UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

PREGNANCY MONITORING IN THE INDO-PACIFIC BOTTLENOSE DOLPHIN (*Tursiops aduncus*) AND PREDICTION OF DELIVERY DATE WITH EMPHASIS ON FETAL BIOMETRY

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Resumo

Monitorização da gestação e previsão da data de parto no golfinho-roaz do Indo-pacífico (*Tursiops aduncus*) com ênfase na biometria fetal

Para manter o bem-estar de uma população de golfinhos-roaz sob cuidado humano, é essencial seguir práticas de rotina de maneio e médicas preventivas. A monitorização do estado reprodutivo dos animais, especialmente do ciclo éstrico das fêmeas, através da ultrassonografia, está na base do sucesso reprodutivo e da gestão populacional. A monitorização ecográfica melhora a compreensão da sequência temporal da organogénese na espécie, permite a identificação de anomalias reprodutivas e malformações fetais, e a previsão da data do parto através da biometria fetal possibilitando a organização da equipa, da população de golfinhos e decisões clínicas em conformidade.

As cinco fêmeas *Tursiops aduncus* grávidas foram seguidas até ao parto. O acompanhamento por ecografia permitiu a identificação da vesícula embrionária e do embrião aos 314±7 dias e 306±10 dias antes do parto. As medidas do CL obtidas variaram entre 2,08 cm e 3,76 cm. A Frequência cardíaca confirmou uma correlação negativa com a gestação com valores médios a < 9 meses de gestação entre 82 bpm e 160 bpm, > 9 meses de gestação entre 71 bpm e 146 bpm, e durante as últimas duas semanas de gravidez entre 61 bpm e 108 bpm.

Mediante as medições do diâmetro biparietal e torácico realizadas no decorrer da gestação e aplicando os modelos de equação criados por Lacave et al. (2004) para *T. aduncus*, foi possível estimar o parto para cada fêmea. A diferença de dias entre a data de parto prevista e a real foi de 8,6±5,1 dias para o diâmetro biparietal e 13,6±5,2 dias para o diâmetro torácico. Horários de observação, e monitorização da temperatura retal e da distância intermamária foram implementados no período final da gestação de forma a reconhecer e preparar o parto iminente. Houve uma diminuição acentuada da temperatura durante os últimos 6 dias de gestação, com uma queda, em relação ao valor de temperatura médio (36,5 ºC), de até 1,3°C ao parto.

A previsão da data de parto com a maior precisão possível é determinante para o sucesso reprodutivo e gestão segura de um parque aquático com golfinhos-roaz.

Palavras-chave: *Tursiops aduncus*; ecografia; data de parto; biometria fetal; temperatura retal;

Abstract

Pregnancy monitoring in the Indo-pacific bottlenose dolphin (*tursiops aduncus*) and prediction of delivery date with emphasis on fetal biometry

Routine husbandry and preventive medical practices are essential for maintaining the welfare of the dolphin population under human care. Monitoring the reproductive status of the animals, especially the oestrus cycle of the females, through ultrasonography is the basis for reproductive success and population management. Ultrasonographic pregnancy monitoring improves understanding of the organogenesis timeline in the species, allows identification of abnormalities and foetal malformations, and prediction of the delivery date through foetal biometry, enabling management of the team and dolphin population accordingly.

The five *Tursiops aduncus* pregnant females in this study were monitored until delivery. Ultrasound follow-up assessments allowed the identification of the embryonic vesicle and embryo at 314±7 days and 306±10 days before parturition. CL measurements ranged from 2.08 cm to 3.76 cm. HR showed a negative correlation with gestation with mean values at < 9 months gestation between 82 bpm and 160 bpm, at > 9 months gestation between 71 bpm and 146 bpm, and during the last two weeks of pregnancy between 61 bpm and 108 bpm.

By taking measurements of foetal biparietal and thoracic diameter throughout the gestation and applying the equation models created by Lacave et al. (2004) for *T. aduncus*, it was possible to estimate the parturition for each female. The day difference between the expected and actual delivery date was 8.6±5.1 days for biparietal diameter and 13.6±5.2 days for thoracic diameter. Observation schedules, rectal temperature, and intermammary distance monitoring were established in the late gestational period to recognize and prepare for the approaching delivery. There was a significant temperature decrease during the last 6 days of gestation with up to 1.3°C drop from the mean temperature value (36.5 ºC) at birth.

Predicting the delivery date as accurately as possible is crucial to enhance the reproductive success of a bottlenose dolphin population in a marine facility.

Key words: *Tursiops aduncus*; ultrasonography; delivery date; fetal biometry; rectal temperature;

Resumo alargado

Monitorização da gestação e previsão da data de parto no golfinho-roaz do Indo-pacífico (*Tursiops aduncus*) com ênfase na biometria fetal

Para manter o bem-estar de uma população de golfinhos-roaz sob cuidado humano, é essencial seguir práticas de rotina de maneio e médicas preventivas. A monitorização do estado reprodutivo dos animais, especialmente do ciclo éstrico das fêmeas, através da ecografia, está na base do sucesso reprodutivo e da gestão populacional. Para além de melhorar a compreensão da sequência temporal da organogénese na espécie, a monitorização ecográfica permite a identificação de anomalias reprodutivas e malformações fetais desde as fases iniciais de desenvolvimento.

Para a aplicação de um protocolo de parto adequado e seguro é essencial estimar a data de parto. Conhecer o intervalo de dias em que o parto deverá acontecer possibilita a organização da equipa de veterinários e treinadores, da população de golfinhos assim como a tomada de decisões clínicas em conformidade. A previsão da data do parto pode ser obtida com base no dia da conceção conhecendo o período de gestação médio daquele indivíduo, população ou espécie, ou recorrendo à biometria fetal.

Atualmente, as medidas fetais que demonstram linearidade e provam ser bons preditores da data de parto em qualquer altura da gravidez são o diâmetro biparietal e o diâmetro torácico. Lacave et al. (2004) mediram o diâmetro biparietal fetal na secção mais larga onde a falx cerebri é visível e o diâmetro torácico ao nível do coração em fêmeas *Tursiops aduncus* residentes no Ocean Park, Hong Kong. As medições obtidas permitiram a construção de modelos de equação para a previsão do parto na espécie. Mais recentemente, outras medidas fetais como o diâmetro da aorta e a espessura da gordura mostraram ser facilmente obtidas e precisas na previsão da idade gestacional.

A medição da temperatura retal e distância intermamária das fêmeas gestantes nos últimos dias de gestação têm demonstrado ser indicadores úteís do parto iminente permitindo a preparação do espaço e dos recursos nas horas que antecedem o parto.

As cinco fêmeas *T. aduncus* gestantes da população estudada foram seguidas desde o momento da identificação da vesícula embrionária até ao parto e período perinatal. O acompanhamento semanal por ecografia permitiu a identificação da vesícula embrionária e do embrião aos 314±7 dias e 306±10 dias antes do parto. Sempre que possível, as dimensões do CL foram determinadas medindo os diâmetros transversais e longitudinais que variaram entre 2,08 cm a 3,76 cm. A frequência cardíaca foi obtida pela primeira vez aos 219 ± 17 dias antes do parto e confirmou uma correlação negativa com a gestação com valores médios a < 9 meses de gestação entre 82 bpm e 160 bpm, > 9 meses de gestação entre 71 bpm e 146 bpm, e durante as últimas duas semanas de gravidez entre 61 bpm e 108 bpm.

A genitália fetal foi reconhecida em duas das cinco gestações. Os fetos da Fêmea 1 e da Fêmea 3 foram identificados como machos, aos 218 dias e 249 dias antes do parto (234 ± 16).

O movimento fetal foi monitorizado ao longo da gestação, e a adoção da posição curvada do feto no útero registada aos 104 ± 9 dias antes do parto.

O parto da fêmea 1 ocorreu em fevereiro de 2019, enquanto as outras quatro fêmeas conceberam em maio do mesmo ano. Dado o número de partos previstos para o mês de maio, foi de extrema importância a aplicação de um método que permitisse estimar um intervalo curto de datas de parto para uma maior probabilidade dos partos serem bem-sucedidos.

Mediante as medições do diâmetro biparietal e torácico realizadas no decorrer da gestação e adotando os modelos de equação criados por Lacave et al. (2004) para a espécie *T. aduncus*, foi possível estimar o parto para cada fêmea. A diferença de dias entre a data de parto prevista e a real foi de 8,6 ± 5,1 dias para o diâmetro biparietal e 13,6 ± 5,2 dias para o diâmetro torácico demonstrando que o diâmetro biparietal é o melhor dos dois na previsão da data de nascimento.

O período gestacional em que as medidas foram mais precisas foi nos primeiros quatro meses de gestação. Os resultados revelam que as medidas torácicas até os 4 meses de gestação foram mais confiáveis que as biparietais, embora a diferença de dias entre a data prevista e a real obtida neste período para os dois diâmetros tenha diferido em apenas 1 dia. Entre as duas, as medidas do diâmetro biparietal foram mais precisas nos intervalos de 4 a 8 meses e > 8 meses de gestação.

Em quatro das cinco fêmeas gestantes, as crias nasceram mais cedo do que o previsto, o que significa que as dimensões do feto no dia do parto eram menores do que o tamanho esperado pela equação. O mesmo foi demonstrado pelas retas de regressão linear.

Observou-se uma diferença discrepante entre os nossos resultados e a linha de regressão padrão nos gráficos 3 e 7 relativos às fêmeas 1 e 3. Os resultados não estão ajustados às retas padrão das equações previstas ainda que o crescimento fetal tenha ocorrido a um ritmo constante. O tórax do feto da fêmea 3 mostrou um crescimento mais lento que qualquer outro feto.

O feto da fêmea 5 foi o que apresentou os melhores resultados, uma vez que a diferença de dias entre a data de parto real e a prevista foi de 1 dia e de 4 dias a partir do diâmetro biparietal e torácico, respetivamente.

As equações utilizadas para prever a data de parto foram baseadas numa população com proveniência diferente das cinco fêmeas estudadas neste trabalho, cuja origem são as Ilhas Salomão, no Sul do Pacífico. Este facto pode explicar o menor diâmetro torácico dos fetos desta população observado nos gráficos de regressão linear.

Os diâmetros biparietal e torácico mostraram ser medidas altamente precisas para prever a idade gestacional na espécie *T. aduncus* (R2>0,96).

Horários de observação, e monitorização da temperatura retal e da distância intermamária foram implementados no período final da gestação de forma a reconhecer e preparar o parto iminente. As fêmeas foram observadas mais atentamente para avaliar o seu comportamento geral, atitude perante os treinadores, a expulsão de leite ou outras secreções e a diminuição de apetite ou anorexia.

As fêmeas apresentaram uma diminuição gradual da temperatura a partir do 14º dia antes do parto, que se tornou mais acentuada durante os últimos 6 dias de gestação. A queda da temperatura até ao parto não foi superior a 1.3 ºC, em relação ao valor de temperatura médio registado (36,5 ºC).

A monitorização deste parâmetro no final da gestação ajudou a prever a aproximação do parto, pois permitiu detetar a queda da temperatura esperada nas 12 a 24 horas que antecedem o parto.

A distância intermamária mostrou ser um parâmetro pouco conclusivo. Embora tenhamos conseguido identificar um ligeiro aumento da distância intermamária, esta medida não contribuiu para a identificação do início do trabalho de parto. A inconsistência dos valores obtidos pode dever-se a possíveis movimentos, instabilidade ou contração da fêmea durante o procedimento, o que aumenta a probabilidade de erro do operador.

Os resultados obtidos validam os protocolos de monitorização de gestação e parto seguidos no Dolphin Bay, Atlantis the Palm, Dubai. A detalhada avaliação ecográfica semanal do trato reprodutor nas fêmeas sexualmente maduras revelou-se eficaz no diagnóstico precoce da gravidez.

Por muito precisa que seja a previsão do nascimento, o parto é um acontecimento complexo que exige que toda a equipa esteja preparada para prestar assistência em caso de complicações. A previsão da data de parto com maior precisão possível assim como a implementação de métodos rigorosos de monitorização e prevenção é determinante para o sucesso reprodutivo, saúde e o bem-estar de um grupo de golfinhos sob cuidados humanos.

Palavras-chave: *Tursiops aduncus*; ecografia; data de parto; biometria fetal; temperatura retal;

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LIST OF ABBREVIATIONS

% - Percentage

BPD - Biparietal Diameter

bpm - Beats per Minute

CA - Corpora Albicantia

CL - Corpus Luteum

cm - Centimetres

ºC – Celsius degrees

DF - Dominant Follicle

HR - Heart Rate

h – Hours

mm - Millimetres

min - Minutes

m – Meters

P4 - progesterone

PGF2α - Prostaglandin F2α

s - Seconds

y – Years

I. INTERNSHIP ACTIVITIES

Internship in Acquario di Genoa, Italy

I was a trainee in acquario di genoa from September to December 2018 under the Erasmus+ program.

The aquarium is home to 600 animal species. Some of the main exhibits are: The shark bay; the Icy kingdom of Gentoo and Magellanic penguins (*Pygoscelis papua; Spheniscus magellanicus*); the Cetacean pavilion, which houses *Tursiops truncatus*; the mermaid lagoons with manatees (*Trichechus manatus)*; the seal island housing Common seals (*Phoca vitulina)*; the tropical fish tanks and a coral reef reconstruction section; the Mediterranean fish tanks; the rainforest area with freshwater fish species, frogs, turtoises, snakes, geckos, among others.

I transitioned through all the teams of trainers and caretakers. I learned about the tank cleaning routine, diet, and feeding of each exhibition.

Laboratory: Preparation of medications; preparation of material for the sampling of blowhole, gastric and faecal of dolphins; analysis of gastric and faecal contents (pH, colour, odour, quantity); preparation and interpretation of cytology; faecal flotation test; preparation of instruments for blood sampling; blood analysis; analysis of water tanks under the microscope; water treatment; organization of the lab and pharmacy, and verification of expired medicines.

Imaging diagnosis: General and reproductive ultrasound of the Common bottlenose dolphins (*T. truncatus),* and of manatees (*T. manatus)*.

Husbandry: Feeding of manatees and penguins; applying identification facets to penguins; training dolphins for ultrasound desensitizing and blood collection presentation.

Clinical cases: Some clinical cases concerned the Scarlet ibis (*Eudocimus ruber*) with skin lesions; freshwater rays with skin/muscle lesions (*Potamotrygon sp*); seal with eye lesions (*P. vitulina*); Common eagle ray *(Myliobatis aquila)* with muscle lesions; seahorses (*Hippocampus hippocampus)* force-feeding; the sandbar shark (*Carcharhinus plumbeus*) skin and muscle lesions; septicemic cutaneous ulcerative disease in *pyxis sp.* and *emys sp;* among others.

Necropsies: Seahorses (*H. hippocampus)*; Pacu (*Piaractus brachypomus);* Chromis *(Chromis viridis)*; Salema (*Sarpa salpa)*; Red piranha (*Pygocentrus nattereri)*; Common eagle ray *(M. aquila)*; Loggerhead sea turtle (*Caretta caretta)*, among others.

Other: Surgery in *C. caretta*; quarentine and preventive medicine of newly arrived animals; sedation of red porgy (*Pagrus pagrus*) and black sea bass (*Centropristis striata*); transportation procedure of two dolphins to another facility; literature research and review;

Internship at Atlantis, the Palm veterinary services, Dubai

The internship had a duration of 6 months, from March until September, 2019.

The veterinary services at Atlantis, the Palm are responsible for the health and care of:

- Dolphin bay: *Tursiops aduncus* population;
- Sea lion point: South African Fur seals (*Arctocephalus pusillus pusillus)*;
- Lost Chambers Aquarium: One of the largest aquariums in the world, holding more than 250 different animal species;

Routine procedures (part of the daily routine): Preparation of supplements and medication for Dolphin Bay and Sea Lion point animals; dolphin voluntary blood collection, analysis, and reports; cytology preparation and interpretation; observation and evaluation of faecal and gastric content of dolphins and sea lions; reproductive ultrasounds to dolphins by voluntary behaviour; lab cleaning protocols, organization, and records;

Laboratory: Blood analyses (complete blood count, microhematocrit, differential, chemistry and logging, manual cell count of white blood cells and red blood cells in Neubauer chamber); blowhole, faecal, and gastric cytology preparation and interpretation (Slides preparation, logging, and staining, and microscopic visualization and record); faecal flotation test; pipetting and microscope operating skills.

Imaging diagnosis: Routine ultrasound in all animals; ultrasound of the pregnant females; X-Ray of teeth (assembling and positioning); endoscopy (assembly and operating);

Clinical cases: Involuntary blood collections of the calves and the mothers, and in sick animals not responding to the trainers; preparation of milk formula; gastric tube feeding of calves and adults (Fig. 1); endoscopy examination in dolphins with gastric ulcers and stones; following several clinical cases in the Indo-Pacific bottlenose dolphin (*T. aduncus)*, Red-tail catfish (*Phractocephalus hemioliopterus*), zebra shark (*Stegostoma fasciatum*), bowmouth guitarfish (*Rhina ancylostoma*), hammerhead shark (*Sphyrna lewini)*, longhorn cowfish (*Lactoria cornuta*), among others.

Necropsies: Several fish species, stingrays, sharks, among others.

Others: Seminars on animal welfare, reproduction, and pre-post labour; measurement of pregnant females' rectal temperature and intermammary distance; observations (mother and calf respiration and neonate nursing records); water quality sampling; learning how to work with the software used in the facility; body condition score; aquarian operations in small and large exhibits; aquarium species identification; beach release of Arabian carpet sharks (*Chiloscyllium arabicum*), and Marbled rays (*Taeniura meyeni*) into the Arabian golf;

Fig. 1- Photograph taken during supplementation procedure of a neonate in the medical pool at Dolphin Bay indoor facility. Courtesy of Dra. Ana Salbany, August 2019.

II. LITERATURE REVIEW

1. The Indo-Pacific bottlenose dolphin – *Tursiops aduncus*

1.1. Taxonomy

The Indo-Pacific bottlenose dolphin, *Tursiops aduncus* [Ehrenberg 1832], classifies into the Delphinidae family, within which belongs to the genus Tursiops (Committee on Taxonomy 2022; Fordyce and Perrin 2023).

T. aduncus was first described in 1832 in a published paper reporting the findings of zoologists Hemprich and Ehrenberg in the Red Sea. In 2007, Perrin et al. (2007) confirmed, through genetic and morphological analyses of the skull found by the two zoologists almost two centuries earlier, that it was indeed a specimen of an Indo-Pacific bottlenose dolphin.

Presently, there are two widely recognised, genetically and osteologically confirmed species (Kemper 2004; Wang et al. 1999). The *Tursiops aduncus* with the common name Indo-Pacific bottlenose dolphin for its distribution along the coastline of the Indian and Pacific oceans, and the *Tursiops truncatus*, known as the Common bottlenose dolphin, inhabiting off-shore and in-shore, temperate, and tropical waters worldwide (Möller and Beheregaray 2001).

Different species and subspecies of bottlenose dolphins have been proposed over the years, such as, most recently, *T. australis* in South Australia (Charlton-Robb et al. 2011). Significant genetic differences of *T. aduncus* from South Africa and China are also prompting a revision of taxa (Natoli et al. 2004). Further changes to the genus *Tursiops* are expected in the future (Perrin et al. 2007; IWC 2021).

1.2. External Features - T. aduncus vs T. truncatus

T. aduncus is morphologically distinct from *T. truncatus* primarily in its smaller size, proportionally longer and narrower beak, and leaner overall body figure (Jedensjö et al. 2020). The maximum reported adult length and weight are 2.7m and 200kg, respectively. In some regions, however, they are noticeably smaller, not growing above 2.3m. The variations in size between populations reflects an adaptation to environmental conditions, specifically to the water temperature of the region. *T. aduncus* from colder waters is longer and more robust (Aswegen et al. 2019; Cockcroft & Ross 1990).

