^3)
Main	10.90	5.50	1975.00	3316.00
1	7.90	4.00	93.00	396.00
2	7.90	4.00	93.00	396.00
3	7.93	2.70	63.00	206.00
4	8.00	2.80	75.00	234.00
5	6.86	1.68	49.00	92.00

The water turnover rate for Dolphin University is 3,900 gallons per minute, total turnover rate is 1.6 - 2.0 hours. The sand filter radius is 1.45 m; the filtration area is 6.6 m^2. The depth of sand in the filter is 1.6 m.

There are 6 interconnected pools at 1 of Ocean Park's cetacean facilities called Dolphin University, where the subjects of the study are maintained. The facility is indoors and semi-covered. Each tank is equipped with solid metal gates. Pool layout is shown in Figure A1.2. Tank dimensions are shown in Table A1.2.

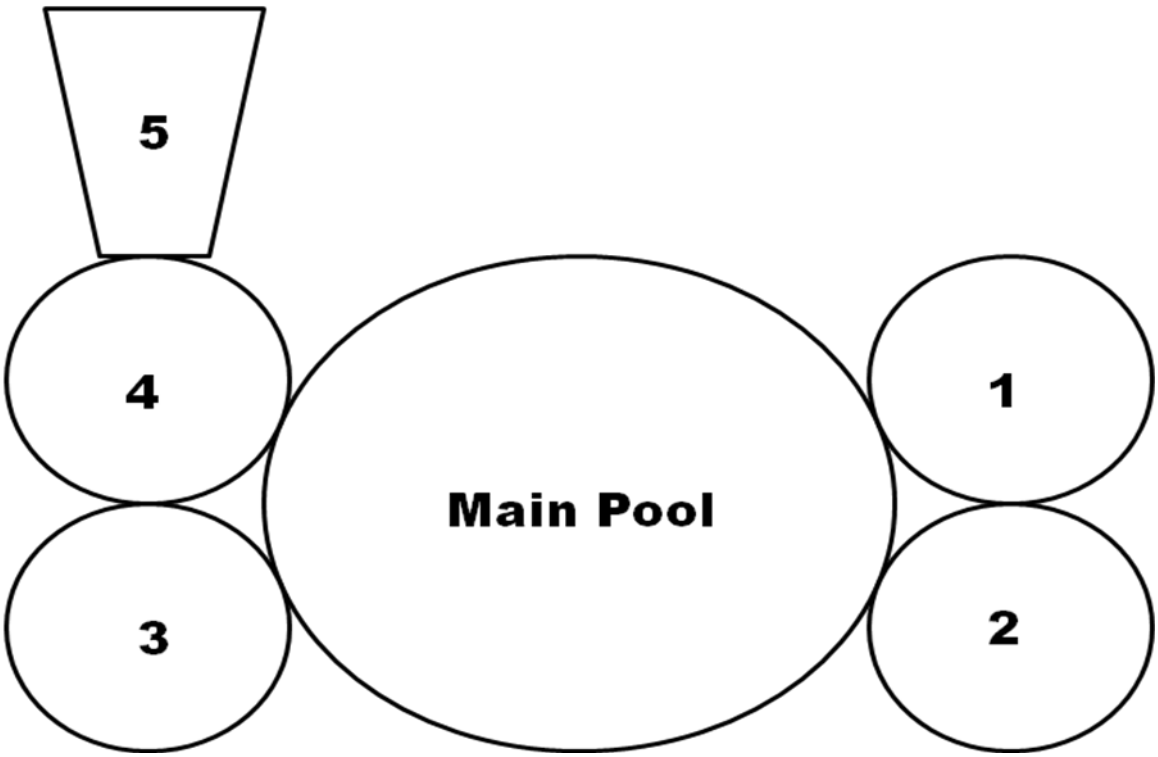

Figure A1.2: Configuration of pool layout at Dolphin University.

Table A1.2: Dimensions of the tanks at the Dolphin University facility.

Tank	Minimum Horizontal Dimension (m)	Minimum Depth (m)	Surface Area (m^2)	Volume (m^3)
Main	18.29	3.66	263.29	794.94
1 - 4	9.14	3.05	65.59	151.42
5	7.62	1.52	30.38	37.85

The water turnover rate for Dolphin University is 4,600 gallons per minute, total turnover rate is 3.5 - 4.0 hours. The sand filter radius is 1.45 m; the filtration area is 6.6 m^2. The depth of sand in the filter is 1.4 m.

Appendix 2

Diet of the *T. aduncus* at Ocean Park, Hong Kong

The composition of the diet of all dolphins at Ocean Park is the same, although individual animals may eat a different total weight of fish per day. All dolphins also receive daily vitamin and mineral supplements. Diet composition is shown in Table A2.1. Supplement regimen is shown in Table A2.2.

Table A2.1: Diet of *T. aduncus* at Ocean Park.

Feed	Percentage of Diet Composition
Capelin	55%
Squid	20%
Sardine	15%
Herring	10%

Table A2.2: Regimen of supplement of *T. aduncus* at Ocean Park.