The fins are larger and broader, relative to body size, and have more teeth and fewer vertebrae than *T. truncatus* (Wang et al. 2000). A typical feature of *T. aduncus* is the presence of speckling in the ventral region of the body (appendix 1) correlated to sexual maturity, which *T. truncatus* does not exhibit (Wang 2018). This speckling first appears in the genital area and increases in number and density with age, spreading to the neck, and lateral belly area, near the pectorals (Kryszczyk & Mann 2011). The onset of spotting during puberty has also been documented in the *Stenella* genus (Mignucci-Giannoni et al., 2003).

1.3. Ecology and conservation

1.3.1. Distribution and habitat

The Indo-Pacific bottlenose dolphin has coastal distribution, inhabiting the shallow waters of the Indian and West Pacific oceans, usually less than 100m deep, from the southern tip of South Africa to as far east as the Fiji archipelago and from the northern waters of Japan to southern Australia, including the islands in between and the Red Sea (Fig. 2) (Dulau-Drouot et al. 2008). It is known to occupy warm temperate to tropical waters around 20ºC to 30ºC even though some populations in Northern China, South Korea, South Australia, and South Africa live in colder waters, with Japan holding the lowest water temperature reported where the species is found, 12°C (Wang and Yang 2009). *T. aduncus* is mainly found in reefs, sandy bays, seagrass bed areas, and island proximities. Its distribution may coincide with that of *T. truncatus* in some regions (Hale et al. 2000).

It is mostly a resident species, yet occasional migration and ocean crossing is reported (Peddemors 1999; Wang and Yang 2009). Females seem to be the more philopatric and males the more dispersing sex. The reproductive success of males may depend on their dispersal to new areas, while female bottlenose dolphins benefit from familiarity with other females, their habitat, its food resources, and the development of specialized foraging skills (Möller and Beheregaray 2004).

1.3.2. Feeding habits and Foraging techniques

The bottlenose dolphin is at the top of the food chain as one of the largest predators of the marine environment. They feed on a wide variety of animals, mainly small pelagic fish, cephalopods, crustaceans, and small rays and sharks (Amir et al. 2005). Because of the narrow beak and the direct ingestion of food without chewing, it prefers to forage on smaller prey up to 30 cm in length (Wang and Yang 2009). According to Kaiser (2012), the *T. aduncus* populations from the coast of Zanzibar and KwaZulu-Natal feed on at least 94 species of prey, even though most of the diet consists of 6 species of fish and 2 of cephalopods.

The bottlenose dolphin can behave as an individual and a group predator (Shane et al. 1986) feeding on shoals or solitary fish. They develop various complex foraging techniques depending on geographic location and available food resources, such as: "Sponging" when dolphins take advantage of marine sponges to protect their beak from the sea floor and potential injuries, "Bottom grubbing" when they poke their beak into seagrass flats looking for fish, "Kerplunking" when they heavily splash the water surface with the fluke disturbing hidden fish that get exposed, "Beach hunting" when they become partially or fully stranded on the beach shore to catch single isolated fish, among others (Connor et al. 2000b; Mann and Sargeant 2003; Patterson et al. 2015; Sargeant et al. 2005; Wild et al. 2020).

The Indo-Pacific bottlenose dolphins of South and South-Western Australia benefit from the high concentration of giant cuttlefish (*Sepia apama*) in the area, representing a significant part of these dolphins' diet (Smith and Sprogis 2016). The prey-handling of cuttlefish and octopus are two other specialized foraging techniques of the bottlenose dolphins, although the second has proved to be highly risky, having already caused the death of two adult dolphins who choked on the prey (Sprogis et al. 2017).

The species has also learned to take advantage of human activity by feeding on stolen fish from fishing gear, following fishing boats, interacting with tourists on sighting ships, and going near fishing villages (Sargeant and Mann 2009; Smith 2012; Wiszniewski et al. 2009).

Females that overlap in home range and are in similar reproductive status tend to aggregate to obtain food, help nurse and rear the calves, to better protect their offspring and themselves against predators such as sharks and avoid aggressive approaches from adult males (Frère et al. 2010; Fury et al. 2013; Heithaus et al. 2017).

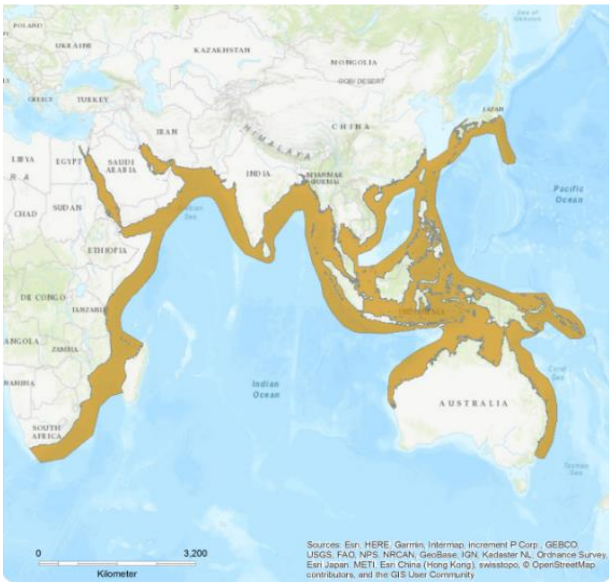


Fig. 2- Distribution map of the Indo-Pacific bottlenose dolphin, T. aduncus. Image adapted from the IUCN Red List of Threatened Species: Tursiops aduncus, 2019.

1.3.3. Conservation status

The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (2019) classified the *T. aduncus* as “Near Threatened”.

According to the IUCN, the small and restricted near-shore distribution, the intensive fisheries and the high risk of entanglement in fishing gear, and the coastal habitat destruction from increasing anthropogenic activities, are the main reasons why the Indo-pacific Bottlenose dolphin is classified as a “Near Threatened” species (Amir et al. 2005; Donaldson et al. 2010; Kiszka et al. 2008a, 2008b; Smith 2012).

Studies showed significantly less feeding, resting, and socializing and an increase in the travelling behaviour of the dolphins when boats were within 50 meters of the population. These approaches to dolphin groups disturb their well-being and may compromise reproductive success and conservation of the species in the long term (Christiansen et al. 2010; Steckenreuter et al. 2011).

2. Anatomy and physiology of the reproductive system

2.1. Male reproductive organs

In cetaceans, the genital slit of males is separate from and located cranially to the anal orifice (Fig. 3). The distance between the two openings is approximately 10% of the body length (Berta et al. 2006).

The penis is fibroelastic, positioned internally, and curved into a sigmoid flexure inside the genital slit. Upon erection, the penis straightens and becomes external. A retractor muscle originating from the surface of the rectum and attached to the ventral surface of the penis withdraws it back into the body, keeping it inside, similarly to ruminants (Cozzi et al. 2017; Saviano 2013; Schroeder 1990).

The testis are elongated and located in the abdominal cavity, slightly caudal to the kidneys (Brook et al. 2000; Robeck et al. 1994). Testicular size varies with age and sexual maturity, which is reached between ages ten and fifteen, sometimes earlier. Testis from sexually mature individuals are larger than immature males with lengths between 164 and 292 mm (Kemper et al. 2014). A large prostate partially surrounds the urethra, where it empties (Plön and Bernard 2007; Yuen 2007).

2.2. Female reproductive organs

Females have a ventromedial slit, denominated anogenital slit, containing the vaginal orifice and the anus. Anterior to the anus is the vulva, constituted of underdeveloped major and minor labia, the urethra cranially, and the clitoris on the anterior end. The two elongated nipples, 1-2 cm on either side of the genital slit, open to the respective mammary gland, which extends from slightly caudally to the umbilicus almost to the anus (Fig. 3) (Kamolnick et al. 1992; Robeck et al. 1994).

The ovaries have an oval to elliptical shape that can vary and acquire different sizes depending on age, reproductive status, and weight of the female (Plön and Bernard 2007).

The uterus is bicornuate, meaning that each oviduct enters its ipsilateral uterus horn, which then fuses to form the body of the uterus, similar in appearance to those of dogs and horses (Schroeder 1990). The ovary, oviduct, and uterine horn are held in position by the broad ligament. The body of the uterus is short, the wall is thick, and the lumen is narrow, terminating at the cervix. The cervix is a well-muscled sphincter that connects the uterus to a 10cm to 15cm long vagina (Robeck et al. 1994). The lower part of the vaginal canal presents longitudinal folds, while the upper part consists of numerous circular folds, which form vaginal plugs caudal to the “true” cervix, often called “pseudocervix”, and a spermathecal recess (Stewart REA and Stewart BE 2009). The pseudocervix may function as a sperm retainer and in preventing seawater from entering the cervix (Saviano 2013).

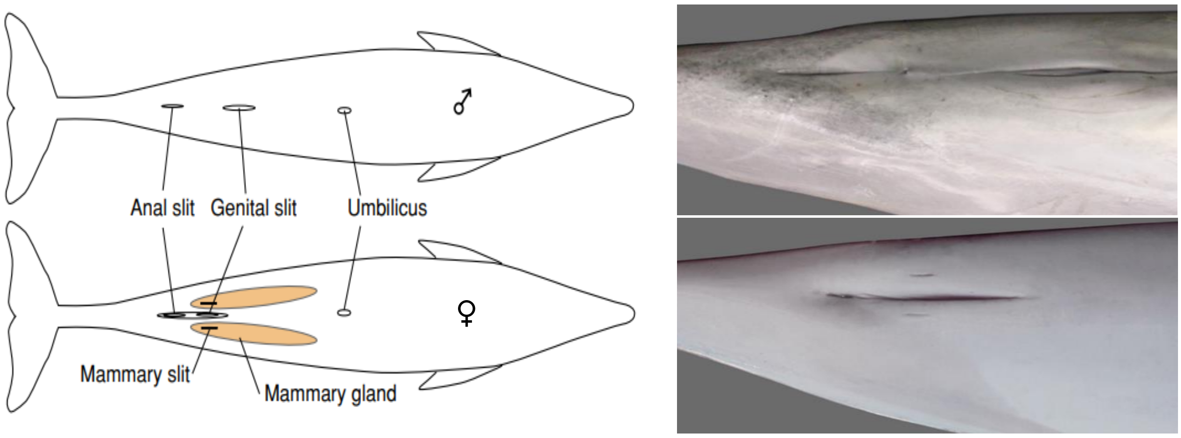


Fig. 3- Representative image of the male and female external genital differences (on the left). Source: Berta et al. (2006). Real image of the T. truncatus male (top) and female (bottom) genital slit (on the right). The two small slits parallel to the genital slit in the female, are the nipples. Source: Cozzi et al. (2017).

3. Reproduction

3.1. Reproductive cycle

The bottlenose dolphin is seasonal polyoestrous (Robeck et al. 2001). The reproductive season covers the months from late winter and early spring until the beginning of autumn (Kirby and Ridgway 1984) though there are records of births that occur in all months of the year, which means several factors can influence the reproductive state, such as captivity, water temperature, and food availability (Fedak et al. 2009).

The age of sexual maturity cannot be established with certainty, though the onset of speckles in the genital area of *T. aduncus* seems to correlate with sexual maturity (Kryszczyk and Janet 2011). A case of pregnancy in a captive bottlenose dolphin female at four and another at seven years of age are described (Lacave 1991; Tavolga and Essapian 1957). Brook (1997) also reported the first-time ovulation in 2 captive *T. aduncus* of 6 and 7 years of age and Zhang et al. (2020) confirmed the reach of sexual maturity from age 6 through hormone analyses in the same population as Brook (1997).

During each breeding season, females experience one up to ten consecutive oestrus cycles with a single ovulation per cycle (Cozzi et al. 2017). Brook (1997) observed that younger females had more ovulations than older individuals and the average time between successive ovulations was 30 days (28-36).

Periods of anoestrus are characterized by the absence of ovulation and ovary stillness. Brook (1997) documented anoestrus periods between non-conceptive cycles of two to 19 months, but it is described as lasting up to 27 months in this species, although the significance of this is unclear (Robeck et al. 2001).

3.2. Mating strategies

Cetaceans are known to adopt some form of polygynous or polygynandrous mating system. Mating strategies include the commonly observed male-male competition, but cases of male association are also reported. Adult males may form cohesive subgroups of two, or three, usually long-term cooperative coalitions with kin members. These subgroups associate with other nearby males and form temporary alliances that help them gain access to, chase, and sequester females from other males (Berta et al. 2006; Diaz-Aguirre et al. 2018). When females are in oestrus, it is common to find new fresher rakes around the genital region, fluke, peduncle, and beak resulting from male attacks (Shane et al. 1986).

Although overlooked in cetacean research, there are some described female strategies to increase control over paternity. Polyestry and mating with multiple males is a mechanism adopted by *T. aduncus* females trapped in aggressive male alliances to control paternity. This tactic can induce sperm competition, avoid consanguinity, lower the risk of breeding exclusively with infertile males, reduce sexual harassment, and confuse paternity and reducing infanticide (Orbach 2019; Connor et al. 2019).

Copulation is internal. The male deposits the sperm inside the spermathecal space. The withdrawal of the penis causes the pseudo cervix to contract, expelling any saltwater that represents an obstacle to the viability of the sperm (Saviano 2013; Schroeder 1990).

3.3. Fetomaternal development

Pregnancy in the bottlenose dolphin lasts about twelve months (Schroeder 1990). The gestation period in *T. truncatus* is longer, around 377 days (O'Brien and Robeck 2010), than in *T. aduncus*, which was 369 ± 5 days in Zhang et al. (2021). Brook (1997) reported a gestation length range from 352 - 359 days in *T. aduncus* from Indonesia, and a larger duration of gestation in *T. aduncus* from the Taiwanese waters, 371 - 384 days. The placenta that cetaceans develop is diffuse epitheliochorial, which facilitates detachment at birth (Reidenberg and Laitman 2009)(appendix 2).

In the foetal period, the loss of the bulbs and olfactory nerves occurs, the tooth buds appear, differentiation of the sexual organs follows, and hair grows on both sides of the rostrum near the tip. This hair, which falls shortly after birth, appears to have tactile properties important in the first days of life (Reidenberg and Laitman 2009).

A vascular plexus, which allows heat exchange by countercurrent, develops around the uterus to avoid overheating the genital organs and the foetus during pregnancy (Plön and Bernard 2007; Rommel et al. 1993; Saviano 2013).

3.4. The mother-calf dyad

The first nursing attempts begin shortly after birth. There is no coupling and suckling of the nipples of the mother. Instead, milk is ejected under pressure when the calf is in a suitable position and grasps the mammary slit with its tongue (Sommer 1998; Tavolga and Essapian 1951). The muscle involved in the expulsion of milk is a part of the cutaneous trunci, which compresses the mammary gland when stimulated by the calf (Thewissen 2009).

Females with calves tend to cooperate in rearing their offspring. For such, they arrange groups referred to as "nurseries". Allomothering behaviour may also occur in wild and captive dolphins affecting non-lactating adult females that, when placed together with unrelated orphaned calves, are induced to lactate after repeated attempts at suckling by the calf (Muraco 2015; Ridgway et al. 1995; Sakai et al. 2016).

The mother-calf dyad is the strongest interaction within the social organization of the bottlenose dolphins. Soon after being born, the calf adopts the echelon position swimming very close, parallel just above the mid-lateral flank, near the mother's dorsal fin (Fig. 4A). The neonate benefits energetically from the pressure wave of the mother, which minimizes the cost of transport for the calf, enabling it to travel at adult speed. Some behaviours such as respiratory synchrony, regular contact, and rubbing with the mother are expected and indicators of newborn wellness (Connor et al. 2000a; Connor et al. 2019). Calves usually transition from swimming in echelon to the infant position (Fig. 4B) (Noren et al. 2008). In this swimming mode, the calf is under the peduncle, close to the abdomen and nipples of the mother, which also increases hydrodynamic and provides protection and nursing access. Echelon swimming has proven crucial to neonate survival. Otherwise, calves are incapable of accompanying the mother during travel (Mann 2019).

Young dolphins continue to nurse until they reach 3 to 4 years of age, during which they stay close to their mothers and the other calves. Occasional nursing has been observed in calves up to 6 years old (Gibson and Mann 2008; Mann et al. 2000; Wells et al. 1987).

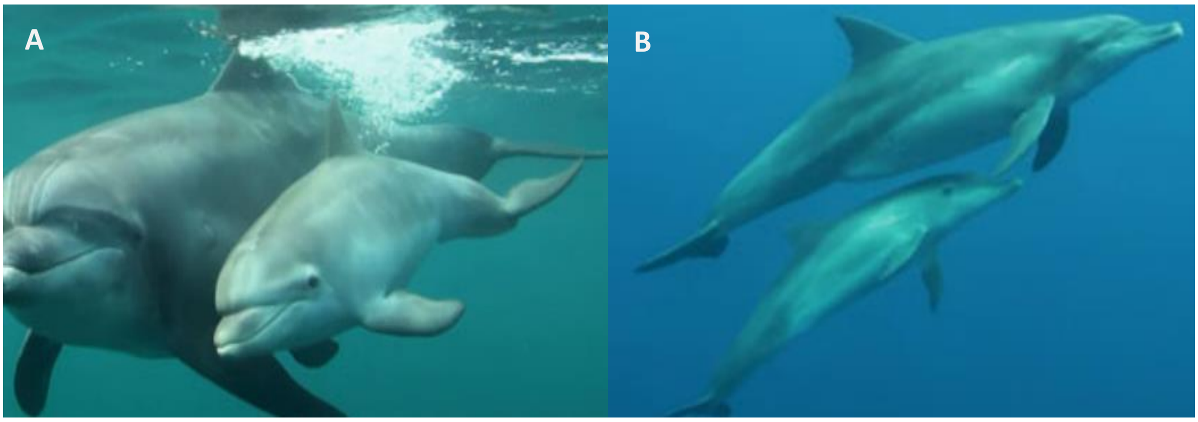


Fig. 4A- T. aduncus calf and mother dyad with the calf in echelon position 4B- T. aduncus calf in infant position below its mother (photos: M. Sakai and T. Morisaka). Source: Connor et al. (2019).

4. Bottlenose dolphins in human care facilities

Over the years, a growing number of countries began building and enforcing laws protecting dolphins, both in the wild and in captivity, to ensure their welfare and conservation (Wells 2009). AZA (Association of Zoos and Aquariums), EAAM (European Association for Aquatic Mammals), and AMMPA (Alliance of Marine Mammals Parks and Aquariums) are associations focused on maintaining a high level of animal care and strict legislative control of marine mammal facilities around the world.

The merging of long-term in-the-wild and dolphins in aquaria research provides a higher understanding of marine mammals biology and behaviour. Dolphins are under close supervision of staff responsible for providing the best care and a safe environment. Any uncommon behaviours, changes in group dynamics, and health-related problems are early recognized and subsequently managed (Moore et al. 2007; Waples and Gales 2002).

Dolphinariums usually include one larger outdoor pool or lagoon for performance shows or "swim with dolphin" programs, one or more holding pools for the training and movement of animals, and at least a pool for medical procedures, isolation, and maternity support, ideally equipped with a lifting platform (Rose et al. 2017; Shyan et al. 2002).

Facility design should meet species-specific requirements and physiological needs such as water and air temperature, salinity, lighting, humidity, and vertical air space controlled regularly. Optimal water parameters such as temperature must be maintained between 10ºC and 28ºC for the *Tursiops* genus, pH between 7.2 and 8.4, and salinity above 28 ppt, analysed at least daily (EAAM 2019).

5. Monitoring of ovulation

Monitoring the oestrus cycle and the timing of ovulation is critical for managing a captive population. Urinary LH and FSH, serum and urinary progesterone, cytology of the vaginal cells, and ultrasonography in conditioned dolphins enable the detection of this fleeting event (Muraco 2015).

Urinary LH is a valuable tool as it reduces the need for blood collection. The genital slit is cleaned with gauze, and the dolphin is conditioned to reflexive urinate by light pressuring the bladder with the hand (Muraco 2015). For the LH surge to be accurately detected, it requires analysis twice daily, since it lasts an average of 32 hours, peaking at approximately 24 hours pre-ovulation (Robeck et al. 2005). After pinpointing this event, the ovulation is confirmed by loss of pre-ovulatory follicle on transabdominal ultrasound (Muraco et al. 2004; Muraco 2015).

Vaginal cell cytology has proven to be an inexpensive and effective method to monitor oestrus. When oestrogen levels increase during oestrus, epithelial cells become superficial, cornified, and anuclear, often called “keratinized”. The female is in optimal breeding time at 80-100% cornification. The decrease in oestrogen and increase in progesterone that follows causes new, parabasal, and intermediate cells to appear (Muraco et al. 2004; Varela et al. 2007).

6. Pregnancy diagnosis

6.1. Progesterone Levels

Progesterone levels in serum, faeces, urine, and milk have enabled the detection of pregnancy in bottlenose dolphins under human care.

The most common method is serum analysis. Progesterone increases exponentially during the first month of pregnancy and peaks by week seven post-ovulation (O’Brien and Robeck 2012). A female exhibiting progesterone levels above 3 ng/ml is tested repeatedly and must sustain the rising levels of the hormone over six to eight weeks to confirm it is due to pregnancy and not to a cystic CL, or pseudopregnancy (Sawyer-Steffan et al. 1983).

P4 concentrations below 1 ng/ml belong to non-pregnant or in anoestrus females (Leatherwood and Reever 1989; Zhang et al. 2021).

Other alternative methods:

Progesterone concentration in faeces - The sampling method is minimally invasive. Samples are obtained by inserting a probe directly into the rectum to minimize contact with pool water. Results of faecal progesterone concentration in pregnant dolphin females are always >10pmol/g and demonstrate a similar pattern to serum progesterone (Biancani et al. 2009).

Progesterone concentration in urine - Urinary progesterone can be detected and analysed together with LH around ovulation time. At LH surge, average urinary progesterone is 0.27 ng/ml, reaching levels above 1.0 ng/ml by day eleven to seventeen post-ovulation, which confirms an active CL (Muraco 2015).

Progesterone concentration in milk – Dolphins can become pregnant while nursing a calf. When milk progesterone is greater than 8ng/ml, the female is pregnant, whereas females in anoestrus have concentrations below 1ng/ml (West et al. 2000).

6.2. Ultrasound assessment

In the 1980's, ultrasound gained widespread attention and relevance for being a non-invasive diagnostic tool for early detection of pregnancy in horses, cattle, sheep, dogs, and cats (Beck et al. 1990; Kähn 1992; Palmer and Driancourt 1980).

In 1987 the Seaworld California and Seaworld Florida described the results of a breeding program using ultrasound as one of the tools for early detection of pregnancy and monitoring of foetal development in the bottlenose dolphin (Cornell et al. 1987).

As a reliable non-invasive medical procedure, ultrasound assessments have become one of the most important tools for maintaining a healthy group of dolphins and an ongoing facility, as it provides immediate supplementary information on each individual.

A well-planned reproductive ultrasound routine is especially relevant in females, as it facilitates the confirmation of pregnancy (Hildebrandt and Saragusty 2014). Fetomaternal ultrasound monitoring helps in the early detection of pregnancy complications and in predicting a successful outcome or, on the contrary, a failed gestation or delivery (Ivančić et al. 2020).

Nowadays, ultrasonography description of the female and male reproductive organs is available, and prediction of the delivery date is possible through ultrasound measurements of the foetus (Brook 2001; Brook et al. 2000; Lacave et al. 2004).

7. Ultrasonography of the reproductive system

In a zoological setting, marine mammals are trained to cooperate voluntarily with medical examinations. Training the animals for ultrasound is now a routine constituent of husbandry and management of dolphins under human care. The particular behaviour of presenting the flank to the trainer is part of the training routine of most parks around the world. With consistent training, animals quickly get used to the ultrasound waves around them and begin to cooperate (Brook et al. 2001; Brook et al. 2000).

On the poolside, the animal adopts a lateral recumbency position floating on the surface of the water while supported by the trainer on the peduncle for stability during the assessment (Yuen et al. 2009) (Appendix 3). Because the procedure usually takes several minutes, interruptions and breaks of the position allow the animal to breathe.

The veterinarian and the equipment are well protected by water splashing and sunlight, some shade is usually provided. However, in case of emergency and impossibility in accessing the animal by voluntary behaviour, the procedure is performed outside water with due care and management (Saviano 2013).

7.1. Ultrasonographic description of the male reproductive organs

Ultrasound imaging has become a valuable practice in evaluating the reproductive health and sexual maturity of male bottlenose dolphins in captivity (Yuen et al. 2009).

To find the testicles, the transducer is positioned transversely at the level of the genital slit and then moved dorsally and cranially until they come into view as a rounded structure (Brook et al. 2001).

The longitudinal examination of the testicles enables measurements of the length, width, and evaluation of the testicular parenchyma. If the testicles are longer than the maximum width of the ultrasonographic field of view available the length is estimated by identifying both ends of the testicles on the screen and locating them on the skin surface (Appendix 4) (Brook et al. 2000; Yuen et al 2009). In longitudinal images, the testicles are large, elongated structures located caudoventrally to the kidneys (Yuen et al. 2009). It is surrounded by a tunicae visualized as a well demarked hyperechoic outline, bordering the testicular parenchyma. The testicular mediastinum is easily visible, as a marked hyperechoic line, which runs from side to side of the testicle in the centre of the parenchyma.

The bladder is visible when it is filled with urine as a round, anechoic structure located ventrally between the testicles serving as a reference (Brook 1997).

7.2. Ultrasonographic description of the female reproductive organs

The repeated identification of the ovaries and their structures in the same female over time provides an understanding of the typical shape and organization of the noncyclic and cycling ovary of that individual. It is, therefore, possible to make ultrasonographic descriptions of the normal female reproductive organs in their different stages (Robeck et al. 2001).

As soon as the animal is in position, the transducer is oriented transversely, placed on the abdomen above the middle of the anogenital slit, and moved dorsally until the transverse axis of the ovary is seen. They are rather superficial, located in the area of union between the lateral border of the rectus abdominus muscle (ram) and the rounded visceral surface of the hypaxialis lumborum muscle (hlm) which is noticed externally as an easily felt depression in the flank (Fig. 5). Once the ovary is found, the transducer should rotate 90º to obtain the longitudinal image. Small movements of the probe in the area help achieve the best possible image of the long axis of the ovary (Brook 2001).

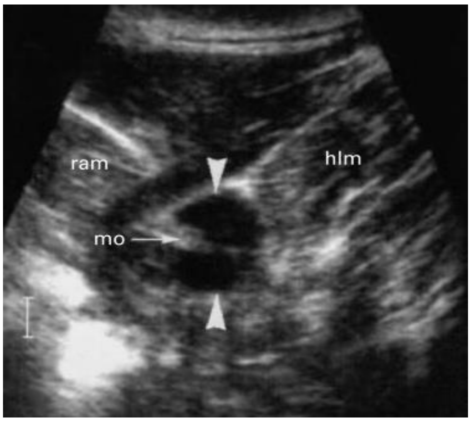


Fig. 5- Ultrasound image showing a cross-section of the left ovary in T. aduncus. Visualization of the ovary between the arrowheads lying between the rectus abdominus (ram) and hypaxial (hlm) muscles, and the insertion of the mesovarium (mo). Source: Brook (2001).

7.2.1. The ovaries

In longitudinal scanning, the ovaries have an almond shape with a more angular cranial end and a more rounded caudal end. The position can vary significantly, especially in females that have given birth. The ovaries are visualized anywhere from the end tip of the kidney to the level of the uterus (Brook et al. 2001).

Ovaries of reproductively active females are easy to identify, showing growing follicles, corpus luteum (CL), or persistent cystic structures. In females that are lactating, in anoestrus, or are sexually immature, the ovaries can be challenging to visualize.

By evaluating the ovarian aspect of *T. aduncus* mature females over time, it seems clear that ovaries get longer when females are cycling and shorten when they are in anoestrus. Brook (1997) also demonstrated that the left ovary was always either the same size or longer than the right ovary and that the length of the ovaries is strongly correlated to the body length.

Identifying the mesovarium is often the easiest way to locate the ovary due to its very particular appearance which resembles a “tram line”, two parallel echogenic lines in the hilar region of the ovary. The ovarian cortex is an ovoid structure with low-intensity echogenicity outlined by a hyperechoic line that represents the tunica albuginea.

In cross-section, the ovary has a rounded shape and a hyperechoic centre. The echogenicity and organization of the parenchyma vary between animals and the cycle moment. However, the cortex of older females tends to be more echogenic and less delineated than that of younger individuals. Images become less clear and sharp as females get older and heavier (Saviano 2013). The more echogenic appearance of the ovary in older females seems to result from accumulated fat and corpora albicantia (CA) from previous ovulations.

Small antral follicles are often seen present in cycling and non-cycling ovaries. A follicle that grows larger than 3 mm is classified as ‘developing’. The dominant follicle (DF), if it exists, is easily identified as completely anechoic, rounded, cyst-like structure (Fig. 6). Saviano (2013) described a variation of the preovulatory follicle diameter between 18mm and 28mm in the *T. truncatus*.

The CL develops posteriorly to ovulation, in the same place where this occurred, and is seen as a rounded, consistent mass, hypoechoic, isoechoic, or slightly hyperechoic in relation to the ovarian parenchyma. The diameters of the CL range from 24 mm to 39 mm.

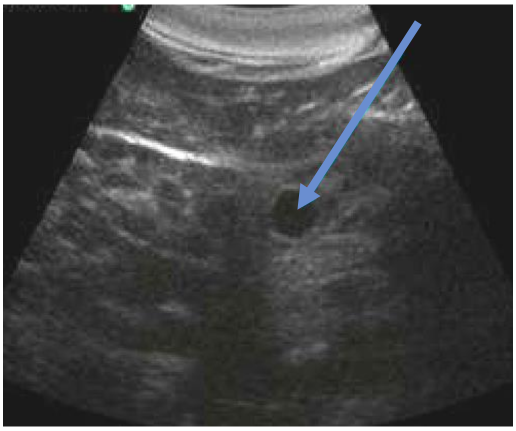


Fig. 6- Ultrasound image of the ovary and preovulatory follicle (arrow) in a *T. aduncus* female. Source: Courtesy of Dr. Ana Salbany, 2020.

The hypoechoic CL can be differentiated from a DF in that it is larger, has less defined and thicker margins, and has no posterior acoustic enhancement (Brook et al. 2002; Brook 1997). After fertilization, the CL that remains throughout pregnancy is hypoechoic, larger, and more organized than a CL from a non-conceptive cycle. It is better delimited and can sometimes cavitate. Identifying a CL from a conceptive cycle may allow for early diagnosis of pregnancy before the gestational sac is seen in the ultrasound.

It is possible to correlate the number of CA found in the ovary with the number of pregnancies. After birth, the CL from the conceptive cycle differentiates into a CA that remains throughout the female's life, which does not happen from CA of infertile ovulation (Brook et al. 2002).

7.2.2. The uterus

The bicornuate uterus is visualized as a curved and malleable s-shape structure with a homogeneous and hypoechoic echo pattern, usually not seen in its entirety due to the surrounding intestine. The transducer is positioned longitudinally above the genital slit, and the cervix is found dorsally on one side of the urinary bladder. From there, the probe must move cranially, and gentle dorsoventral movements of the transducer should enable the visualization of the uterine body. If there is mucus in the cervix canal, it may be possible to differentiate the pseudocervix and spermathecal recess (Brook et al. 2001).

After delivery, uterine involution should be followed by ultrasound to rule out the possibility of postpartum uterine infections. The observation of a dilated uterine lumen with heterogeneous content and different echo patterns is indicative of infection (Saviano 2013).

7.3. Pregnancy detection

Ultrasonography has allowed the dolphin facilities to follow the health of the mother and the foetus from the moment of pregnancy detection until the moment of delivery and make close predictions of the expected date of birth.

Pregnancy is diagnosed when the embryonic vesicle is visualized for the first time by ultrasound. According to Robeck et al. (2001) more than 68% of pregnancies in the bottlenose dolphin develop in the left uterine horn and result from ovulation from the left ovary. The embryonic sac can be first spotted 3 weeks after ovulation as an anechoic fluid-content rounded structure (Saviano 2013). Ivančić et al. (2020) identified the sac at approximately 29 days of gestation and the embryo in the following week (Fig. 7).

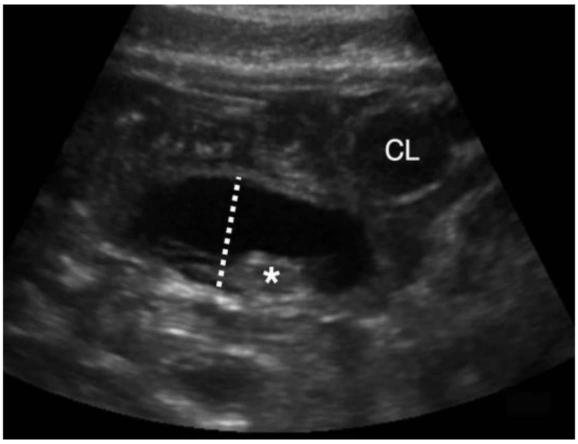


Fig. 7- Ultrasound image of early pregnancy in T. truncatus. Identification of the gestational sac, small pocket of anechoic uterine fluid. Dotted line represents the maximum fluid depth, * the embryo, and CL the corpus luteum. Source: Ivančić et al (2020).

7.4. Prenatal Development

The distinction between head and trunk is visible approximately 68 days post-ovulation, and at approximately 92 days, the diaphragm can be seen separating the abdomen from the thorax (Fig. 8A) (Saviano et al. 2020).

The umbilical cord is visible in cross-section, composed of 2 arteries and 2 veins, and the allantoic duct in the centre. It is observed clearly as a hyperechoic coiled structure at around 119 days of gestation, reaching lengths of 30cm to 40cm. Ivančić et al. (2020) consecutively measured the umbilical cord diameter near the foetal insertion and described a positive correlation with gestational age. The continuous evaluation of the umbilical cord throughout gestation, using colour flow and pulse doppler mode, is crucial to identify any malformation and flow deficiency (Sklansky et al. 2010) (Fig. 8B). Brook et al. (2007) reported 3 cases of *T. truncatus* females with excessively long umbilical cords wrapped around the peduncle in stillbirths.

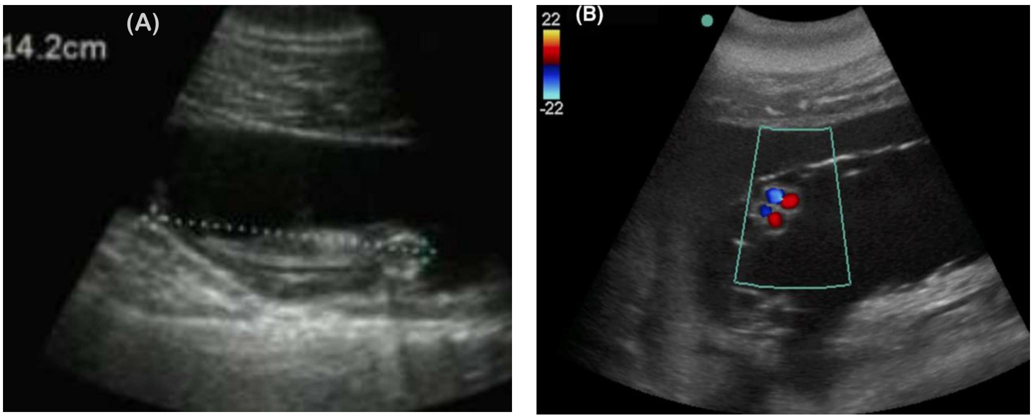


Fig. 8(A)- Ultrasound image of a T. truncatus foetus at 3 months of pregnancy. It is possible to distinguish the head, trunk, tail and spine. Source: Semenov et al. (2020). Fig. 8(B)- Transverse view of the umbilicus using Colour Doppler. The two blue vessels represent the umbilical veins and the two red vessels represent the umbilical arteries. Source: Ivančić et al (2020).

Saviano (2013) and Ivančić et al. (2020) observed heart movement at 68 days and 70 days post-ovulation. Heart rate (HR) is measured from the first time it is detected until parturition, showing a strong negative correlation with age. During the first trimester, HR is not easy to access. Until the ninth month of gestation, the HR is expected to stay between 155bpm and 198bpm, decreasing to 140bpm during the last trimester. In the final 2 weeks of pregnancy, the HR lowers to approximately 85bpm.

Ivančić et al. (2020) studied the relationship between foetal lung and liver echo patterns. Both parenchyma is clearly distinguished at approximately 112 days post-ovulation. The liver is homogeneously hypoechoic, presenting anechoic vessels with hyperechoic borders throughout the parenchyma. The lung parenchyma is homogeneous and about twice as echogenic as the liver. Decreased lung/liver echogenicity ratio indicates distress and bronchopulmonary irregularities in neonates (Ivančić et al. 2020; Saviano et al. 2020).

The urinary bladder and stomach are visible if filled with fluid as rounded, anechoic structures surrounded by a linear hyperechoic wall. They are seen for the first time at the beginning of the second trimester. The kidneys present the same shape as in adults and a similar homogeneous echogenicity of the parenchyma, bordered by a thin hyperechoic wall. Saviano et al. (2020) identified the kidneys for the first time 3 months before parturition.

The eye is visible as an anechoic cavity, and movement of the lens can be seen later in gestation. The ribs, the teeth, and the bent dorsal fin are identified around mid-pregnancy.

Genitalia is observed, and the sex of the foetus recognized. Males present a tri-lobed structure, whereas females show a rounded, apricot-like appearance (Ivančić et al. 2020) (Fig. 9).

The Amniotic and allantoic fluids develop different echogenicities as pregnancy progresses. The amniotic fluid appears hyperechoic with identifiable free-floating echogenic particles in the second half of pregnancy, while the second is distinguished as an anechoic fluid (Saviano et al. 2020; Ivančić et al. 2020). Ivančić et al. (2020) recorded a higher maximum depth of amniotic fluid than allantoic fluid, and demonstrated that the fluids have no correlation with gestation.

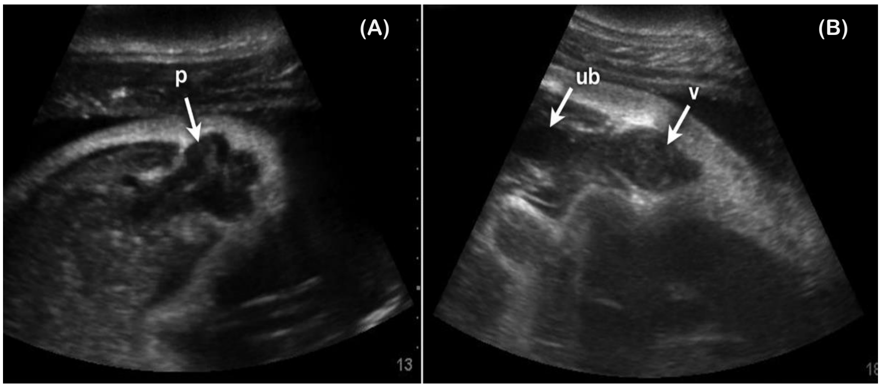


Fig. 9(A)- Male **foetal** ***genitalia, tri-lobed structure (p). Fig. 9 (B)- Female*** **foetal** ***genitalia, apricot appearance, showing the vagina (v) and urinary bladder (ub). Source: Ivančić et al. (2020).***

Foetus tail-first presentation at birth is the most common in *Tursiops* (Ivančić et al. 2020) (Appendix 5). The rudimentary pelvis and fusiform shape of cetaceans, the flattened placement of the dorsal fin and flippers, and the lateral u-shape position of the foetus before labour, seem to favour the sliding of the tail out of the vagina with tail emerging first (Fig. 10) (Reidenberg and Laitman 2009; Terasawa et al. 2021). However, records of successful deliveries of head foetal presentation, both in the wild and under human care, describe the cephalic presentation as a natural variation rather than a pathology although with increased risk of premature rupture of the umbilical cord and potential drowning of the calf (Muraco 2015; Saviano et al. 2020).