Supplement	Regimen
MMT	1 tab per 2.2kg fish fed; daily
Vitamin B1	1 tab for dolphins < 100kg; 2 tabs for dolphins > 100kg; daily
Protexin	2 capsules for dolphins < 100kg; 3 capsules for dolphins > 100kg; daily from May 1st to October 31st
Folic Acid	2 tabs for pregnant or pre-pregnant dolphins; daily

Appendix 3

Water Quality Parameters

Water quality parameters for both Ocean Theater and Dolphin University are shown in Table A3.1. The water was ozone treated on a regular basis.

Table A3.1: Water analysis and bacterial counts at Ocean Theater and Dolphin University.

Water Analysis	**Measurement**
Temperature	< 24 ℃
pH	7.2 - 8.2
Turbidity	< 0.25 NTU
Salinity	27 - 35 ppt
Ammonia / Ammonium	< 0.1 ppm
Nitrate	< 15 ppm
Free Bromine	0.4 - 0.7 ppm
Total Bromine	0.8 - 1.5 ppm
Color	< 1 CU
Oxidation Reduction Potential	550 - 750 mV
Total Alkalinity - CaCO3	100 - 140 ppm
Bacterial Counts	
Blood Agar	< 10 cfu/100mL
Coliform	0 cfu/100mL

Appendix 4

Hormone assay protocols

A 4.1 Total Thyroxine (T4) evaluation

Approximately 10 mL of blood was collected in a non-heparinized, disposable syringe after dorsal venipuncture of tail fluke vessels, using a 22 G butterfly needle. The blood was left to stand to clot in a plain tube for 30 minutes at room temperature, and serum was harvested by centrifugation at 4500 RPM for 10 minutes. Serum was divided to allow analysis of free T4, total triiodothyronine (T3) and free T3.

For serum total T4 concentration evaluation, commercial test kit VIDAS total T4, (bioMérieux sa, France), coupled with an automated VIDAS analyzer (bioMérieux sa, France) were used. The total T4 test requires 200 µL of serum sample. The standards are of human serum origin.
The information provided by the manufacturer of the kit is as follows.

A 4.1.1 Principle

The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (ELFA).

A 4.1.2 Measurement range

The measurement range of the VIDAS total T4 kit extends between 6 and 320 nmol/l.

A 4.1.3 Detection limit

The detection limit of these hormones was defined as the smallest concentration of total T4 which is significantly different from the zero concentration with a probability of 95%: 6nmol/l.

A 4.1.4 Accuracy

A 4.1.4.1 Dilution test

Three samples were diluted in human T4-free serum and each dilution was tested in 3 runs. The measured mean values compared to the expected mean values are shown below as the mean recovery percentages (Table A4.1).

Table A4.1: Recovery concentration after dilution.

Sample	Dilution factor	Expected concentration (nmol/l)	Measured concentration (nmol/l)	Recovery percentage
1	1:1	127.7	127.7	100.0
	1:2	63.8	68.0	106.5
	1:4	31.9	36.6	114.7
2	1:1	251.2	251.2	100.0
	1:2	125.6	121.4	96.6
	1:4	62.8	66.2	105.4
3	1:1	183.2	183.2	100.0
	1:2	91.6	90.1	98.4
	1:4	45.8	53.4	116.6

A 4.1.5 Precision

A 4.1.5.1 Intra-assay precision

Five samples were tested 30 times in the same run and P values determined to assess intra-assay precision. Mean concentrations and coefficients of variation were determined. Results are shown below.

Table A4.2: Mean concentrations and coefficients of variation of samples in intra-assay precision test.

Sample	1	2	3	4	5
Mean concentration (nmol/l)	33.0	61.1	93.8	166.6	238.5
Coefficient of variation (%)	9.0	5.1	4.7	5.0	5.9

A 4.1.5.2 Inter-assay precision

Five samples were tested in singlet over an 8 week period on the same VIDAS instrument. Mean concentrations and coefficients of variation are shown below.

Table A4.3: Mean concentrations and coefficients of variation of samples in inter-assay precision test.

Sample	1	2	3	4	5
Mean concentration (nmol/l)	27.5	55.3	85.4	150.8	233.1
Coefficient of variation (%)	8.6	6.2	6.0	5.0	7.6

A 4.1.6 Specificity

The antibody used in the VIDAS® T4 assay was tested for cross-reactivity against a number of compounds. The results in the table below are represented as the

percentage ratio between the T4 concentration and the cross reactant concentration at 50 % binding.

Table A4.4: Specificity of the anti-T4 antibody used in the assay.

Tested compound	Cross-reactivity (%)
L-Thyroxine	100
D-Thyroxine	83
L-Triiodothyronine	2.3
D-Triiodothyronine	1.8
Diiodotyroxine	<0.01
Diiodothyronine	<0.01
Diphenylhydantoin	<0.01
Propylthiouracile	<0.01
Sodium salicylate	<0.01

A 4.2 Free Thyroxine (T4) evaluation

For serum free T4 concentration evaluation, commercial test kit VIDAS free T4, (bioMérieux sa, France), coupled with an automated VIDAS analyzer (bioMérieux sa, France) were used. The free T4 test requires 100 µL of serum sample. The standards are of human serum origin.

The information provided by the manufacturer of the kit is as follows.

A 4.2.1 Principle

The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (ELFA).

A 4.2.2 Measurement range

The measurement range of the VIDAS total T4 kit extends between 1 and 70 pmol/l.