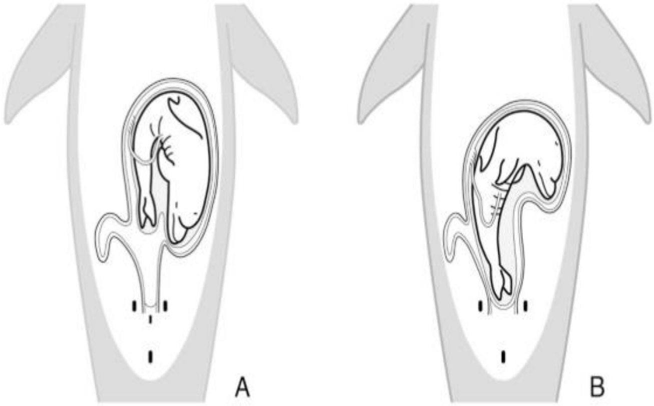


Fig. 10- Illustration of the* foetal *position in the uterus. A- Position adopted from 90 days pre-partum until parturition. B- Movement of the* foetus *at parturition with tail-first presentation. Source: Terasawa et al. (2021).

It is possible by observing the position of the foetus during ultrasound examinations of the last trimester to predict the presentation of the calf at birth. In the more common tail-first birth, the head and tail are directed caudally, but the beak is adjacent to the ovary at the tip of the uterine horn near the CL on the image (as shown further on in Fig. 13), while the fluke is oriented towards the cervix due to flexion of the uterine horn (Reidenberg and Laitman 2009; Saviano 2013). If, on the other hand, the tail fluke is seen near the CL, it is likely to result in a cephalic birth (Saviano et al. 2020).

7.4.1. Reproductive abnormalities and Foetal malformations

Brook (1994) identified a disproportionately small skull and a large cranial defect suggestive of anencephaly during an ultrasound evaluation of a *T. aduncus* pregnant female. The gestation resulted in a spontaneous delivery of a stillborn anencephalic male at 359 days. In 2013, the ultrasound assessment of *T. truncatus* female allowed the diagnosis in utero of an omphalocele and an umbilicus with 3 vessels (Smith et al. 2013). In 2000, Lacave et al. (2002) detected a twin gestation in a *T. truncatus* female by ultrasonography. The pregnancy was followed by ultrasound until abortion. Osborn et al. (2012) reported the only successful birth of a twin gestation in cetaceans in a beluga.

7.5. Foetal biometry and prediction of delivery date

Parturition can be predicted based on day of conception and in-utero foetal measurements. For an adequate and safe delivery protocol to be applied, it has become essential to estimate the date of birth by employing specific ultrasound foetal measurements.

The first reports of foetal biometry and growth curves of foetal structures for animals date back to 1975 in the rhesus monkeys, in which the pregnant females underwent regular ultrasound examinations to measure the biparietal diameter (BPD) of the foetuses throughout pregnancy (Sabbagha et al. 1975). After that, several studies were published for dogs, cats (Beck et al. 1990), horses, cattle, and other animals such as dolphins (Williamson et al. 1990; Kähn 1992).

Currently, the measurements that have shown linearity and proven to be good predictors of the delivery date at any time during pregnancy are the BPD and thoracic diameter (Fig. 11). Lacave et al. (2004) measured the foetus's BPD at the widest section where the *falx* is visible and the thoracic diameter at the heart level over 14 gestations in *T. truncatus* and 12 in *T. aduncus*. Several measurements were taken in each assessment and then analysed to determine the most accurate according to anatomical understanding. Those allowed the construction of equation models that used the BPD and thoracic diameter values to predict the birth date for each species. Comparing the predicted delivery dates to the actual dates of birth in both species, the BPD has proven to be a more accurate parameter in predicting the delivery date (Ivančić et al. 2020; Lacave et al. 2004).

Ivančić et al. (2020) showed that other fetal measurements such as the aorta diameter and blubber are easily obtained and accurate in predicting gestational age.

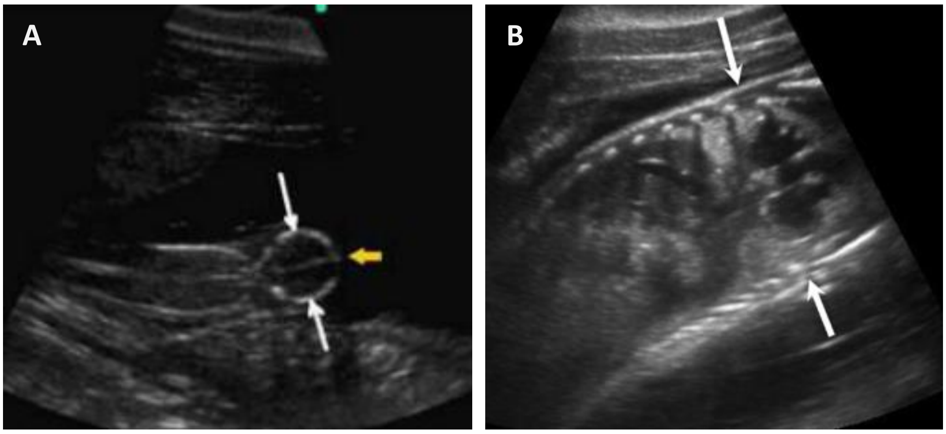


Fig. 11 A- Biparietal diameter in early pregnancy. Yellow arrow indicates the "falx". Source: Saviano (2013). B- Thoracic width (dorsal plane). Source: Ivančić et al. (2020).

8. Parturition under Human Care

The trainers and veterinarians follow a birth protocol for bottlenose dolphins in an aquaria context, intended to help with late pregnancy clinical decisions and delivery. Some required materials such as a scale, measuring tape, emergency medication box, a floating mattress, blood collection tools, a mammary breast pump adaptable to the dolphin anatomy, calf formula ingredients, gastric tubes, syringes for tube feeding, and a bottle for hand feeding, are prepared in advance (Baumgartner et al. 2018).

The pregnant females have a previous desensitizing training to adapt to the medical pool and the lifting floor movement.

8.1. Imminence of labour

Approximately 1 month from the expected delivery date, some signs of onset labour are observed and recorded. Observation shifts are organized, and the trainers keep record of (Biancani et al. 2021): General behaviour of the female (seeking physical contact with the trainers, restless attitude, and decreased appetite) (Blanchet et al. 2009); Frequency, and intensity of contractions and distensions (Bortolotto and Stanzani 1998); Vaginal discharges and rupture of the amniotic sac; Milk expulsion;

Ultrasound assessments must continue until birth to confirm foetal viability, position, and HR, which is expected to decrease progressively during the last month of pregnancy (Baumgartner et al. 2018).

8.1.1. Rectal temperature

Monitoring the rectal temperature twice daily for at least the last two weeks of pregnancy is a valuable predictor of the impending birth. The digital thermometer probe is inserted 10cm to 12cm into the rectum until the temperature stabilizes (Biancani et al. 2021). A drop of, on average, 1 ºC usually indicates the onset of labour within the next 12 to 24 hours (Blanchet et al. 2008; Katsumata 2010; Terasawa et al. 1999).

8.1.2. Intermammary distance

As in humans, prolactin levels increase as pregnancy progresses, causing the mammary gland of dolphins to enlarge in the last months of pregnancy. The mammary gland development can be followed by ultrasound of the region and the noticing of swelling of the genital area and an increase in the distance between the two nipples (Cornell et al. 1987). An increase in this distance is indicative of milk production and approaching parturition (Blanchet et al. 2009; Muraco 2015). According to Baumgartner et al. (2018), this method does not bring great value in detecting the approaching birth. During the last month of pregnancy, the intermammary distance, although slightly larger than in the previous stages of gestation, did not variate significantly (Baumgartner et al. 2018). Intermammary gland distance is measured using a ruler, considering the cranial or middle point of the mammary gland slit (Biancani et al. 2021).

8.2. Delivery

Normal labour should last anytime between 20 min to 2 hours (Schroeder 1990). In a tail-first birth, flukes emerge from the female genital slit for a few minutes to hours before complete delivery. If birth is taking longer than expected, intervention is indicated, and the veterinary team proceeds with ultrasonographic exams to check the viability of the calf. When applicable, the administration of oxytocin is used to promote labour progression (Lacave 1991).

Any complications such as placental retention or foetal death need treatment, which usually involves drug administration of oxytocin and $PGF_{2\alpha}$, intra-uterine washes, frequent blood samples, and ultrasound assessments to monitor the female and control a possible infection. Sometimes, it is necessary to intervene by manually extracting the deceased calf. Material for fetotomy should also be available for critical cases (Baumgartner et al. 2018).

8.3. Perinatal period

The highest rate loss of *Tursiops* occurs in the age category of calves up to 30 days postpartum. Close monitoring of the mother and calf after birth reveals good or poor development of the neonate and recovery of the mother, and is the basis for any clinical decisions (Biancani et al. 2021).

8.3.1. The mother

The mother continues to experience powerful contractions and distensions that cease after the placenta gets expelled. The placenta should be delivered in the first six hours after birth and collected for analyses in the laboratory. In case of incomplete delivery of the placenta, oxytocin injection is in order.

The mother’s food intake should increase by about 50% of the typical baseline consumption for maintenance so that the calf's nutrition is not compromised. The applied strategy for an increase in food consumption is a 24h on-demand diet (Baumgartner et al. 2018).

8.3.2. The calf

The wobbly dorsal fins, tail flukes, and pectorals become rigid within a few hours post-parturition. The distinct foetal folds/lines around the calf's midsection, from the curled position in the uterus, are an excellent visual indicator of neonate age, development, and health. *T. aduncus* can present these marks for up to 3 months (Mann 2019) (Appendix 6).

The nursing parameters are the most valuable information for the calf health assessment. The first suckling attempt is expected within the first 8 hours post-parturition. The 24-hour post-partum mark is critical, after which nutritional intervention through milk formula hand or tube feeding (Annex 1) for a calf that has not started nursing is required. A record sheet is created to monitor the nursing frequency, duration, and cumulative seconds of nursing per hour for at least the first two weeks of life. The resulting nursing performance curve enables the assessment of the calf's development and to decide on further medical procedures (Sweeney et al. 2010).

Attention to weight gain and body conformation is essential. There is an anticipated weight loss in the first couple of days. In the case of a calf does not fill in the peduncle, abdominal areas, and the "peanut head" appearance between the seventh and tenth day after birth, medical intervention is highly considered (Sweeney et al. 2010).

Another indicator of poor calf development is related to skin phasing. At birth the skin is dark grey, gradually changing to light grey dorsally and pink/white ventrally. Typically, it is completed by day fourteenth, but water temperature can influence the mottling progression.

Evaluating the calf's respiratory pattern is an integrated part of monitoring methodologies. Calf breaths are counted for 5-minute periods. The expected respiratory rate is 12 to 25 respirations over 5 min. The calf and mother, while swimming together, are expected to match breathing patterns. The observation of a neonate persistently on the surface strongly suggests poor health (Sweeney et al. 2010).

The introduction of calves to solid food starts earlier than in nature. Muraco (2015) reported the beginning of fish ingestion by captive dolphin calves at approximately six months old as a supplement to nursing and weaning occurring from 18 months post-partum (Peddemors et al. 1992; Ridgway and Benirschke 1977). However, nursing in *T. truncatus* has been observed in animals aged seven and over, and gastric samples reveal that some calves occasionally nurse until much later (Wells and Scott 1999).

III – PREGNANCY MONITORING IN THE INDO-PACIFIC BOTTLENOSE DOLPHIN (*Tursiops aduncus*) AND PREDICTION OF DELIVERY DATE WITH EMPHASIS ON FETAL BIOMETRY

1. Aims of the study

The arrival of a new dolphin calf to a group leads to social changes in the population. Males may exhibit aggressive behaviour towards calves, and females may attempt to steal neonates from their mothers (Lacave unpublished observations, Brasseur et al. 1998).

Assessing the accuracy of commonly used methods for long- and short-term prediction of parturition in conditioned bottlenose dolphins is crucial for the best pregnancy monitoring protocols to be applied.

At Dolphin Bay, predicting the delivery date enables the management and preparation of the medical and training staff in advance for a safe and successful pregnancy, delivery, and development of calves born.

The main objectives of the present study are:

- **Within the scope of foetal ultrasound**
  - ✓ To diagnose pregnancy in five *T. aduncus* females through ultrasound;
  - ✓ To ultrasonographic monitor and describe the foetal development in five *T. aduncus* gestations;
  - ✓ To predict the delivery date from the obtained foetal biometrics (biparietal and thoracic diameters) of five pregnant *T. aduncus* females;
  - ✓ To test the accuracy of the delivery prediction equation models used;
  - ✓ To test which of the two foetal measurements gives the most accurate delivery predictions;
  - ✓ To test which gestational period foetal measurements provide the most accurate delivery predictions;

- **Within the scope of physical and physiological examinations**
  - ✓ To record the rectal temperature and intermammary distance and assess the value of the two methods in estimating an approaching delivery;

2. Material and Methods

2.1. Dolphin Bay, Atlantis the Palm, Dubai

Dolphin Bay is the habitat of a group of Indo-Pacific bottlenose dolphins located in the resort Atlantis the Palm, Dubai.

In 2007, Dolphin Bay welcomed a pod of 28 *T. aduncus* from the Solomon Islands. It was the first facility in the Middle East to offer “swimming with dolphin” programs while taking as primary interest the welfare and protection of its animals and the conservation of the species.

The dolphin area installations comprise 4.5 hectares, including seven interconnected indoor pools and three exterior interactive lagoons.

The water system of the facility is semi-closed. Fresh seawater is supplied to Dolphin bay through 1 of 3 intake pipes located outside and inside the breakwater surrounding Palm Jumeirah Island. There are also storage tanks for seawater that undergoes treatment and where the water circulates and is mixed with fresh seawater. The turnover rate for the entire water volume in the dolphin area is 3.5 hours.

The temperature, turbidity, salinity, pH, free and total chlorine, ORP, and HBr levels are monitored every day by the water quality staff, and microbiological analyses are performed once weekly at various points around the dolphin area. Monitoring of air, sand, soil, and surface quality and hygiene is done in-house or resorting to external laboratories if needed.

Atlantis, The Palm is a member of WAZA and is currently AZA-accredited. To maintain this high-level animal welfare status, it must go through the entire demanding accreditation process every five years (AZA 2022).

At Dolphin bay, the veterinary team, multilevel staff trainers, water quality and diving team, and the general maintenance staff are responsible for the marine mammals’ daily husbandry. The dolphins are cared for under preventive medicine protocols, which include: daily monitoring of food and hydration; daily observation and report of behaviour; individual medication and supplements; blood, faeces, gastric, and blowhole sampling for analyses, and cytology; ultrasound and assessment of physical parameters, among others. These procedures are routinely scheduled, and weekly veterinary reports are elaborated.

The species fed to the animals are whole frozen thawed capelin, squid, and smelt. Akwavit dolphin supplement is specially developed for dolphins to provide vitamins and minerals depleted in frozen fish. Each dolphin has a specific diet according to age, weight, health and physiological status, and general characteristics

2.2. Studied population

The Indo-Pacific bottlenose dolphins included in this study consist of five pregnant Dolphin bay females assessed by serial voluntary transabdominal ultrasonography, physical, and physiological examinations.

The females will be identified as Females 1 to 5, with Female 1 being the first to deliver and Female 5 being the last. For ease of reference, females may be referred to as F1 to F5 throughout this document.

All females were wild born. Their estimated age at parturition was 30, 17, 17, 37, and 13 years old for females 1, 2, 3, 4, and 5, respectively. They all gave birth between February and May 2019.

Females 1, 4, and 5 have had successful births before, whereas females 2 and 3 were primiparous. At the time of arrival at the facility in 2007, females 2,3, and 5 were sexually immature. No history of disease or past complications was known for any of the mothers.

As part of the protocol, the five females were supplemented with lactobacillus and received an extra vitamin B complex with folic acid supplement starting from the time of pregnancy diagnosis. Females were monitored throughout gestation and maintained their regular daily routine until the final stages of pregnancy.

The facility has two pools designated as maternity pools, located within good visual reach for staff and direct access to the medical pool, which is equipped with a lifting floor used for deliveries, neonatal interventions, or emergencies.

2.3. Ultrasonography techniques

The ultrasound routine carried out at Dolphin Bay allows for the identification and monitoring of estrus cycles without them going unnoticed. Ultrasound assessments are routinely scheduled every two weeks for all immature or under-contraception females. When the females are cycling, ultrasound exams become more frequent, at least once a week, to assess the overall health of the reproductive tract, closely follow follicular growth and anticipate the moment of ovulation.

When ovulation is observed or suspected and a CL is detected, and there is a possibility that male-female interaction may have occurred prior to CL identification, the female is evaluated attentively for pregnancy diagnosis.

Dolphins adopt a lateral position for examination under operant conditioning training, and scanning is performed using the pool water for acoustic coupling. However, working with animals requires adaptability. It is not always possible to comply with the schedule if, for some reason, the animal does not cooperate. When the animal did not present the behaviour for ultrasound assessment, the procedure was paused briefly and then repeated. After a few failed attempts, the exam was scheduled for the upcoming days.

The assessments were conducted in the morning as early as possible. As the day progresses, the gastrointestinal tract becomes filled with faeces, interfering with and making it difficult to see other abdominal structures (personal communications from Salbany, 2019).

2.3.1. Equipment

The ultrasound examinations were performed by two experienced veterinary doctors individually or together using a MyLab™DeltaVET device from Esaote®, with the convex probe AC2541 VET (Fig. 12A). The images were digitally captured and saved in the internal digital memory of the device before being transferred to a computer folder. This machine is portable, easy to clean, and splashproof, which is advantageous to the dolphin facility scenario.

The ultrasound machine was placed inside a transportable black plastic box near the poolside where the animal was (Fig. 12B). The purpose of the box is to cover the device protecting it from accidental contact with water and sunlight during the procedure.

Fig. 12A- Ultrasound machine used by the vet team at Dolphin Bay. Fig. 12B- Black transportable box used during ultrasound assessments at Dolphin Bay. Original photographs courtesy of Dra. Ana Salbany, 2020.

2.3.2. Pregnancy monitoring

Pregnancy monitoring begins from the moment of first-time identification of the embryonic vesicle. When pregnancy was confirmed, routine ultrasound monitoring became approximately once a week for all pregnant females.

The females stationed in lateral recumbency floating by the poolside with the trainer supporting the peduncle for stabilization, similar to what is described by Brook et al. (2001).

The ovary was found placing the transducer longitudinal to the body, using the tip of the dorsal fin as a reference. A slight depression of the body was easily seen and felt where the transducer should be placed.

The operator proceeded with the general observation of the foetus, body movements, position, and internal organs. The first visualization of the gestational sac and the embryo was recorded. The CL was also identified, and longitudinal and transversal diameters were measured whenever they could be found in the ultrasound assessment (Fig. 13). Heart movement was monitored, and HR assessed using M-mode. Maximum and minimum HR values were registered and three gestational periods (≤9 months, >9 months, and the last two weeks of pregnancy) were created to assess HR evolution. Foetal genitalia recognition and the first observation of the foetus in the curved (U-shaped) position were also recorded. The first identification of these structures was described in days until delivery.

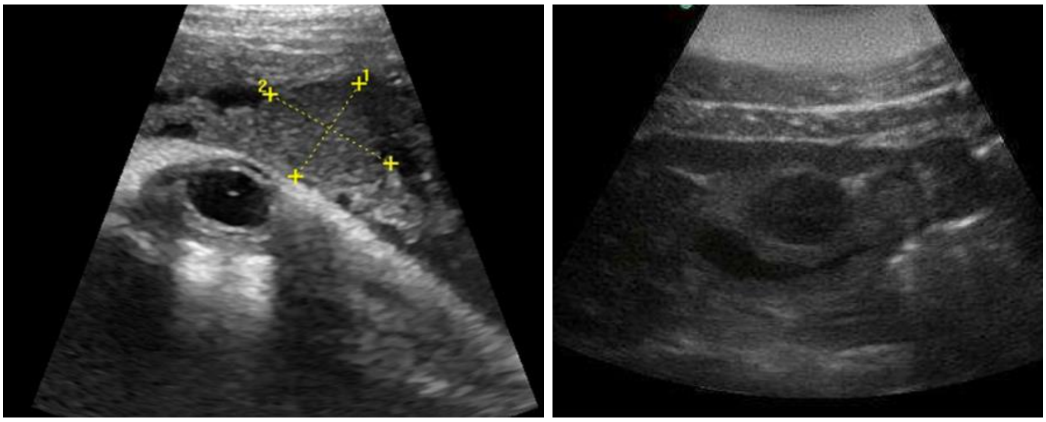


Fig. 13- Ultrasound image of a T. truncatus foetal head (the eye is visible) and CL measurements. Tail-first presentation at birth is expected (on the left). Source: Saviano et al. (2020). Ultrasound image showing a CL of a T. aduncus female (on the right). Source: Courtesy of Dr. Ana Salbany, 2020.

The images in which the embryo/foetal head and thorax were best visualized were saved and subjected to a more rigorous assessment after the medical procedure. Whenever possible, ultrasound cine loops of relevant fetomaternal structures were recorded to minimise the duration of the exame.

Foetal measurements were performed from the first possible assessment when the foetal head and thorax were clearly differentiated until the foetus became too large for reliable diameters to be obtained. The head is visible as a symmetrical ovoid, and the biparietal diameter was measured at the level where the midline echo of the *falx cerebri* between the parietal bones was seen and at the widest section (Fig. 14A and 14C).

In Dolphin Bay, one calliper is placed at the outer edge of the hyperechoic line and the inner edge of the hyperechoic line of the opposite parietal bone. The thoracic diameter was measured using the same technique, at the widest thoracic level, which is the level of the heart (Fig. 14B and 14C).

Regarding these measurements, different authors refer to distinct techniques (Ivančic et al. 2020; Saviano 2013; Semenov et al. 2020; Williamson et al. 1990). Accuracy of results requires consistency in the technique used.

Errors and variability between the two operators are possible but seen as minimal considering their experience, shared method, and collaborative work in evaluating the scans.

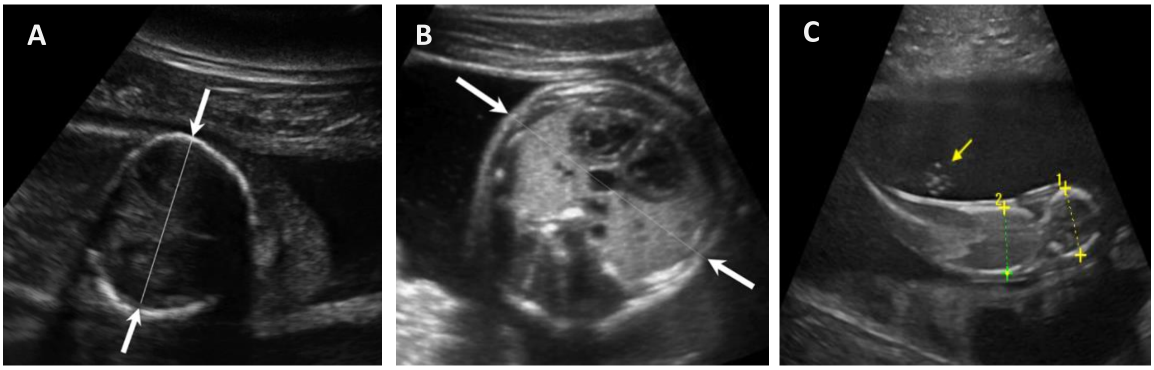


Fig. 14A- Ultrasound image showing the foetal biparietal diameter measurement (dorsal plane). Fig. 14B- Ultrasound image of the foetal thoracic width (transverse). Source: Adapted from Ivančić et al (2020). Fig. 14C - Early gestation ultrasound image. Clear distinction of the head and thorax and measurement of biparietal and thoracic diameters. Identification of the umbilical cord (yellow arrow). Source: Adapted from Saviano et al. (2020).

2.4. Prepartum approach

Close observation of the pregnant females began in the last month of pregnancy.

An observation sheet was created for every female for the purpose of monitoring the last stages of pregnancy. These sheets were completed at each moment by the assigned marine mammal trainers and included: contractions and distensions, urine and defecation, respirations, swim patterns, unusual behaviour, and unusual secretions.

Two weeks before the predicted date of birth the protocol is narrowed:

- ✓ Twenty-four-hour continuous observations;
- ✓ Daily ultrasound assessments;
- ✓ Rectal temperature twice a day, morning and evening until parturition;
- ✓ Intermammary distance twice a day, morning and evening until parturition;

The staff looked attentively for signs of approaching labour, such as the descent of rectal temperature, the increase of intermammary distance, anorexia, loss of liquid, mucous, or membranes, milk expulsion, and shift of position head/tail in the ultrasound (Semenov et al. 2020).

For the rectal temperature, the female adopted a dorsal decubitus position at the poolside while supported by the trainer at the peduncle. The veterinarian used a digital thermometer connected to a flexible probe. The probe end was covered with lubricant, inserted into the anal orifice of the female about 10cm, and kept in place until the temperature value stabilized on the device's panel.

For the intermammary distance measurement, the female adopted the same position (belly-up) as for rectal temperature. The veterinarian used a translucent ruler, which enables the view of the mammary surface through it. The measurement was performed in the middle of the mammary slit (Fig. 15). The general appearance of the mammae was noted, as well as the presence or absence of milk or abnormal secretions near the nipples.

Rectal temperatures and intermammary distance were obtained in all five females.

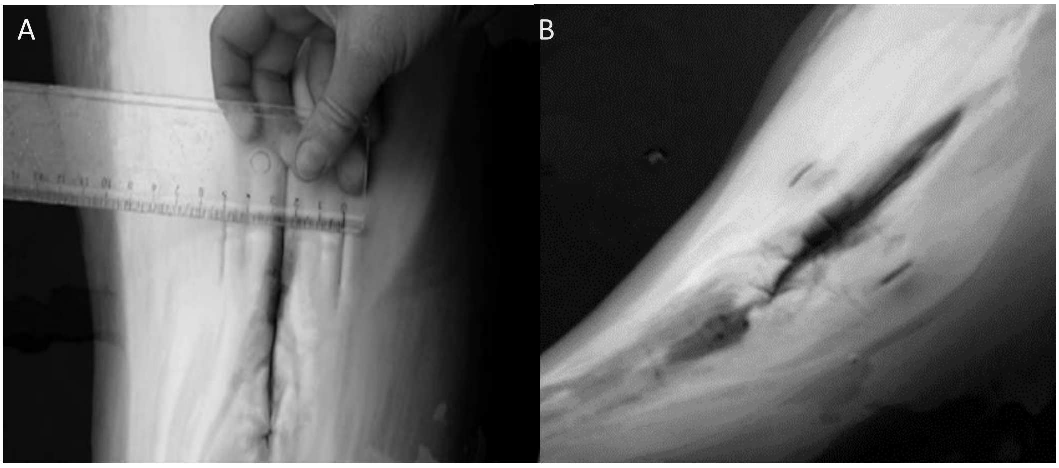


Fig. 15 (A)- Measurement of Intermammary distance in a female harbour porpoise. Intermammary distance is 5cm. (B)- Genital slit of a female harbour porpoise two days before parturition. Source: Blanchet et al. (2008).

2.5. Prediction of the delivery date

To obtain the predicted delivery date, it was applied the formula created by Lacave et al. (2004) for the *T. aduncus* species. The author provided us with the Excel program sheet that performs the estimated delivery date calculation by entering the ultrasound assessment date and respective biparietal and thoracic diameters in millimetres on the chart.

The specific equations for *T. aduncus* used data from seven pregnancies at first, and five further pregnancies helped update and validate the delivery prediction program. In total, 97 measurements of the biparietal diameter and 97 measurements of the thoracic diameter were used for the development of the *T. aduncus* formula (Lacave et al. 2004).

Using the referred equation program, it was possible to predict the delivery date for the five pregnant females in our studied population. For assessment dates where more than one biparietal or thoracic diameter was obtained, the average value was determined and entered into the delivery date prediction table.

The *T. aduncus* equation model created by Lacave et al. (2004) is the following:

- **Biparietal diameter (mm)** = 0,411 x (days before parturition) + 135,612 ($R2=0.979$; $P<0.0001$)
- **Thorax diameter (mm)** = 0,474 x (days before parturition) + 155,079 ($R2=0.971$; $P<0.0001$)

2.6. Statistical analysis

The data were entered and stored into Microsoft Excel 2019 database, where all graphs were made.

Descriptive statistics, including mean and standard deviation, are presented as $\overline{X} \pm SD$. Mean days until parturition, considering the actual birth date of each female, were obtained for first-time identification and detection of the gestational sac, embryo, foetal HR, and curved position of the foetus in the uterus. Mean minimum and maximum CL transversal and longitudinal diameters were calculated.

The values were analysed by linear regression to assess the relationship between days until parturition and foetal HR, biparietal and thoracic measurements, rectal temperature, and intermammary distance.

For a dolphin facility, time management is crucial, and therefore reducing the period in which foetal measurements are performed is an added value. In this regard, we divided the gestation into three periods in an attempt to determine in which of them the foetal diameters provided more accurate delivery date predictions. This time frames were determined assuming a gestation of 371 days, which is the average gestation length recorded by Brook (unpublished observations) for the *T. aduncus* population from Ocean Park, Hong Kong, where the delivery prediction formula originated (Lacave et al 2004), and biparietal and thoracic measurements were divided based on each female's actual parturition date (<4 months, 4 to 8 months, and > 8 months). The same was accomplished for HR, but by splitting the gestation into ≤ to 9 months, > 9 months, and the last two weeks of pregnancy as mentioned above.

Descriptive statistics, including mean and standard deviation ($\overline{X}$±SD), minimum and maximum values were calculated for HR, temperature, and intermammary distance.

Statistical analysis was done using SPSS statistics, version 29.0. Data were checked for normality using the Shapiro-Wilk test. Subsequently, the parameters were tested for the non-parametric Wilcoxon signed-ranks paired test. HR was checked for correlation. A significance level of 5% was used for all analyses.

3. Results

3.1. Pregnancy detection and development

The detection of the embryonic sac in the population studied occurred at 314 ± 7 days before parturition, and identification of the embryo happened approximately one week later at 306 ± 10 days before delivery. The days until parturition from the first sighting of the embryonic sac and embryo (Fig. 16) are detailed in table 1.

FEMALE	embryonic sac (days until parturition)	Embryo (days until parturition)
F1	310	303
F2	309	304
F3	318	318
F4	306	290
F5	326	313

Table 1– First-time ultrasonographic identification of the embryonic sac and embryo in the five *T. aduncus* females.

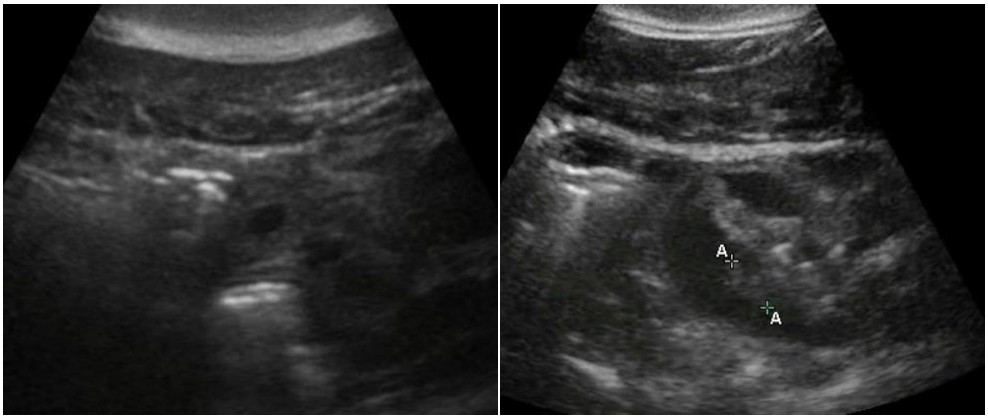


Fig. 16- First-time ultrasonographic identification of the embryonic sac (on the left) and embryo (on the right) in the female 1. Source: Courtesy of Dr Ana Salbany, 2019.

The size of the CL was determined by measuring the two furthest points from right to left and from top to bottom. The CL size varied between 2.08cm and 2.82cm for transversal diameter and between 2.11cm and 3.76cm for longitudinal diameter. Average minimum and maximum transversal and longitudinal CL diameters (N=40) are presented in Table 2.

Female (N=40)	CL transversal diameter (cm)		CL longitudinal diameter (cm)	
	Min.	**Max.**	**Min.**	**Max.**
F1 (11)	2.16	2.68	2.44	2.98
F2 (10)	2.41	2.82	2.61	3.00
F3 (9)	**2.08**	2.32	2.28	2.69
F4 (4)	2.10	2.29	2.11	2.88
F5 (6)	2.68	2.82	2.98	**3.76**
$\bar{X}$	***2.29***	***2.59***	***2.48***	***3.06***
SD	0.23	0.24	0.30	0.37

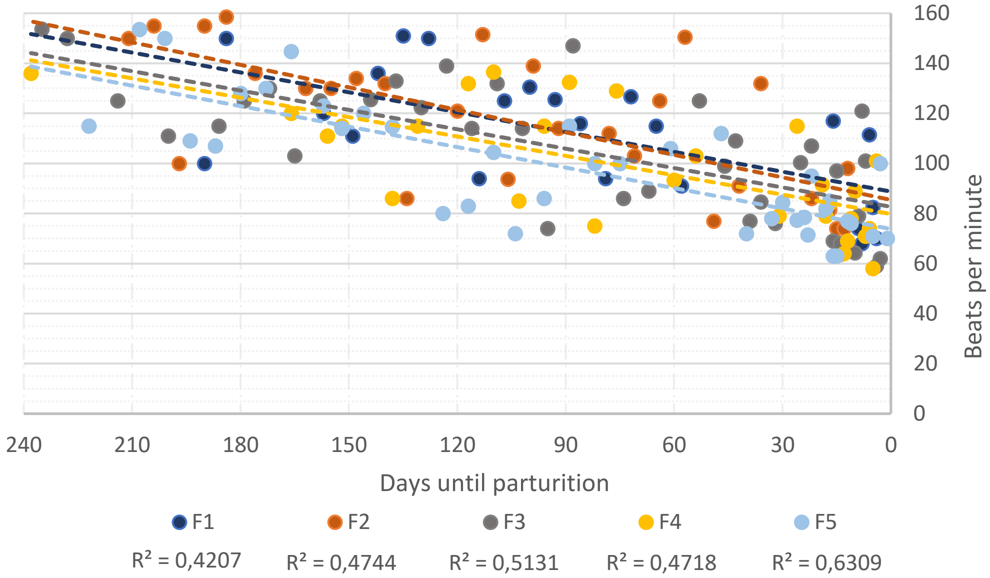
Table 2- CL minimum, maximum, mean and standard deviation diameters throughout gestation of the five T. aduncus females.

The first-time measurement of HR occurred between 190 days and 238 days before delivery date, 219 ± 17 (Table 3).

FEMALE	HR (days until parturition)	HR (bpm)
F1	190	100
F2	211	150
F3	235	158
F4	238	136
F5	222	115
$\bar{X} \pm SD$	219 ± 17	132 ± 22

Table 3– First-time detection of Heart rate of the foetus in the five pregnant females in days until parturition and corresponding HR values in bpm.

Foetal HR showed a negative regression trend with gestational age (Graph. 1) a correlation value of 0.7 (p-value< 0.001), and was not normally distributed (0.963, sig<0.001). The measured values (n=246) ranged from 167 bpm and 58 bpm during gestation with a reported mean of 106.89 ± 28.37 bpm.



Graph. 1- Scatter plot demonstrating the relationship between foetal HR and days until parturition in the five gestations.

Up to the 9th month of pregnancy, HR remained between 72 bpm and 167 bpm (average interval 82 bpm and 161 bpm). During the last trimester, the lowest and highest HR obtained were 62 bpm and 156 bpm (average interval 71 bpm and 146 bpm), respectively. In the last two weeks before birth, HR values varied between 58 bpm and 121 bpm (average value 61 bpm and 108 bpm). There was a progressive decline during the course of pregnancy (Table 4).

FEMALE	N (246)	≤ 9 months gestation			> 9 months gestation			Last 2 weeks of gestation		
		Min.	*Max.*	*n*	*Min.*	*Max.*	*n*	*Min.*	*Max.*	*n*
F1	38	94	167	18	91	150	10	65	117	10
F2	49	86	167	26	62	156	13	60	100	10
F3	62	74	167	24	64	150	27	59	121	11
F4	36	85	144	12	75	136	16	58	101	8
F5	61	72	157	30	63	136	23	63	100	8
$\bar{X}$±SD		82 ± 8.16	160.4 ± 8.28		71 ± 11.05	145.6 ± 8.14		61 ± 2.61	107.8 ± 9.24	

Table 4- Foetal HR levels during pregnancy. Minimum and maximum until 9 months of gestation, after 9 months of gestation, and in the last 2 weeks of gestation.

Foetus genitalia was recognized in two of the five pregnancies. The foetus from Female 1 was identified as a Male, 218 days before delivery, and the foetus from Female 3 as also a Male, 249 days before delivery (234 ± 16).

The foetal movement was monitored throughout gestation, and its adoption of the curved position was registered for all females. The foetus was folded at 104 ± 9 days before delivery. From females 1 through 5 was seen folded for the first time at 114, 92, 109, 96, and 110 days before parturition, respectively. Table 5 assembles the organogenesis timeline.

First-time identification	Days until parturition
Embryonic vesicle	314 ± 7
Embryo	306 ± 10
Genitalia (N=2)	234 ± 16
Heart rate	219 ± 17
Folded position (u-shape)	104 ± 9

Table 5- First-time identification of fetal structures in the Indo-Pacific bottlenose dolphin (Tursiops aduncus) studied population.

3.2. Prediction of delivery date

This study included 110 ultrasound assessments. However, it was not always possible to obtain both foetal measurements. A total of 98 values of biparietal diameters and 88 thoracic diameters were analysed.

The actual and predicted delivery dates from biparietal and thoracic diameters for each female are demonstrated in table 6. The interval of days between the actual and predicted delivery date was also determined.

Female	***Actual delivery date***	***Biparietal diameter***				***Thoracic diameter***			
		N=98	PDD	SD (days)	≠ (days)	N=88	PDD	SD (days)	≠ (days)
F1	06/02/2019	19	**19/02/19**	8	*13*	16	**22/02/19**	10	*16*
F2	15/05/2019	20	**28/05/19**	8	*13*	18	**28/05/19**	9	*13*
F3	17/05/2019	20	**21/05/19**	7	*4*	19	**05/06/19**	16	*19*
F4	20/05/2019	18	**01/06/19**	6	*12*	15	**05/06/19**	7	*16*
F5	25/05/2019	20	**24/05/19**	9	*1*	19	**29/05/19**	9	*4*
≠ x̄ ± SD					***8.6 ± 5.1***				***13.6 ± 5.2***

Table 6- Actual and predicted delivery dates (SD) from biparietal and thoracic diameters for each female. ≠ is the day difference between the actual and predicted delivery dates. Mean day difference was determined for the two measurements (≠ $\bar{x}$ ± SD).

Two scans of the thoracic and biparietal diameters obtained during examinations of female 1 are presented in Fig. 17. Table 7 shows, in detail, all the ultrasound assessments performed and respective diameters obtained for female 1. This chart was adapted from Lacave et al. (2004). The tables referring to the other four females are presented in the annexes of this dissertation (annex 2).