A 4.2.3 Detection limit

The detection limit of these hormones was defined as the smallest concentration of total T4 which is significantly different from the zero concentration with a probability of 95%: ≤ 1 pmol/l.

A 4.2.4 Precision

A 4.2.4.1 Intra-assay precision

Five samples were tested 30 times in the same run and P values determined to assess intra-assay precision. Mean concentrations and coefficients of variation (CV) were determined. Results are shown below.

Table A4.5: Mean concentrations and coefficients of variation of samples in intra-assay precision test.

Sample	1	2	3	4	5
Mean concentration (pmol/l)	4	12.1	26.9	43.7	59.2
Coefficient of variation (%)	11.4	6.5	5.1	6.0	4.4

A 4.2.4.2 Inter-assay precision

Five samples were tested in singlet over an 8 week period on the same VIDAS instrument. Mean concentrations and coefficients of variation (CV) are shown below.

Table A4.6: Mean concentrations and coefficients of variation of samples in inter-assay precision test.

Sample	1	2	3	4	5
Mean concentration (pmol/l)	3.8	11.9	26.8	42.2	57.6
Coefficient of variation (%)	10.3	8.2	8.8	6.0	6.6

A 4.2.5 Specificity

The cross-reactivity percentage is the ratio between the thyroxine concentration and the compound concentration to be tested at 50% binding. No cross-reactivity in the VIDAS FT4 assay was observed with the substances tested.

Table A4.7: Specificity of the anti-T4 antibody used in the assay.

Tested compound	Cross-reactivity (%)
L-Thyroxine	100
D-Thyroxine	100
L-Triiodothyronine	1.2
D-Triiodothyronine	2.1
Diiodotyrosine	<0.1
Diiodothyronine	<0.1
Diphenylhydantoin	<0.1
Propylthiouracile	<0.1
Sodium salicylate	<0.1

A 4.3 Total Triiodothyronine (T3) evaluation

For serum total T3 concentration evaluation, commercial test kit VIDAS total T3, (bioMérieux sa, France), coupled with an automated VIDAS analyzer (bioMérieux sa, France) were used. The total T3 test requires 100 µL of serum sample. The standards are of human serum origin.

The information provided by the manufacturer of the kit is as follows.

A 4.3.1 Principle

The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (ELFA).

A 4.3.2 Measurement range

The measurement range of the VIDAS total T4 kit extends between 0.4 and 9 nmol/l.

A 4.3.3 Detection limit

The detection limit of these hormones was defined as the smallest concentration of total T4 which is significantly different from the zero concentration with a probability of 95%: < 0.4 nmol/l.

A 4.3.4 Accuracy

A 4.3.4.1 Dilution test

Three hyperthyroid samples were each diluted in sera with normal T3 concentrations and tested in singlet in 3 runs. The measured mean values compared to the expected mean values are shown as the mean recovery percentages in the table below.

Table A4.8: Recovery concentration after dilution.

Sample	Dilution factor	Expected concentration (nmol/l)	Measured concentration (nmol/l)	Recovery percentage
1	1:1	6.33	6.33	100
	1:2	4.07	4.44	109
	1:4	2.93	3.13	107
	1:8	2.37	2.57	109
2	1:1	7.53	7.53	100
	1:2	4.53	5.22	115
	1:4	3.02	3.63	120
	1:8	2.27	2.55	112
3	1:1	3.99	3.99	100
	1:2	2.76	2.90	105
	1:4	2.14	2.15	101
	1:8	1.83	1.83	100

A 4.3.5 Precision

A 4.3.5.1 Intra-assay precision

Five samples were tested 30 times in the same run and P values determined to assess intra-assay precision. Mean concentrations and coefficients of variation (CV) were determined. Results are shown below.

Table A4.9: Mean concentrations and coefficients of variation of samples in intra-assay precision test.

Sample	1	2	3	4	5
Mean concentration (nmol/l)	0.68	1.8	3.16	6.17	8.71
Coefficient of variation (%)	11.0	5.3	4.4	2.7	2.3

A 4.3.5.2 Inter-assay precision

Five samples were tested in singlet for 29 different runs over an 8 week period on the same VIDAS instrument. Mean concentrations and coefficients of variation (CV) are shown below.

Table A4.10: Mean concentrations and coefficients of variation of samples in inter-assay precision test.

Sample	1	2	3	4	5
Mean concentration (nmol/l)	0.76	1.93	3.34	5.84	6.42
Coefficient of variation (%)	12.4	5.7	4.2	2.8	3.2

A 4.3.6 Specificity

The antibody used in the VIDAS® T3 assay was tested for cross-reactivity against a number of compounds. The results in the table below are represented as the percentage ratio between the T3 concentration and the cross reactant concentration at 50 % binding.

Table A4.11: Specificity of the anti-T3 antibody used in the assay.