Prediction of delivery date in *Tursiops aduncus*						
FEMALE 1						
US DATE	Diameter (mm)		Based on the Head		Based on Thorax	
	Biparietal	**Thoracic**	Days before birth	**PDD**	Days before birth	**PDD**
09/04/2018	10	10	303	**06/02/2019**	303	**05/02/2019**
23/04/2018	12,6	14,3	297	**13/02/2019**	294	**10/02/2019**
30/04/2018	11,3	14,8	300	**23/02/2019**	293	**16/02/2019**
08/05/2018	19,1	18,2	281	**13/02/2019**	286	**17/02/2019**
14/05/2018	18	19,8	284	**21/02/2019**	283	**20/02/2019**
28/05/2018		25,9			270	**22/02/2019**
05/06/2018	28,7667	30,5333	258	**18/02/2019**	261	**20/02/2019**
11/06/2018	32,4	30,6	250	**15/02/2019**	260	**26/02/2019**
18/06/2018	33,8	35,55	246	**19/02/2019**	250	**23/02/2019**
25/06/2018	36,1	36,1	241	**20/02/2019**	249	**01/03/2019**
03/07/2018		51,2			218	**06/02/2019**
09/07/2018	43,8	47,2	223	**16/02/2019**	226	**20/02/2019**
31/07/2018	53,4	51,8	200	**15/02/2019**	217	**04/03/2019**
06/08/2018	54,7	55,2	197	**18/02/2019**	210	**03/03/2019**
28/08/2018	61,8		180	**23/02/2019**		
02/09/2018	71,9	71,5	156	**04/02/2019**	177	**25/02/2019**
24/09/2018	68,8	79	163	**05/03/2019**	161	**04/03/2019**
15/10/2018	86		122	**14/02/2019**		
12/11/2018	98		93	**13/02/2019**		
19/11/2018	90,5	100	111	**10/03/2019**	118	**17/03/2019**
03/12/2018	105		77	**17/02/2019**		
10/12/2018	102,5		83	**02/03/2019**		
PDD				**19/02/2019**		**22/02/2019**
SD				8 days		10 days
ACTUAL DELIVERY DATE			*6th of February 2019*			

Table 7- Partus prediction for T. aduncus female 1 based on biparietal and thoracic diameters using Lacave et al. (2004) equation chart.

For female 1 the PDD calculated from the biparietal and thoracic diameters of the first assessment (09/04/2018) was 6 February and 5 February 2019, respectively. The PDD from the thoracic diameter of 03/07/2018 was also the actual date of delivery. These were the closest predictions to the actual date of birth.

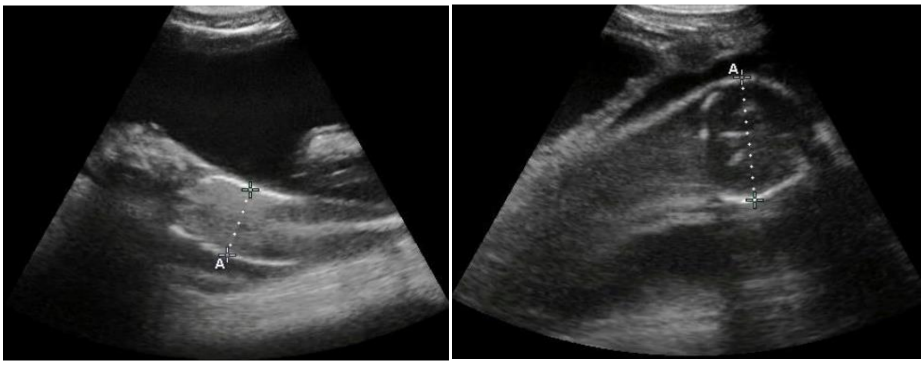


Fig. 17- Measurement of the foetal thoracic diameter at 184 days before delivery (left image) and biparietal diameter at 157 days before delivery (right image) of the female 1. Source: Courtesy of Dr. Ana Salbany, 2019

The 110 assessments were split into three gestational periods: less than 4 months (*n=57*), between 4 and 8 months (*n=101*), and over 8 months of gestation (*n=28*). An average predicted delivery date was obtained from the two measurements for each of these periods in all the females. The day difference between the actual birth date and each mean predicted delivery date was determined (table 8).

Female	Real birth date	Scanning period (months)	N	PDD Biparietal diameter	$\neq \bar{X}$	N	PDD Thoracic diameter	$\neq \bar{X}$
F1	06/02/2019	<4	5	15/02/2019	*9*	6	15/02/2019	*9*
		4 to 8	10	18/02/2019	*12*	10	24/02/2019	*18*
		>8	5	22/02/2019	*16*	1	17/03/2019	*39*
F2	15/05/2019	<4	4	29/05/2019	*14*	5	24/05/2019	*9*
		4 to 8	9	26/05/2019	*11*	10	01/06/2019	*17*
		>8	7	01/06/2019	*17*	3	21/05/2019	*6*
F3	17/05/2019	<4	6	15/05/2019	*2*	6	18/05/2019	*1*
		4 to 8	11	23/05/2019	*6*	12	14/06/2019	*28*
		>8	3	27/05/2019	*10*	1	02/06/2019	*16*
F4	20/05/2019	<4	5	31/05/2019	*11*	6	30/05/2019	*10*
		4 to 8	8	03/06/2019	*14*	6	11/06/2019	*22*
		>8	5	31/05/2019	*11*	3	03/06/2019	*14*
F5	25/05/2019	<4	7	24/05/2019	*1*	7	26/05/2019	*1*
		4 to 8	13	24/05/2019	*1*	12	31/05/2019	*6*

Table 8- Day difference between the mean predicted delivery dates from the biparietal and thoracic diameters for each period and the actual delivery date of each female.

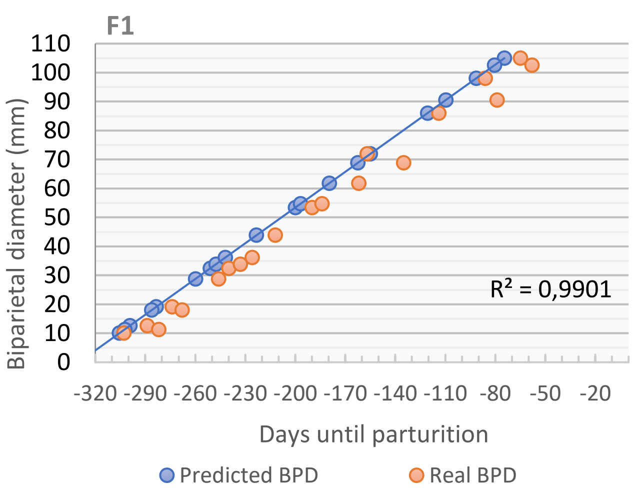
The mean day difference between the predicted delivery date and the actual delivery date from each period and measurement is demonstrated in table 9 (annex 3). The diameter that proved most accurate in each period has been identified and is mentioned in the table as "Best".

Scanning period (months)	x̄ Biparietal diameter	SD	x̄ Thoracic diameter	SD	**Best**
<4	7 days	5	6 days	4	*Thorax*
4 to 8	9 days	5	18 days	7	*Biparietal*
>8	14 days	3	19 days	12	*Biparietal*

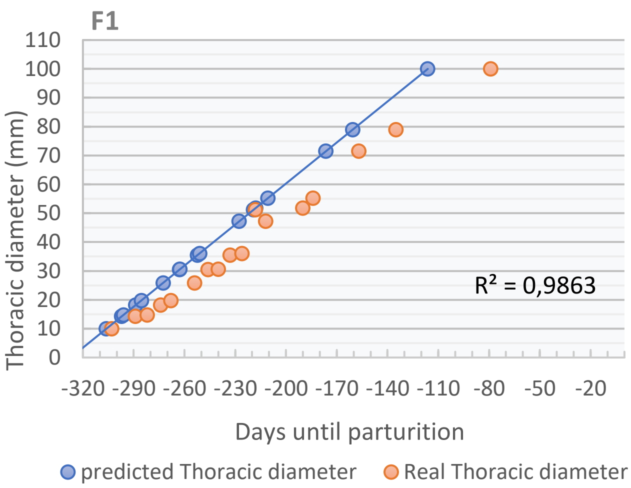
Table 9- Accuracy of each foetal measurement and gestational period in predicting the delivery date.

Linear regression analyses

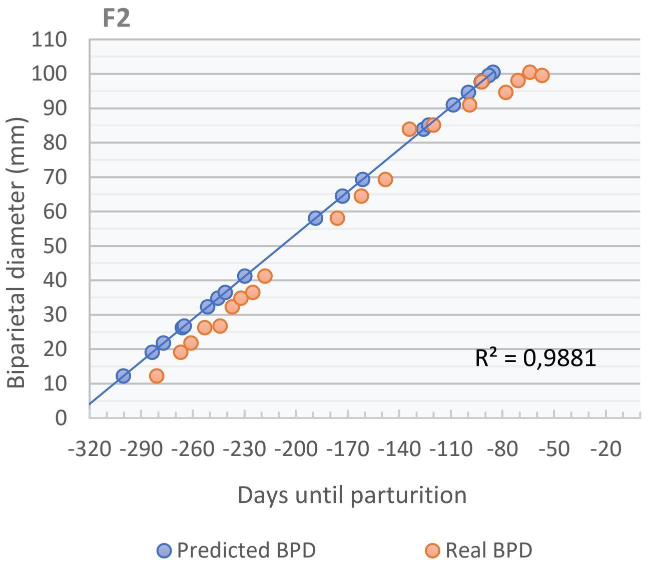
According to the actual delivery date of each female, the real days before parturition were obtained for all ultrasound assessments. A simple regression was applied to the data (graph 2 to 11). This analysis intended to assess the relationship of the biparietal and thoracic diameter with the stage of gestation and compare the straight line corresponding to the delivery prediction equation from the two fetal measurements with respect to the actual days before parturition.



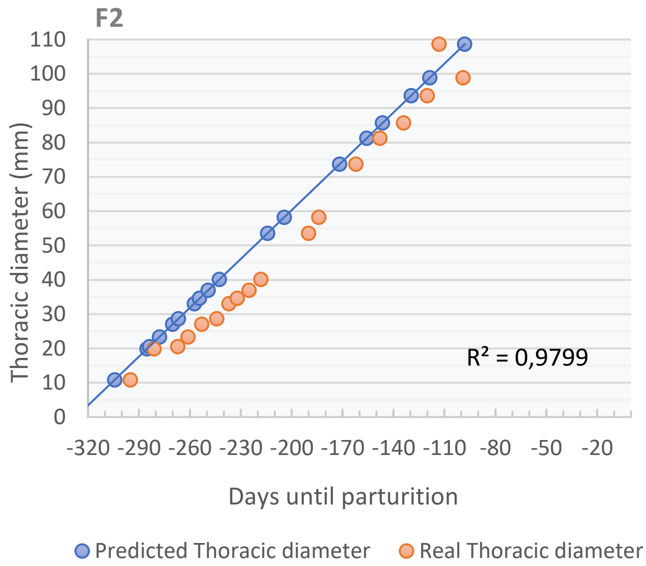
Graph. 2- Linear regression analyses for the days before parturition using the biparietal diameter in F1.



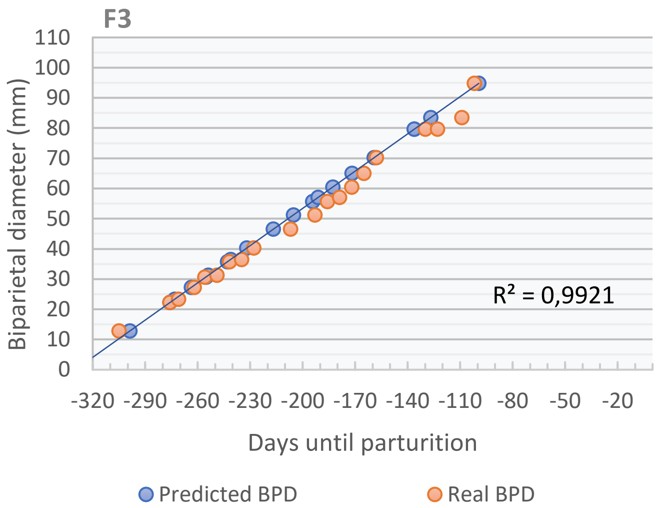
Graph. 3- Linear regression analyses for the days before parturition using the thoracic diameter in F1.



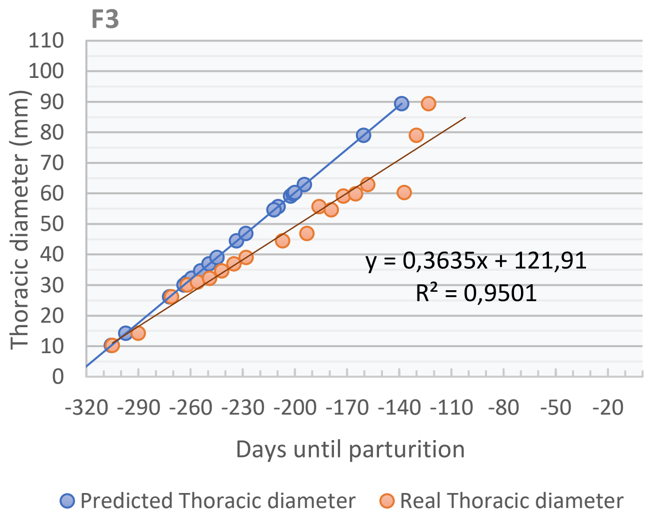
Graph. 4- Linear regression analyses for the days before parturition using the biparietal diameter in F2.



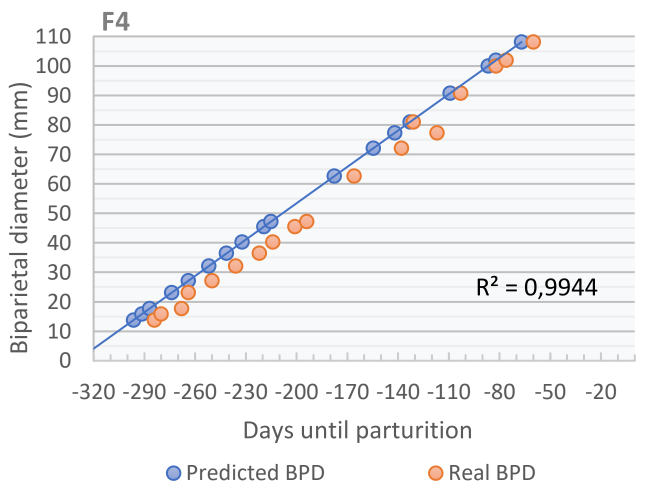
Graph. 5- Linear regression analyses for the days before parturition using the thoracic diameter in F2.



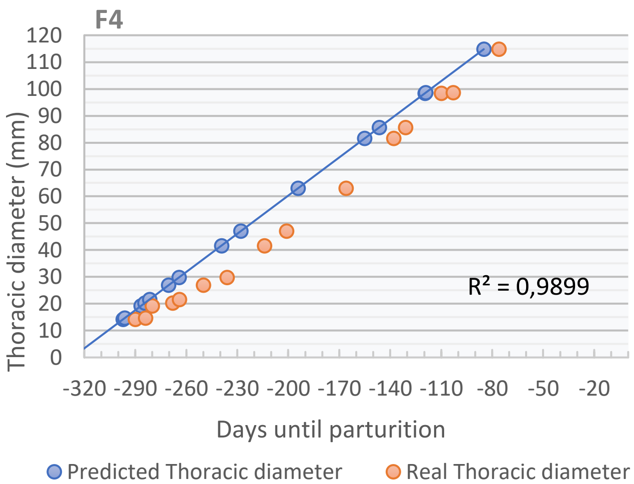
Graph. 6- Linear regression analyses for the days before parturition using the biparietal diameter in F3.



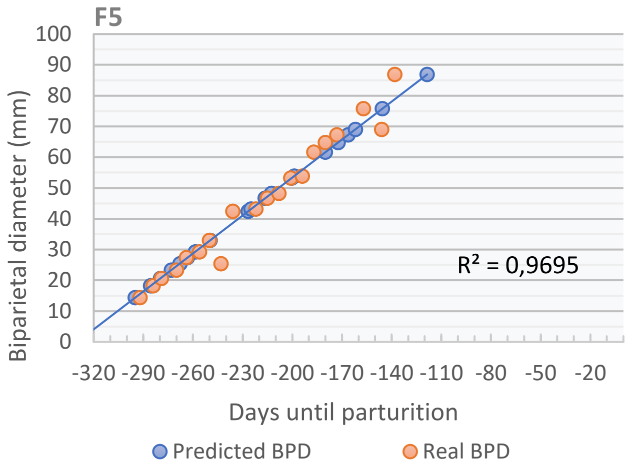
Graph. 7- Linear regression analyses for the days before parturition using the thoracic diameter in F3.



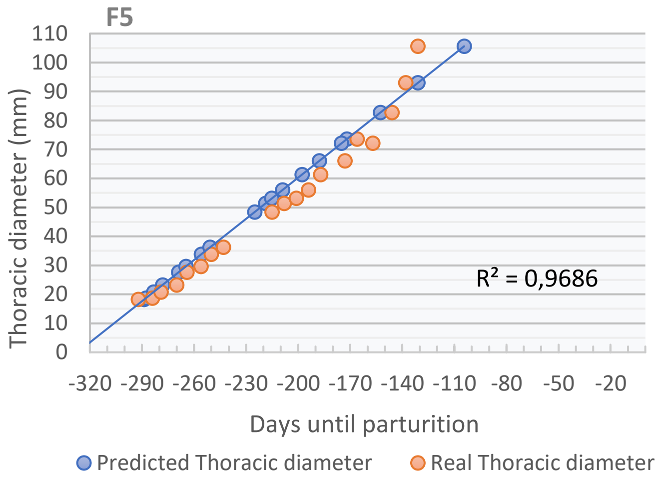
Graph. 8- ***Linear regression analyses for the days before parturition using the biparietal diameter in F4.***



Graph. 9- Linear ***regression analyses for the days before parturition using the thoracic diameter in F4.***



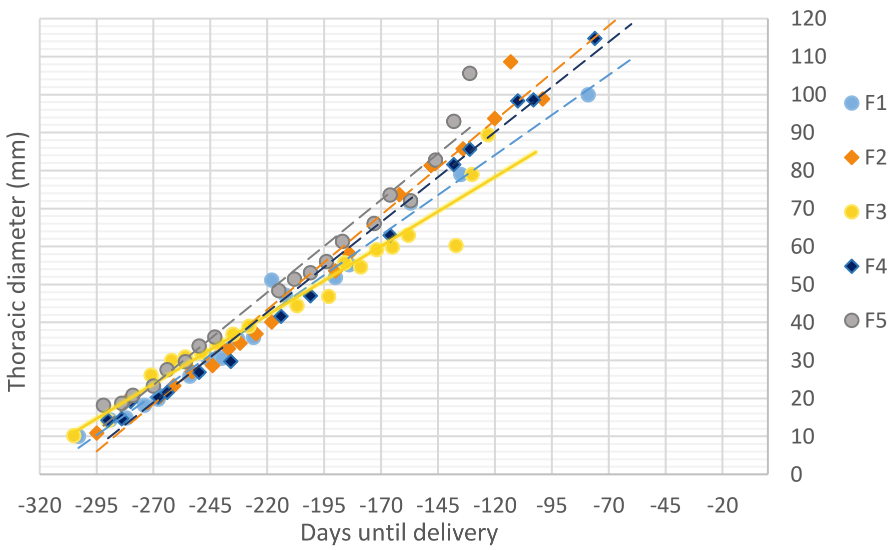
Graph. 10- Linear regression analyses for the days before parturition using the biparietal diameter in F5.



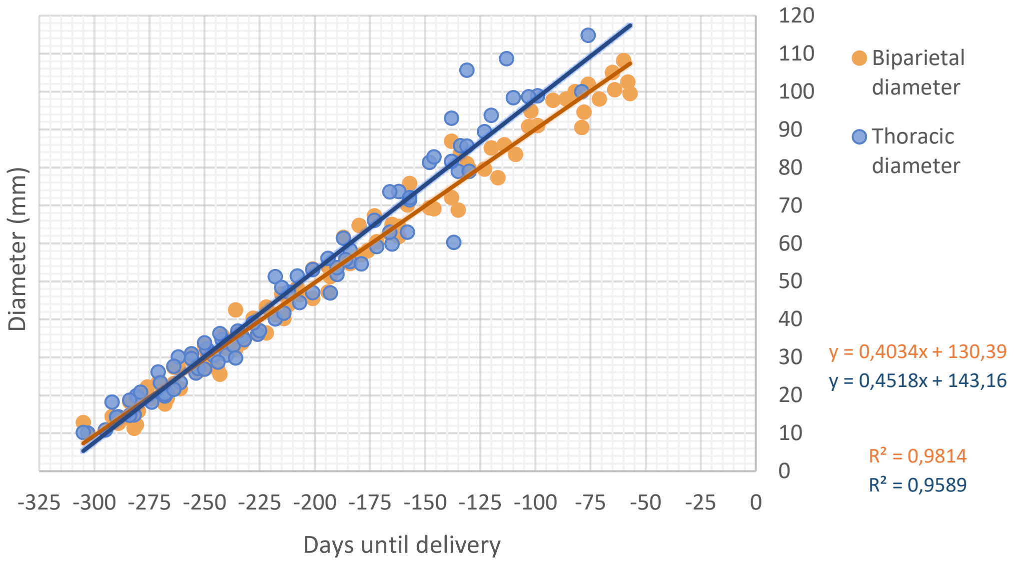
Graph. 11- Linear regression analyses for the days before parturition using the thoracic diameter in F5.

For the studied females, the straight-line representing the real cephalic and thoracic growth until parturition day is slightly below the straight-line predicted by Lacave et al. (2004) biparietal and thoracic diameter equations used to estimate the delivery date in the species. Graph. 12 presents the linear regression straight lines from the thoracic diameter of all females. F3 shows a smaller trend line slope compared to the other four females.

Biparietal and thoracic diameter demonstrated a positive correlation with gestational age (R^2 >0.96) (graph 13). The equations for foetal biparietal and thoracic diameter were determined, based on the five pregnant *T. aduncus* females.



Graph. 12- Linear regression lines from the thoracic diameter of all five females.



Graph. 13- Scatter plot demonstrating relationship between the two ultrasonographic measurements, biparietal and thoracic diameter and days until parturition in five *T. aduncus* foetus.

In the last months of pregnancy, especially after the foetus had adopted a folded position in the uterus, it was difficult to obtain accurate measurements. The last BPD was obtained 57 days before delivery.

According to the Shapiro-Wilk test, the data tested (biparietal diameter, thoracic diameter and actual days before delivery) are not normally distributed (p-value <0,05), so we reject the null hypothesis of normality for all the measurements.

We proceeded to non-parametric tests. Comparison between the predicted days before delivery from the two ultrasound measurements and the actual days until delivery was achieved using the Wilcoxon signed-ranks paired test (Table 10). There was a significant difference between the real days before delivery and the predicted days from biparietal diameter (Z= - 7.559, p-value< 0.001). There was a significant difference between the real days before delivery and the predicted days from thoracic diameter (Z= -8.498, p-value< 0.001).

Statistic test - Wilcoxon

	Actual Days before delivery - Predicted Days before delivery BPD	Actual Days before delivery - Predicted Days before delivery Thoracic diameter
z	-7.854	-8.689
Sig (two-tailed)	<.001	<.001

Table 10- Wilcoxon signed-ranks paired test for actual days before delivery and predicted days before delivery from biparietal and thoracic diameters.

3.3. Parameters for short-term delivery prediction

A total of 167 temperature measurements were performed in the interval of 33 days before delivery and parturition, and 144 intermammary distance measurements were obtained from 24 days before delivery until birth. Descriptive statistics of both measurements are presented in table 11.

Tests for normality were performed for the two parameters (Table 12). We rejected the null hypothesis of normality, since Shapiro-Wilk was significant, meaning the data are not normally distributed.

Values during pregnancy	*N*	$\overline{X}$	*Min.*	*Max.*	*SD*
Temperature (ºC)	167	36.49	35.2	37.1	0.26
Intermammary distance (cm)	144	4.39	3.0	6.5	0.87

Table 11- Descriptive statistics for rectal temperature and intermammary distance of the five pregnant females.

	Shapiro-Wilk		
	Statistics	df	Sig.
Temperature (ºC)	0.879	167	<,001
Intermammary distance (cm)	0.950	144	<,001

Table 12- Shapiro-Wilk test for normality for rectal temperature and intermammary distance.

The average rectal temperature obtained during the latest gestational period was 36.49 ± 0.26 ºC. The graphs of temperature obtained for each female can be consulted in the annexes of this dissertation (annex 4). The average temperature of each female is discriminated in table 13.

By interpreting the graph 14, there is indeed a decrease in temperature in the days preceding parturition. The decrease is not greater than 1.3ºC of the mean temperature value.

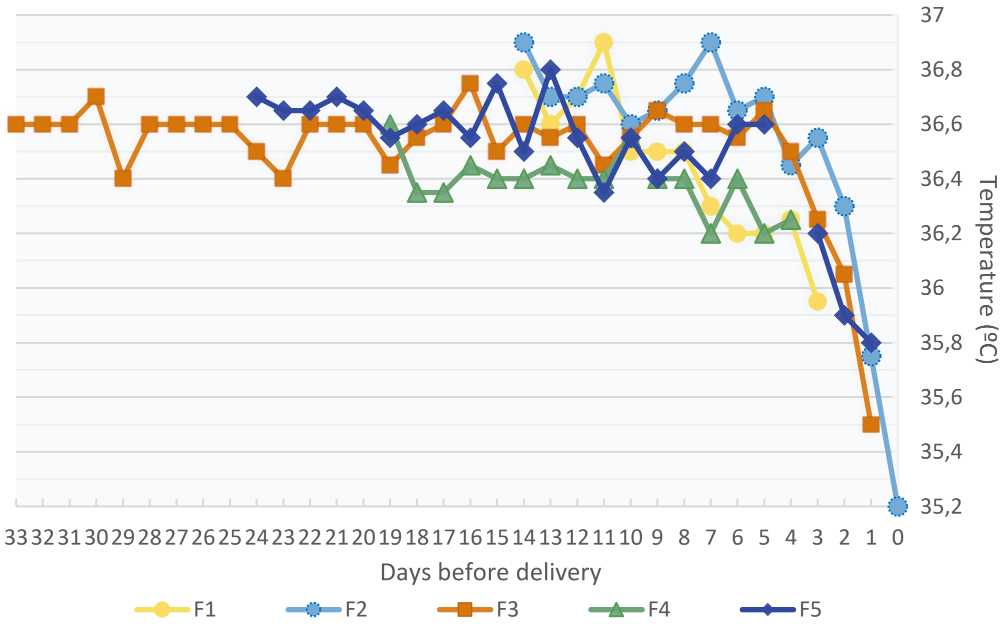
The average intermammary distance for the assessment period was 4.4 ± 0.87 cm. Females 2, 3, 4, and 5 show a slightly positive tendency, with female 4 and 5 reaching distances of 6 cm between the 6th and 4th day before delivery (graph 15).

The values of temperature and intermammary distance obtained on each day before parturition represented in graphs 14 and 15, is the average between the morning and afternoon measurements, when both could be achieved.

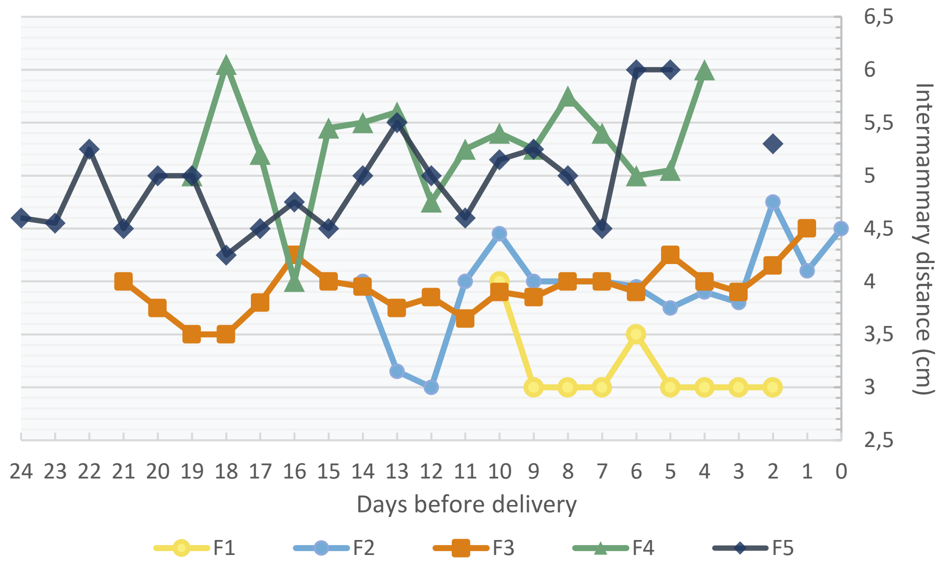
Female	Average rectal temperature [a]	Last rectal temperature obtained and corresponding days prior to delivery	
F1	36.5±0.27 (19)	36.1[c]	3
F2	36.5±0.40 (28)	35.2[b]	0
F3	36.5±0.21 (52)	35.5[b]	1
F4	36.4±0.14 (30)	36.3[c]	4
F5	36.5±0.22 (38)	35.8[c]	1

Table 13- Rectal temperatures of the five pregnant T. aduncus females

a. Values are averages ± standard deviations, with sample size in parentheses.
b. Measurement taken in the morning
c. Measurement taken in the afternoon.



Graph. 14- Temperature variation during the last days of gestation in the five pregnant females. Each value on the graph is the mean of the measurements taken on that day.



Graph. 15- Intermammary measurements during the last days of gestation in the five pregnant females.

4. Discussion

Pregnancy detection and development

The ultrasonographic identification of the embryonic sac and embryo in our population occurred, on average, 314 ± 7 days and 306 ± 10 days before parturition. Although we could determine the interval of days within which ovulation occurred, it was not possible to pinpoint the day of conception. For that reason, the first-time visualization of foetal structures were recorded in days until delivery.

Still, we could compare with the first-time pregnancy detection by other authors. Saviano et al. (2020), detected the gestational sac approximately three weeks after ovulation and the embryo at the end of the first month, which is earlier than we could detect in our females. The earliest embryonic sac and embryo visualization occurred in females 5 (326 days before parturition) and 3 (318 days before parturition), respectively. The latest first-time identification of the same structures happened in female 4, at 306 days (embryonic sac) and 290 days (embryo) before parturition.

In the bottlenose dolphin, the CL is the sole producer of progesterone, at least during the first third of gestation, and actively excretes the hormone until parturition, when it drops drastically. The luteotrophic function of the placenta in the later gestational period has been reported (Bergfelt et al. 2011; Hobson and Wide 1986). However, Robeck et al. (2012) provided evidence that the CL is the primary progesterone source throughout pregnancy and exogenous administration of the hormone to females with low progesterone levels, or no CL can sustain gestation to term (Robeck et al. 2012).

CL size was recorded in all females when clearly seen on the ultrasound. The shortest measurement obtained was 2.08 cm, a few millimetres below the minimum values recorded by Brook (2001) and Saviano (2013), 2.4 cm and 2.5 cm, respectively. The largest diameter obtained in our study, 3.76 cm, was within the measurement range described in Brook (2001) and above the CL size from Saviano (2013), 3.9 cm and 3.2 cm, respectively. The animals studied in Saviano (2013) were *T. truncatus* and *Stenella coeruleoalba*. *T. truncatus*, as mentioned at the beginning of this dissertation, is a larger and heavier species, which most likely translates into larger CLs, since ovary length has been proven to be positively related to body length (Brook 1997). Also, the smaller CL measurements obtained from our females may reveal to be population specific.

The smallest CLs belonged to females 3, with mean transversal/longitudinal diameters of 2.21cm/2.58 cm, and 4, with an average CL of 2.20cm/2.50 cm. It is to be noted that female 4 CL diameters were recorded only twice during pregnancy, once at about 3 months and again at approximately 4 months of gestation, which may not reflect the maximum reached size. Bergfelt et al. (2012) raised the possibility of a progressive and marked decrease in progesterone concentrations during pregnancy being related to stillbirth. The delivery of female 3 resulted in a stillbirth, whereas female 4 had a healthy female calf (table 14). Although progesterone levels were not assessed throughout gestation, all pregnancies were carried to term, which indicate that CLs produced sufficient progesterone concentrations.

Foetal genitalia has been recognized through ultrasound in the bottlenose dolphin (Ivančić et al. 2020). The foetal movement and position during the examination make it challenging to evaluate and determine the gonads. However, the veterinary team of Dolphin bay could identify the foetal sex in females 1 and 3, both as males, at 218 days and at 249 days before delivery, which falls in the 4th and 5th month of gestation, respectively. The previous timeline for genitalia recognition was established at 230 days of gestation (Saviano et al. 2020).

Female	**Age (estimated)**	**Calf**
F1	30 y	Male
F2	17 y	Female
F3	17 y	Stillbirth
F4	37 y	Female
F5	13 y	Male

Table 14- Age of the studied population and sex of the calves born.

Ultrasound M-mode was able to detect and measure foetal HR for the first time at 213±15 days before parturition, which is at approximately four and a half months gestation. According to Cornell et al. (1987), foetal heart rate is reliably measured from about 4-5 months of pregnancy. However, Saviano et al. (2020) earliest recording of foetal HR occurred much sooner, on the 68th day post-ovulation. This earlier detection may result from the significant and rapid development of ultrasound equipment and integrated technology that allows much better visualisation and the performance of these measurements in M-mode today.

This study confirms the significant negative correlation between foetal HR and gestational age (-0.7). The average minimum and maximum foetal HR values were: Up to 9 months of gestation [82 bpm and 160 bpm]; After the 9th month of pregnancy [71 bpm and 146 bpm]; Last two weeks of pregnancy [61 bpm and 108 bpm]. Maximum and minimum HR were 167 bpm and 58 bpm.

These value ranges are in line with what has been mentioned for the genus in previous studies (Ivančić et al. 2020; Saviano et al. 2020; Semenov et al. 2020).

Heart rate variations are normal. Apart from verifying fetal viability, the HR frequency can be an indicative of fetal stress if presenting lower values than the expected mean for the pregnancy stage. Saviano (2013) described the acceleration of foetal HR immediately after foetal movement, which is not observed when a foetus is in distress (Saviano 2013; Stone et al. 1999). In humans and mares, transient increases in foetal HR are also associated with foetal movement and activity periods, with HR accelerations and decelerations becoming more frequent a few days before parturition (Bucca et al. 2005).

The foetus was first seen in a curved position at 104 ± 9 days until parturition. The foetus adopts a laterally bent position to reduce the intrauterine volume that the mother requires to manage foetal growth. Continuous foetal movement and position changes are expected up to three months before delivery, after which they adopt and remain in the position for labour (Terasawa et al. 2021).

Prediction of delivery date

The parturition of female 1 occurred in February 2019, while the other four females delivered in May of the same year. Given the number of births expected for the month of May, it was of utmost importance to apply a method to estimate a short interval of parturition dates, to manage and prepare the teams and space and consequently achieve a higher probability of successful deliveries.

Table 6, shows the actual and predicted delivery days obtained from the biparietal and thoracic diameters for each female. In the case of female 1, both biparietal and thoracic diameters' expected birth dates were delayed concerning the actual date of delivery. The same was also true for females 2, 3 and 4. Female 5 showed the best results as the actual date of parturition was within the range predicted by the equation from each diameter.

It is visible from table 6 that the average difference in days between the actual and predicted delivery dates from both measurements is slightly different. The biparietal diameter showed better results (mean day difference between predicted and actual birth dates of 9 days), than the thoracic diameter (mean day difference of 14 days). These results are in agreement with what has been previously demonstrated by Lacave et al. (2004) and Ivančić et al. (2020), that the biparietal diameter is the most accurate in estimating the parturition date.

By interpreting the table 7 and annex 2, it is noticeable a greater difficulty in obtaining the thoracic diameter from mid-gestation. This challenge in obtaining reliable images is due to the large size of the foetus from this time onwards and the curved position it adopts in the uterine horn. Thoracic diameters were not obtained during the last ten weeks of gestation, as the foetus had grown to no longer fit in the ultrasound window.

The gestational period in which the measurements were most accurate was during the first four months of gestation, followed by the 4^{th} to 8^{th}-month gestational period (table 8). Our results reveal that the thoracic measurements up to 4 months of pregnancy were the most reliable, although the difference from the biparietal measurements in the same period was only 1 day (table 9).

The biparietal diameter measurements were more accurate in the interval from 4 to 8 months and after 8 months of gestation than any thoracic diameter obtained after the 4^{th} month of pregnancy. Similar gestational periods were defined in Lacave et al. (2004) and Ivančić et al. (2020) studies. They both refer to biparietal measurements in the first months as being the best at predicting the birth date in the bottlenose dolphin. Interestingly, we describe slightly contrary results, but considering that the difference between predicted and actual days until delivery obtained in the first gestational period was 7 days for biparietal diameter and 6 days for thoracic diameter, we consider the results as being consensually close to the expected. Further studies of this sort would help to clarify this matter.

In four of the pregnant females, the calves were born earlier than predicted, meaning that the foetal dimensions on the day of delivery were smaller than the foetal size expected by the equation. The same is demonstrated by the linear regression lines (Graphs 2 to 11).

The straight-line referring to the *T. aduncus* Lacave et al. (2004) model used to predict the days before parturition is not the most suitable for our females. For a particular foetal diameter, the days until delivery calculated by the prediction equation were more than the actual days until delivery for that exact measurement. The vast majority of the foetal diameters obtained corresponded to a later gestational age than that estimated by the standard line.

By interpreting the graphics for the five females, the slope is similar to the slope of the standard biparietal and thoracic diameter linear regression lines, but both measurements reveal to be smaller at birth and throughout the pregnancy, especially the thorax. Graphs 6 and 10 referring to the biparietal diameters of females 3 and 5, respectively, are the most fitted.

We can see a discrepant difference between our results and the standard regression line in graphs 3 and 7 concerning females 1 and 3. The trend line from these thoracic measurements is very deviant. The results, are not fitted to the standard straight lines of the predicted equations. However, both regression lines are very satisfactory, indicating foetal growth at a steady pace. As previously cited, female 3 delivered a stillborn, which may explain the unexpected initial development of the foetus. The straight line in the graph suggests an early pregnancy problem that persisted throughout the pregnancy.

The last thoracic diameters obtained from female 3 may have been mis determined by the operator, as they are further away from the trend line. The same can be stated for the last biparietal and thoracic diameters of female 5 and the last two thoracic diameters of female 2, which do not reflect a progressive thoracic growth. These results are in agreement with what was mentioned above. Precise measurements are demonstrably difficult to obtain as the foetus grows in the latest stage of pregnancy (Lacave et al. 2004).

Female 5 (graph 10 and 11) showed the most accurate results, which we could also verify in table 6, since the day difference between the actual and predicted delivery date from biparietal diameter was 1 day and between the actual and predicted delivery date from thoracic diameter was 4 days.

Graph 12 presents all the linear regression straight lines from the thoracic measurements so we could make a better comparison between each foetus trend line slope. It is evident the smaller slope of the foetus of female 3 compared to the other four foetuses. The thoracic size of the foetus of female 3 had slower growth.

Based on what was reported in Pryce et al. (2011), it is hypothesized that neonate size depends on the progenitors' length. If the female or male parents are shorter, fetal growth may be influenced that way. This follow-up and calf monitoring was not covered in the present study but should be considered as a possible influencing factor, which deserves a closer look in the future.

The two equations from graph 13 were based on the measurements performed in all five pregnant females. These are more dispersed in the second half of gestation.

The biparietal and thoracic diameters proved to be highly accurate measurements for predicting gestational age in the *T. aduncus* species (R^2>0.96) (graph 13). Between 300 and 275 days before delivery, the foetal head began to show less accelerated growth than the thorax. The results of Lacave et al. (2004) were similar in that they also reported a slower cephalic growth during pregnancy.

The linear growth rate of the head and thorax in *T. truncatus* was first demonstrated by Williamson et al. (1990) and Stone et al. (1995), and Lacave (2000) continued the work. More recently, Ivancic et al. (2020) reported that the biparietal diameter had the strongest linear correlation with gestational age and presented a highly accurate equation for determining the delivery date (R^2=0.99). They also demonstrated, for the first time, the accuracy of cord diameter, foetal aortic diameter and foetal blubber thickness in estimating birth. Since biparietal and thoracic diameters are not measured accurately and consistently in the last months of gestation, these measurements, whose visualization on ultrasound is possible in their entirety until the end of gestation may be valuable to study in future pregnancies in Dolphin bay.