Tested compound	Cross-reactivity (%)
L-Triiodothyronine	100
D-Triiodothyronine	100
L-Thyroxine	0.21
D-Thyroxine	0.04
Diido-L-thyronine	3.3
Monoiodotyrosine	<0.01
Diiodotyrosine	<0.01
Diphenylhydantoin	<0.01
Propylthiouracile	<0.01
Triiodothyroacetic acid	100
Triiodothyropropionic acid	100
Sodium salicylate	<0.01
Phenyl butazone	<0.01
Propionic acid	50

A 4.4 Free Triiodothyronine (T3) evaluation

For serum free T3 concentration evaluation, commercial test kit VIDAS free T3, (bioMérieux sa, France), coupled with an automated VIDAS analyzer (bioMérieux sa, France) were used. The free T3 test requires 100 µL of serum sample. The standards are of human serum origin.

The information provided by the manufacturer of the kit is as follows.

A 4.4.1 Principle

The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (ELFA).

A 4.4.2 Measurement range

The measurement range of the VIDAS free T3 kit extends between 0.7 and 45 pmol/l.

A 4.4.3 Detection limit

The detection limit of these hormones was defined as the smallest concentration of free T3 which is significantly different from the zero concentration with a probability of 95%: ≤0.7 pmol/l.

A 4.4.4 Precision

A 4.4.4.1 Intra-assay precision

Four samples were tested 30 times in the same run and P values determined to assess intra-assay precision. Mean concentrations and coefficients of variation (CV) were determined. Results are shown below.

Table A4.12: Mean concentrations and coefficients of variation of samples in intra-assay precision test.

Sample	1	2	3	4
Mean concentration (pmol/l)	12.60	7.64	4.66	3.09
Coefficient of variation (%)	3.4	5.3	3.4	7.2

A 4.4.4.2 Inter-assay precision

Four samples were tested in singlet over an 8 week period on the same VIDAS instrument. Mean concentrations and coefficients of variation (CV) are shown below.

Table A4.13: Mean concentrations and coefficients of variation of samples in inter-assay precision test.

Sample	1	2	3	4
Mean concentration (pmol/l)	12.50	7.43	4.49	3.07
Coefficient of variation (%)	3.8	5.6	5.2	6.5

A 4.4.5 Specificity

The cross-reactivity percentage is the ratio between the thyroxine concentration and the compound concentration to be tested at 50% binding. No cross-reactivity in the VIDAS FT3 assay was observed with the substances tested.

Table A4.14: Specificity of the anti-T3 antibody used in the assay.

Tested compound	Cross reactivity (%)
Triiodothyronine	100
L-Thyroxine	0.20
Diiodothyronine	6
Triiodoacetic acid	65
Tetraiodoacetic acid	0.16
3-3'-5' triiodothyronine (T3 reverse)	<0.1
Diiodotyrosine	<0.1
Monoiodotyrosine	<0.1
Phenylbutazone	<0.1
Tyrosine	<0.1
Sodium salicylate	<0.1

Conversion table:

Total T4: nmol/L * 0.0777 = µg/dL

Free T4: pmol/L * 0.0777 = ng/dL

Total T3: nmol/L * 65.1 = ng/dL

Free T3: pmol/L *0.651 = pg/mL

Appendix 5

A comparison of portable ultrasound unit and fully-equipped clinical ultrasound unit in the thyroid size measurement of the Indo-Pacific Bottlenose dolphin, *Tursiops aduncus*

A5.1 Introduction

Ultrasound is a non-invasive, real-time imaging tool that provides high resolution images for soft tissue characterization, and allows repeatable measurements. 2-D ultrasound has a prominent role in evaluating the morphology of the thyroid gland in humans (Hegedüs, 2001; AIUM, 2003; Khati et al., 2003) and companion animals (Cartee et al., 1993; Wisner et al., 2002; Reese et al., 2005; Brömel et al., 2006). The mammalian thyroid gland is critical in regulating metabolic functions including cardiac rate and output, lipid catabolism, skeletal growth, and production of oxygen and heat. Environmental contaminants and local environmental influences have been implicated in thyroid hormone imbalances (Cowan and Tajima, 2006) and development of morphological and histological abnormalities (Schumacher et al., 1993; Mikaelian et al., 2003; Das et al., 2006) leading to calf mortality (Garner et al., 2002). To the best of our knowledge, the formal literature is devoid of any reference to the diagnosis of thyroid abnormalities in living dolphins. In order to accurately diagnose and assess thyroid abnormalities in live animals, reliable methods of assessing the thyroid morphology must be developed so that corrective therapy can be undertaken.

In human medicine, the thyroid volume is a useful clinical measure, particularly in the diagnosis of thyroid diseases and determining the appropriate dosage in radioiodine therapy. Volume measurement of each lobe is usually estimated using the ellipsoid equation developed by Brunn et al. (1981) i.e. volume = $\pi/6$ x craniocaudal x mediolateral x dorsoventral dimensions and its derivatives using the

cross-sectional area (Shabana et al., 2003). Recently, efforts have been made to establish a standardized scanning protocol in evaluating the morphology of the thyroid gland in a group of Indo-Pacific bottlenose dolphins using a fully-equipped clinical ultrasound unit (FCUS) with 3-D ultrasound capabilities (see Section 4.2.1.1 and 4.2.1.2). Using the equations in Brunn et al. (1981) and Shabana et al. (2003), 4 ultrasound thyroid volume measurement methods (Methods A - D) were developed, in which 13 linear and 5 cross-sectional measurements were undertaken in the dolphin thyroid study. Since serial ultrasound measurements of the dimensions of the thyroid gland have been proven to be useful in identifying thyroid diseases and monitoring treatment response, assessment of the aforementioned dimensions of the dolphin thyroid gland is essential, in addition to the thyroid volume itself.

Access to a FCUS, as well as 3-D ultrasound equipment, is always limited at zoological and aquarium settings. Procuring a FCUS is not always feasible in most veterinary settings due to its high start-up and maintenance cost. In addition, its bulkiness makes it unfavourable in various captive animal settings. A portable ultrasound unit (PUS) equipped with basic ultrasound functions for veterinary medicine has a comparatively lower cost that is affordable for most zoological and aquarium settings. Ultrasound studies conducted in various veterinary clinical settings, as well as wildlife research projects, have been mostly performed with different PUSs (Hildebrandt et al., 2000; King, 2006; Adams et al., 2007). However, the miniaturization of the PUS is believed to create compromises in function, and there are concerns regarding the image quality in these smaller and less expensive units. In view of the presently extensive applications of PUS in veterinary imaging, from being a diagnostic tool for routine clinical check-up of a range of species, to conducting disease screening, conservation projects, commercial services, herd management and clinical research, it is important to evaluate the inter-equipment variability between the PUS and FCUS in terms of direct linear measurements as well as cross-sectional areas of specific planes, which are essential parameters for volume measurement of an interested organ. In addition, the intra-operator variability (repeatability) of the individual PUS and FCUS should be further

examined under the same scanning conditions to ensure accurate assessments of the thyroid size in follow-up examinations throughout the course of treatment.

The aims of the present study were to evaluate the inter-equipment variability in dolphin thyroid ultrasound linear and cross-sectional area measurements between a PUS (Aloka SSD 900) and a FCUS (Philips HD 11) under identical scanning conditions, and to assess the repeatability of these measurements using both ultrasound units.

A5.2 Materials and Methods

A5.2.1 Subjects and study design

Fifteen *Tursiops aduncus*, at Ocean Park, Hong Kong (5 males and 10 females) were included in the study. The mean age of the subjects was 15.1 years (range, 2-35 years). All dolphins involved in the study were being trained to cooperate for neck ultrasound examination. Ultrasound images from each dolphin were taken on its thyroid using a PUS Aloka SSD 900 ultrasound unit in conjunction with a 3.5 MHz curvilinear transducer (Aloka Company Ltd., Tokyo, Japan) and a FCUS Philips HD 11 ultrasound unit in conjunction with a 5-2 MHz broadband curved array transducer (Philips Medical System, Bothell, Washington, 98021, USA).

A5.2.2 Technical differences between the PUS and the FCUS

The Aloka SSD 900 ultrasound unit is a miniaturized portable general imaging ultrasound unit that provides 256 shades of gray resolution and dynamic focus. This PUS is more portable than the FCUS because of its comparatively small size and low weight (13.6 kg). Similar to the FCUS, the PUS also offers a full range of measurement functions for clinical ultrasound examinations and incorporates super high density transducers to enhance imaging resolution.

Technical details of the PUS and the FCUS that may influence the thyroid linear and cross-sectional area measurements are listed in Table A5.1.

Table A5.1: Technical details of the portable ultrasound unit (PUS) and the fully-equipped clinical ultrasound unit (FCUS).

	Ultrasound machine	
Technical details	**PUS**	**FCUS**
Transducer frequency (MHz)	5	2 - 5
Frame rate (fps)	max 237	max 785
Gain setting	operator defined	operator defined
Grey scale	operator defined	operator defined
Persistence setting	4 settings	7 settings
No. of depth settings	11	30
No. of focus settings	4 user-selectable focal zones	4 user-selectable focal zones
Image resolution (axial resolution)	At 5 cm depth: 1 mm; At 11 cm depth: 1 mm	At 5 cm depth: 1 mm; At 11 cm depth: 1 mm
Image resolution (lateral resolution)	At 5 cm depth: 2 mm; At 11 cm depth: 4 mm	At 5 cm depth: 2 mm; At 11 cm depth: 4 mm

A5.2.3 Thyroid ultrasound imaging and measurement

Ultrasound measurements using both units were performed by the same operator (BK) and the operator was blinded to the linear and cross-sectional area measurements obtained from both units. There was a time interval of at least 30 minutes between measurements of the 2 sets of images from the same dolphin thyroid gland. Therefore, recall bias of the results for the same dolphin thyroid gland was avoided. The operator had more than 3 years of experience in performing dolphin thyroid ultrasound examinations.

Standardized scanning protocols for dolphin thyroid gland were used in the present study and four 2-D ultrasound thyroid volume measurement methods were used to measure the thyroid volume (see Section 4.2.1.1 and 4.2.1.2).

During the thyroid scanning with each ultrasound unit, time-gain-compensation and depth settings were adjusted to optimize image quality. For both ultrasound units, all measurements were performed using the electronic calipers. For the Aloka SSD 900 ultrasound unit, all images were recorded onto thermal printing paper, scanned and stored into digital format, while the images obtained by the Philips HD 11 were captured and stored digitally.

A5.2.4 Statistical analysis

To analyze the inter-equipment variability of both ultrasound units, different thyroid ultrasound linear and cross-sectional area measurements were assessed by the intraclass correlation coefficient (ICC) and 95% confidence intervals (C.I.). In order to evaluate the intra-operator variability (repeatability) of the different thyroid ultrasound linear and cross-sectional area measurements, intraclass correlation coefficient (ICC) and 95% C.I. were also used to assess the level of agreement of the measurements in a single operator (BK). An ICC > 0.7 is commonly used to indicate sufficient general reliability (Chien and Khan, 2001; Khan and Chien, 2001). All statistical analyses were carried out using SPSS (SPSS for windows 16.0, SPSS Inc., Chicago, Illinois).

A5.3 Results

The inter-equipment variability of the different thyroid ultrasound linear and cross-sectional area measurements is shown in Table A5.2. Overall, the ICC was 0.964 with 95% C.I. range of 0.889 – 0.988. Results demonstrated that the ICC values of all measurements were above 0.85, indicating correlations of over 85% between both ultrasound units. The cross-sectional area measurements yielded a higher inter-equipment reproducibility than the linear measurements. Overall, both ultrasound units yielded a high level of agreement in different thyroid ultrasound linear and cross-sectional area measurements.

Table A5.2: Inter-equipment (reproducibility) variability of the ultrasound thyroid linear and cross-sectional area measurements.

Measurement	ICC[k] (2,1)	95% C.I.[l] of ICC[k] (Lower - Upper)
Max TS[a]	0.969	0.896 - 0.990
L LS[b] (H[h])	0.907	0.752 - 0.967
L LS[b] (W[i])	0.915	0.766 - 0.971
L LS[b] (CSA[j])	0.934	0.821 - 0.977
Mid LS[c] (H[h])	0.939	0.829 - 0.979
Mid LS[c] (W[i])	0.938	0.801 - 0.980
Mid LS[c] (CSA[j])	0.976	0.894 - 0.993
R LS[d] (H[h])	0.958	0.818 - 0.987
R LS[d] (W[i])	0.933	0.813 - 0.977
R LS[d] (CSA[j])	0.949	0.648 - 0.987
L Obl[e] (L[g])	0.943	0.819 - 0.981
L Obl[e] (H[h])	0.936	0.824 - 0.978
L Obl[e] (W[i])	0.877	0.677 - 0.957
L Obl[e] (CSA[j])	0.949	0.859 - 0.982
R Obl[f] (L[g])	0.924	0.796 - 0.974
R Obl[f] (H[h])	0.859	0.638 - 0.950
R Obl[f] (W[i])	0.925	0.758 - 0.976
R Obl[f] (CSA[j])	0.959	0.884 - 0.986

[a]The maximum transverse dimension of the thyroid gland.
[b]The maximum longitudinal scan plane of the left thyroid lobe.
[c]The longitudinal scan plane of the left thyroid lobe.
[d]The maximum longitudinal scan plane of the right thyroid lobe.
[e]The oblique scan plane of the left thyroid lobe.
[f]The oblique scan plane of the right thyroid lobe.
[g]Length; craniocaudal dimension.
[h]Height; dorsoventral dimension.
[i]Width; mediolateral dimension.
[j]Cross-sectional area.
[k]Intraclass Correlation Coefficient.
[l]Confidence Interval.

Equation for calculation of thyroid volume
Thyroid Volume (mL): $\pi/6$ x TS_MAX x mean of craniocaudal dimension in 3 planes (LS_L ,LS_MID and LS_R) x mean of dorsoventral dimension in 3 planes (LS_L ,LS_MID and LS_R)

The intra-operator variability (repeatability) of using the 2 ultrasound units in thyroid ultrasound linear and cross-sectional area measurements is shown in Table A5.3. Overall, the ICC was 0.974 with 95% C.I. range of 0.925 – 0.991 for the PUS and 0.962 with 95% C.I. range of 0.891 – 0.987 for the FCUS. The cross-sectional area measurements yielded a higher intra-operator repeatability than the linear measurements. Results demonstrated that both ultrasound units yielded a high intra-operator repeatability for all thyroid ultrasound linear and cross-sectional area measurements. Compared to the FCUS, the PUS showed a higher repeatability.

Table A5.3: Intra-operator (repeatability) variability of the ultrasound thyroid linear and cross-sectional area measurements.

	PUS[m]		FCUS[n]	
Measurement	ICC[k] (3,1)	95% C.I.[l] of ICC[k] (Lower - Upper)	ICC[k] (3,1)	95% C.I.[l] of ICC[k] (Lower - Upper)
Max TS[a]	0.974	0.924 - 0.991	0.954	0.870 - 0.984
L LS[b] (H[h])	0.949	0.854 - 0.982	0.722	0.351 - 0.897
L LS[b] (W[i])	0.890	0.705 - 0.962	0.863	0.640 - 0.952
L LS[b] (CSA[j])	0.927	0.797 - 0.975	0.904	0.738 - 0.967
Mid LS[c] (H[h])	0.965	0.900 - 0.988	0.856	0.624 - 0.949
Mid LS[c] (W[i])	0.914	0.765 - 0.970	0.835	0.577 - 0.941
Mid LS[c] (CSA[j])	0.981	0.945 - 0.994	0.884	0.691 - 0.960
R LS[d] (H[h])	0.973	0.921 - 0.991	0.887	0.697 - 0.961
R LS[d] (W[i])	0.854	0.619 - 0.948	0.851	0.613 - 0.947
R LS[d] (CSA[j])	0.974	0.925 - 0.991	0.951	0.861 - 0.983
L Obl[e] (L[g])	0.984	0.952 - 0.994	0.867	0.650 - 0.953
L Obl[e] (H[h])	0.934	0.815 - 0.977	0.898	0.724 - 0.964
L Obl[e] (W[i])	0.928	0.800 - 0.975	0.878	0.676 - 0.957
L Obl[e] (CSA[j])	0.956	0.873 - 0.985	0.928	0.799 - 0.875
R Obl[f] (L[g])	0.950	0.857 - 0.983	0.939	0.829 - 0.979
R Obl[f] (H[h])	0.930	0.806 - 0.976	0.709	0.327 - 0.892
R Obl[f] (W[i])	0.896	0.720 - 0.964	0.802	0.508 - 0.929
R Obl[f] (CSA[j])	0.975	0.927 - 0.992	0.851	0.614 - 0.948

[a]The maximum transverse dimension of the thyroid gland.
[b]The maximum longitudinal scan plane of the left thyroid lobe.
[c]The longitudinal scan plane of the left thyroid lobe.
[d]The maximum longitudinal scan plane of the right thyroid lobe.
[e]The oblique scan plane of the left thyroid lobe.
[f]The oblique scan plane of the right thyroid lobe.
[g]Length; craniocaudal dimension.
[h]Height; dorsoventral dimension.
[i]Width; craniocaudal dimension.
[j]Cross-sectional area.
[k]Intraclass Correlation Coefficient.
[l]Confidence Interval.
[m]Portable ultrasound unit.
[n]Fully-equipped clinical ultrasound unit.

Equation for calculation of thyroid volume

Thyroid Volume (mL): π/6 x TS_MAX x mean of craniocaudal dimension in 3 planes (LS_L ,LS_MID and LS_R) x mean of dorsoventral dimension in 3 planes (LS_L ,LS_MID and LS_R)

A5.4 Discussion

Ultrasound is considered as a safe, non-invasive and well-tolerated imaging method in non-sedated animals (King, 2006). Diagnostic ultrasound enables serial examinations to monitor the progress of clinical condition and treatment response. The results of the present study demonstrated that ultrasound is an effective and reliable tool for measuring thyroid parameters. To the best of our knowledge, there has been no previous research investigating dolphin thyroid measurements using 2 different ultrasound machines, therefore the current study reflects the potential of detecting changes that exceed measurement error, for clinical and research applications.

There was a high level of agreement between the 2 ultrasound units in dolphin thyroid measurements, with the ICC values ranging from 0.859 to 0.976. Theoretically, the reproducibility (ICC) has a maximum value of 1. In most papers, a reproducibility of 0.7 and higher for labeling methods or units is considered to be sufficient (Chien and Khan, 2001; Khan and Chien, 2001). Thus, the results supported a high degree of agreement between the PUS and FCUS to quantify dolphin thyroid volume.

Results of the present study demonstrated that both the PUS and FCUS had a high intra-operator repeatability in thyroid measurements, with the ICC values of the PUS ranging from 0.854 to 0.984, and the ICC values of the FCUS ranging from 0.709 to 0.954. These results supported that the measurements yielded by the PUS are not only comparable to that of the FCUS, but that each unit can be used to perform thyroid volume measurements in a consistent manner.

Overall, the inter-equipment and intra-operator variability was minimal due to a number of reasons. The presence of a well-defined capsulated thyroid gland improved visualization on ultrasound scanning, enabling a higher precision while performing linear and cross-sectional area measurements. Since the dolphin thyroid gland was situated at the thoracic inlet, midway between the insertions of the pectoral flippers, this minimized measurement variation caused by the effect of

physiological activity such as heart beats and breathing during the scan. In the present study, a standard scanning protocol for the four 2-D ultrasound thyroid volume measurement methods was implemented, allowing the operator to have a clear and a precise sense of the procedures, facilitating the consistency of measurements during the ultrasound scanning. A single operator performed the present study enabling familiarity and greater experience with the established protocol. All dolphins involved in the study were trained to cooperate for neck ultrasound examination in a dorsal recumbence position, with their neck straightened and remaining still at the poolside. This prevented the distortion of the thyroid gland and thus allowed higher consistency with measurements during the ultrasound scanning.

These findings are in accordance with the results of the previous *in vivo* and *in vitro* studies which have incorporated ICC as a statistical test to assess agreement. Li et al. (2004) reported a high correlation in the inter-operator and intra-operator measurements of the mean splenic length (ICC value of 0.89 and 0.94), whereas Alshami et al. (2009) demonstrated a high correlation in the inter-operator and intra-operator measurements of the cross-sectional area of the tibial nerve at the tarsal tunnel (ICC values $\geq$ 0.86). For inter-equipment variability, previous studies reported that measures obtained using both PUS and FCUS were not significantly different and were equally repeatable (Magnussen et al., 2006; Hing et al., 2009; Legerlotz et al., 2010). However, the direct comparisons must be treated with caution. Our present study focused on the agreement between the 2 compared ultrasound units, rather than the accuracy of the portable ultrasound unit itself. Comparison of dolphin thyroid volume measurement accuracy using the 2 captioned ultrasound units is not possible due to the lack of a standard of reference. In our previous study, 3-D ultrasound thyroid volume measured by the FCUS was compared with the 2-D ultrasound thyroid volume measurement with the identical ultrasound unit and settings (Chapter 4). 3-D ultrasound thyroid volume measurement cannot be used as the standard of reference in the present study, since 3-D ultrasound is a functional capability of the FCUS. The PUS measurements have a substantially different image quality, and thus would result in a bias in favour of the FCUS measurements. As such, instead of looking into the accuracy of both

ultrasound units on their own, the present study investigated the agreement between these 2 ultrasound units (with the FCUS measurement accuracy validated in our previous study).

In the present study, the PUS yielded a higher intra-operator repeatability than the FCUS. Compared to the FCUS, the PUS has less precise calipers, limiting the measurements to 1 decimal place. In contrast, the FCUS gives the measurements to 2 decimal places, making it less prone to rounding error. This may give the PUS a higher intra-operator repeatability since the measurements had a higher degree of estimation with more measurements demonstrating absolute agreement.

The cross-sectional area measurements were found to have a higher inter-equipment reproducibility and intra-operator repeatability than that of the linear measurements. In a previous study, Warner et al. (2008) reported that the cross-sectional area measurements of the custom-made tissue phantoms had a higher inter- and intra-operator reliability than that of the linear measurements. Additionally, Shabana et al. (2003) found that the inter-operator variability for calculating thyroid volume was statistically significant using the formula with linear measurements, but was not statistically significant using the formula with cross-sectional area measurement. In the present study, for Methods A and B, the maximum cross-sectional area measurements from all 3 maximum longitudinal dimension scan planes yielded a higher reliability than the linear measurements (craniocaudal and dorsoventral dimensions). However, there may be difficulties in consistently estimating the linear measurements on the maximum longitudinal dimension scan plan between the 2 ultrasound scans. Since the thyroid gland was not a true oval shaped structure for the measurement on the longitudinal planes in Methods A and B and the transverse planes in Methods C and D, the determination of maximum long axis dimension was highly subjective, which possibly resulted in a larger variation on the linear measurements. In contrast, the determination of the maximum cross-sectional area relied on manual free-hand tracing of the thyroid borders, which was considered to be a relatively easier and more straight-forward procedure, resulting in a higher reproducibility and repeatability on the measurements. The same issues applied for

Methods C and D, in which the maximum cross-sectional area measurements in the scan plane 90 degrees to the craniocaudal dimension also yielded a higher reliability than the linear measurements (mediolateral and dorsoventral dimensions). Moreover, it is possible that there are different measurements of the mediolateral and dorsoventral dimensions on the same image plane; however, the cross-sectional area based on the same image plane would not change, resulting in a higher reliability than the linear measurements.

Even though this study has the undeniable merit of offering valuable insight into the agreement between the PUS and the FCUS in the application of dolphin thyroid measurements, there are some limitations. The transducers of the compared units were not in the identical frequency range. This is virtually unattainable since the FCUS in this study utilizes the latest transducer technology, which provides a broad range of frequencies rather than a single frequency emitted by the PUS compatible transducer. Image resolution may be degraded due to the frequency differences, and thus may affect the measurement accuracy. To minimize this difference in technology, the transducer frequency of the FCUS was set to the "middle to high" range between 5-2 MHz, which should be comparable to the 5 MHz used in the PUS transducer. In addition, the issue of image quality comparison between the captioned ultrasound units had not been mentioned in the present study. Rosenthal (2005) believed that the image quality is undoubtedly a component of the diagnostic ability of a system, but it is only one facet in determining an optimal system. Though we believe that the measurement accuracy may possibly be affected by the different image quality yielded, the degree of influence should be insignificant in our case, due to the presence of a well-defined capsulated thyroid gland in the dolphin which allows for an accurate linear measurement on different thyroid dimensions. Despite the controversy in objectively defining the image quality (Blaivas et al., 2004; Shrimali et al., 2009), there is no doubt that differential diagnosis was confirmed when a more advanced clinical ultrasound unit was used, which inevitably produced higher quality ultrasound images for clinical diagnosis. Studies have suggested that PUS provides a significant benefit that can drastically alter the disposition and treatment in patients at Accident and Emergency Departments, Intensive Care Units, small-scale hospitals and remote location

settings (Ryan et al., 2002; Blaivas et al., 2004; Stamilio et al., 2004; Blaivas et al., 2005). In view of the concerns raised from zoological and aquarium settings, a PUS could play an adequate role in improving a variety of veterinary procedures by providing a real-time, non-invasive clinical tool. Further studies in objectively evaluating the difference in image quality between the PUS and the FCUS in a zoological or aquarium setting is suggested to reinforce confidence of using PUS in veterinary medicine.

A5.5 Conclusion

There was no substantial inter-equipment variability between PUS and FCUS in thyroid size measurements. Both systems had high intra-operator repeatability in thyroid size measurements, substantiating further application of PUS for quantitative analyses of dolphin thyroid gland in research and clinical practice at an aquarium setting, when FCUS is not available.