Although the equations for the two diameters revealed in graph 13 are specific to Dolphin bay females, our sample size is too small to obtain reliable results. Data from upcoming successful pregnancies in the facility, may be used to update and improve the formula.

Ultrasonography was helpful for overall foetal health and development assessment. Movement, position, and amniotic and allantoic fluid characteristics were examined. It was observed from mid-gestation that female 3 had a smaller pocket fluid depth than expected in a normal pregnancy. No other parameters led us to suspicion of a complicated labour. This aspect was closely evaluated by the team and observations, physical and physiological assessments such as rectal temperature begun earlier than in the other females. It would have been interesting to compare other parameters between females whose delivery was normal and female 3. Haematological parameters such as haemoglobin, immature neutrophils and alanine aminotransferase (ALT), or the age of the pregnant female, have shown to be good indicators of the chances of a physiologically normal and successful birth (Semenov et al 2020).

The equations we used to predict the date of birth were built on the basis of 9 *T. aduncus* females resident at Ocean Park, Hong Kong. The Park's original population included females from Taiwan, the Pacific coast of central and western Japan, and Indonesia (Reeves et al. 1994). Recent Ocean Park studies on its Indo-Pacific female population refer to a large proportion being from Taiwan, China and Indonesia (Zhang et al. 2021). The females of Dolphin Bay originate from the Solomon Islands in the South Pacific Ocean, whose characteristics bring them closer to populations from Indonesia and China and differentiate them from those found off the South African coast and Red Sea (Wang and Yang 2009).

The *T. aduncus* groups from both parks came from different wild habitats. This can explain the smaller thoracic diameter of our foetus observed in the linear regression graphs. Western North Pacific dolphins, inhabit colder waters, and are larger, with longer bodies of up to 2.7m (Kogi et al. 2004; Wang and Yang 2009). Cheal and Gales (1992) reported substantial variations in body mass depending on water temperature, which was supported by Ross and Cockcroft (1990) (Appendix 6), and Ridgway and Fenner (1982) earlier findings that dolphins from colder regions were heavier than those from warmer tropical waters.

It is also relevant to mention that gestation length of females in Dolphin bay, considering past pregnancies from which ovulation day was determined, is on average 355 days. The difference in days between the average gestation period of our females and the average gestation length of the females used for the creation of the Lacave et al. (2004) equation models (371±9) may explain the obtained late prediction of delivery date in females 1, 2, 3 and 4.

As mentioned, Ocean Park females are a mixture of populations which may influence to a large extent, the mean gestation period obtained in their studies (Brook unpublished data; Zhang et al. 2021). It is also relevant to note that the taxonomy of the genus *Tursiops* is currently under discussion and re-evaluation. A new species has been proposed in south-eastern Australia, T. australis (Charlton-Robb et al. 2011). Proximity to the Solomon Islands could suggest a relationship between the two populations. However, genetic studies at the mitochondrial level have reported significant differences between individuals from the Solomon Islands and neighbouring populations from New Caledonia, China, Taiwan, and eastern Australia (Oremus et al. 2013).

Questions have been raised regarding the taxonomy of the species. Even though the captive setting, particularly in terms of nutrition (high-level nutritional support) and energy expenditure (no foraging or predator avoidance costs), influences reproduction and the development of a population under human care (Brook 1997; Cheal and Gales 1992), further research on the dolphins surrounding the Solomon Islands may help to understand the biology and genetics of the Dolphin bay population.

Parameters for short-term delivery prediction

Temperatures obtained between day 33 to day 14 before parturition fluctuated but were never below 36.3°C. Females showed a gradual decrease in temperature from day 14 before parturition, with a marked drop in the last 6 days of gestation (graph 14).

The female with the most pronounced decrease was female 2, whose temperature, dropped by 1.1°C between the 2nd day before delivery and parturition day. Female 3 had a decrease of 0.7 ºC between the 3rd and last day before parturition. Female 5 also showed a decline of 0.5ºC between the 3rd day before parturition and the last day of gestation. The decrease was not greater than 1.3 ºC of the mean temperature value (36.5ºC).

The last rectal temperature measurement of female 4 was 36.2ºC four days before parturition. Considering the results observed in the other females, it is likely that a similar sharp dropping had also occurred in female 4 but could not be detected. Previous studies advise the measurement of this parameter to monitor the late gestation since, as we also demonstrated here, the temperature drops critically a few hours before delivery (Katsumata et al. 2006; Terasawa et al. 1999).

Katsumata (2010) described the influence of progesterone and temperature decrease on parturition in killer whales. However, this temperature drop did not occur before a stillbirth delivery that was being monitored, suggesting that the mother's rectal temperature decrease prior to parturition may be crucial in the species' normal birthing process. We report contrary ideas in the Indo-Pacific bottlenose dolphin, as female 3 experienced the second largest temperature drop observed within our population after female 2.

Starting the rectal temperature measurements 15 days before the estimated birth date seems sufficient to detect the expected drop in temperature at parturition. Our results support the value of measuring rectal temperature on the days close to the predicted delivery date, as it is a short-term aid in managing and preparing for imminent birth.

A slight positive correlation can be noticed between intermammary distance and imminence of delivery, although the results from female 1 are not in agreement (graph 15). This relationship is not as evident as what was demonstrated for temperature results. Blanchet et al. (2008) reported a linear increase in the intermammary distance of a female harbour porpoise starting at around 10 days before delivery. A visible increase in this distance can be a useful indication of approaching birth. However, this parameter in our females was not very conclusive and had minimal contribution in predicting the onset of parturition. The inconsistency of the values obtained might be due to possible movement, instability, or contraction of the female during the procedure, which increases the operator's chances of error. Errors between veterinarians may have occurred but are unlikely due to the objective and shared technique.

The engorgement of the genital and mammary areas increases as the pregnancy comes to term, but as demonstrated here, that does not necessarily seem to translate into an increase in the intermammary distance. In the graph 15 we observe that these increases and decreases are more or less random in the last days of pregnancy.

5. Conclusions

The operant techniques adopted, such as long-term ultrasound and rectal temperature assessments, along with the direct observation of behaviours and events indicative of labour approach, were of great importance in predicting the timing and success of delivery in our population. The team operating as a collective unit and following strict pregnancy monitoring and birth protocols has managed the successful delivery of four calves due to be born in 2019.

The results of this study indicate that the most accurate prediction of the delivery date comes from measurements obtained during the first four months of pregnancy. Thoracic diameter showed slightly better accuracy than biparietal diameter during the early gestational period. However, our results showed that the head increases more slowly than the thorax suggesting that biparietal diameter is more reliable, at least from mid-gestation onwards. Thoracic measurements from the time of foetal folding should not be used in predicting the delivery date as they are easily mis determined.

The results obtained validate the monitoring and delivery protocols followed at Dolphin bay. Weekly detailed ultrasonographic evaluation of the reproductive tract in sexually mature females proves successful in early pregnancy diagnosis.

Improving the *T. aduncus* equation model through biparietal and thoracic measurements of upcoming pregnancies in the facility should provide a more accurate estimation of the parturition date. Since the average gestational period of this population is 355 days, determining the exact day of ovulation may be the best alternative to predict the delivery date of our females.

Rectal temperature monitoring during at least the last two weeks of pregnancy aided in predicting the approaching parturition, as it enabled the detection of the pronounced temperature drop expected in the hours prior to delivery. Though we could identify a slight enlargement of the intermammary distance, this measurement did not help predict labour.

Some limitations of our study are worth mentioning, such as the small sample size, the dependence on behavioural cooperation and potential stress of the animal, the movement and position of the foetus during assessments, and errors between operators.

No matter how highly accurate the birth prediction is, parturition is a complex event that requires the whole team to be prepared to assist in case of complications. Implementing strict monitoring and preventive methods is the foundation for the health and welfare of a group of dolphins under human care.

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V. ANNEXES

Annex 1 – Milk formula used in Dolphin bay in case of a calf that is not nursing effectively.

Dolphin Bay
ATLANTIS

MILK FORMULA FOR BABY DOLPHINS

Adapted from CRC Marine Mammal Medicine

Milk formula should be prepared daily and keep refrigerated no longer than 24 hours.

Fish selection for the formula should be done carefully and taking in account the known sensitivity of this population of *Tursiops aduncus* to fish fat content.

Formula should be sent to caloric analysis for feeding frequency and volume calculation.

COMPOSITION:

Capelin and/or school whiting fillets plus viscera	- 750 ml
Zoologic milk® Matrix 30/55 (Pet-ag)	- *2.5 cups (600ml)*
Salmon oil (or sardine menhaden or safflower oil	- *50 ml*
Lecithin	- *1 table spoon (5 to 10 ml)*
Lactobacillus tablets	- *3 tablets*
Osteoform powder (Vet-mix)	- *1 table spoon (5 to 10 ml)*
Multivitamin with Zinc	- *1 capsule*
Taurine	- *250 mg*
Bottled water	- *1100 ml*

Annex 2 – Tables of predicted delivery date based on biparietal and thoracic diameters for females 2, 3, 4 and 5 using Lacave et al. 2004 equation chart (all assessments).

PREDICTION OF DELIVERY DATE IN TURSIOPS ADUNCUS						
FEMALE 2						
US DATE	Diameter (mm)		Based on Head		Based on Thorax	
	Biparietal	**Thoracic**	Days before birth	**PDD**	Days before birth	**PDD**
24/07/2018		10,9			301	**20/05/2019**
07/08/2018	12,2	19,9	298	**31/05/2019**	282	**16/05/2019**
21/08/2018	19,1	20,6	281	**29/05/2019**	281	**28/05/2019**
27/08/2018	21,7667	23,3667	275	**28/05/2019**	275	**29/05/2019**
04/09/2018	26,3	27,05	264	**26/05/2019**	268	**29/05/2019**
13/09/2018	26,7	28,7	263	**03/06/2019**	264	**04/06/2019**
20/09/2018	32,3	33,1	250	**27/05/2019**	255	**02/06/2019**
25/09/2018	34,8	34,6	244	**26/05/2019**	252	**04/06/2019**
02/10/2018	36,5	37	240	**29/05/2019**	247	**06/06/2019**
09/10/2018	41,2	40,1	229	**25/05/2019**	241	**06/06/2019**
06/11/2018		53,6			213	**07/06/2019**
12/11/2018		58,2			204	**03/06/2019**
20/11/2018	58,1		188	**27/05/2019**		
04/12/2018	64,5	73,7	173	**26/05/2019**	172	**25/05/2019**
18/12/2018	69,36	81,3	162	**28/05/2019**	156	**23/05/2019**
01/01/2019	83,9	85,7	127	**08/05/2019**	147	**28/05/2019**
15/01/2019	85,15	93,7	124	**19/05/2019**	131	**26/05/2019**
22/01/2019		108,633			100	**02/05/2019**
05/02/2019	91	98,9	110	**26/05/2019**	120	**05/06/2019**
12/02/2019	97,65		94	**17/05/2019**		
26/02/2019	94,6		102	**07/06/2019**		
05/03/2019	98,05		93	**06/06/2019**		
12/03/2019	100,5		87	**07/06/2019**		
19/03/2019	99,5		90	**16/06/2019**		
PDD				***28/05/2019***		***28/05/2019***
SD				8 days		9 days
REAL DELIVERY DATE			*15th of May 2019*			

PREDICTION OF DELIVERY DATE IN *TURSIOPS ADUNCUS*						
FEMALE 3						
US DATE	Diameter (mm)		Based on the Head		Based on Thorax	
	Biparietal	**Thoracic**	Days before birth	**PDD**	Days before birth	**PDD**
16/07/2018	12,8	10,2	296	**08/05/2019**	302	**14/05/2019**
31/07/2018		14,2			294	**20/05/2019**
14/08/2018	22,2		274	**14/05/2019**		
19/08/2018	23,3	26,15	271	**17/05/2019**	269	**15/05/2019**
28/08/2018	27,2667	30,05	262	**16/05/2019**	261	**16/05/2019**
03/09/2018	30,6667	30,9333	254	**14/05/2019**	260	**20/05/2019**
10/09/2018	31,25	32,15	252	**20/05/2019**	257	**25/05/2019**
17/09/2018	35,7	34,6333	242	**16/05/2019**	252	**27/05/2019**
24/09/2018	36,5	36,9	240	**21/05/2019**	247	**29/05/2019**
01/10/2018	40,2667	39,0333	231	**19/05/2019**	243	**01/06/2019**
22/10/2018	46,5	44,4	216	**26/05/2019**	232	**11/06/2019**
05/11/2018	51,2	46,9	205	**28/05/2019**	227	**19/06/2019**
12/11/2018	55,7	55,7	194	**25/05/2019**	209	**08/06/2019**
19/11/2018	57	54,6	191	**29/05/2019**	211	**18/06/2019**
26/11/2018	60,45	59,15	183	**27/05/2019**	202	**15/06/2019**
03/12/2018	64,95	59,8667	172	**24/05/2019**	200	**21/06/2019**
10/12/2018	70,15	62,925	160	**18/05/2019**	194	**22/06/2019**
31/12/2018		60,3			200	**18/07/2019**
07/01/2019	79,6667	79	137	**24/05/2019**	161	**17/06/2019**
14/01/2019	79,6	89,4	137	**31/05/2019**	140	**02/06/2019**
28/01/2019	83,45		128	**05/06/2019**		
04/02/2019	94,8		101	**16/05/2019**		
PDD				***21/05/2019***		***05/06/2019***
SD				7 days		16 days
REAL DELIVERY DATE			*17th of May 2019*			

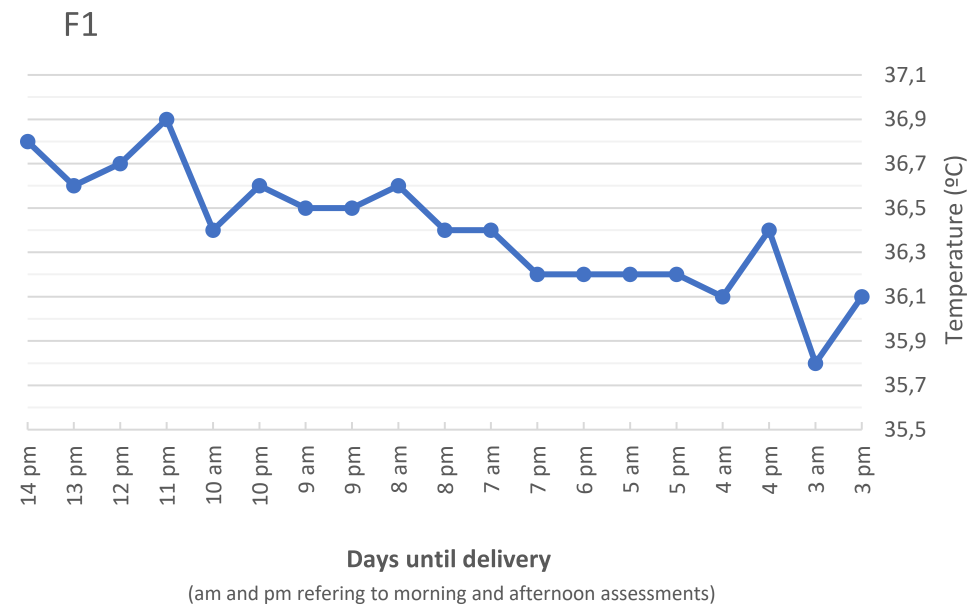
PREDICTION OF DELIVERY DATE IN *TURSIOPS ADUNCUS*						
FEMALE 4						
US DATE	Diameter (mm)		Based on the Head		Based on Thorax	
	Biparietal	**Thoracic**	Days before birth	**PDD**	Days before birth	**PDD**
03/08/2018		14,25			294	**23/05/2019**
09/08/2018	13,8	14,7	294	**29/05/2019**	293	**28/05/2019**
13/08/2018	15,8	19,2	289	**29/05/2019**	284	**23/05/2019**
25/08/2018	17,7	20,3	285	**05/06/2019**	281	**02/06/2019**
29/08/2018	23,05	21,6	272	**27/05/2019**	279	**03/06/2019**
12/09/2018	27,1	26,9	262	**01/06/2019**	268	**06/06/2019**
26/09/2018	32,1	29,8	250	**03/06/2019**	262	**15/06/2019**
10/10/2018	36,4		240	**07/06/2019**		
18/10/2018	40,2	41,65	231	**06/06/2019**	238	**12/06/2019**
31/10/2018	45,4667	47,05	219	**06/06/2019**	227	**14/06/2019**
07/11/2018	47,1667		215	**09/06/2019**		
05/12/2018	62,6	63	178	**31/05/2019**	194	**16/06/2019**
02/01/2019	72,1	81,6	155	**06/06/2019**	156	**06/06/2019**
09/01/2019	81	85,65	134	**22/05/2019**	148	**05/06/2019**
23/01/2019	77,3		143	**14/06/2019**		
30/01/2019		98,4			121	**31/05/2019**
06/02/2019	90,75	98,6	111	**27/05/2019**	121	**07/06/2019**
27/02/2019	100		89	**26/05/2019**		
05/03/2019	101,9	114,8	84	**28/05/2019**	88	**31/05/2019**
21/03/2019	108,1		69	**29/05/2019**		
PDD				***01/06/2019***		***05/06/2019***
SD				6 days		7 days
REAL DELIVERY DATE			*20th of May 2019*			

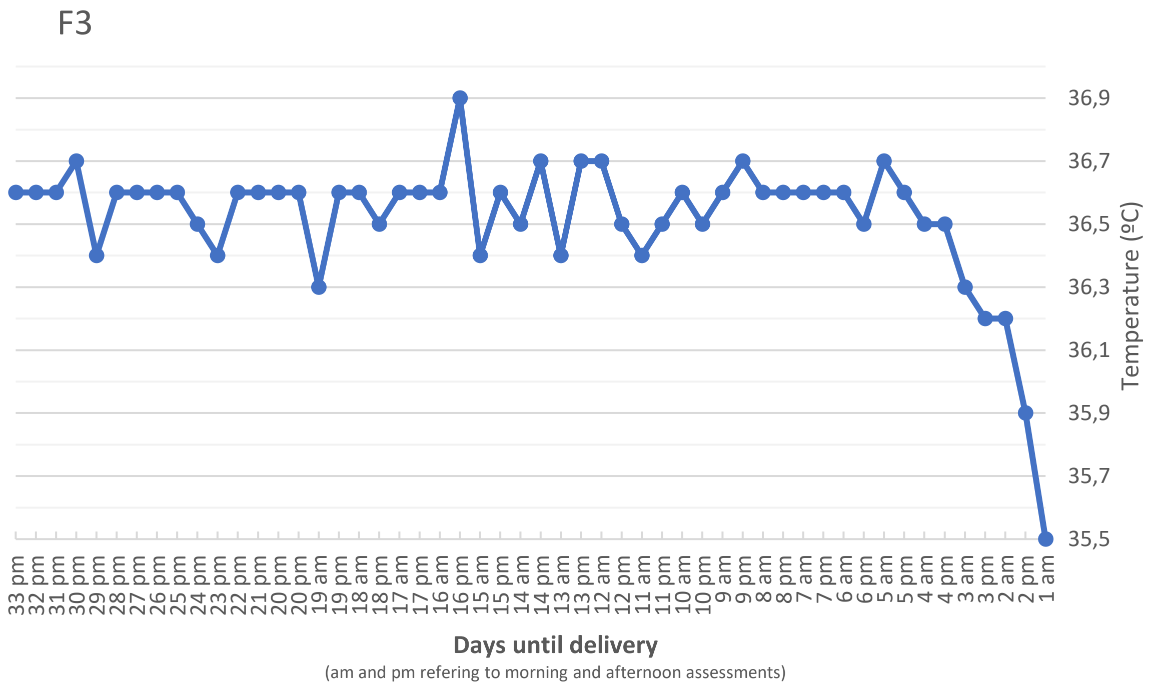
PREDICTION OF DELIVERY DATE IN *TURSIOPS ADUNCUS*						
FEMALE 5						
US DATE	Diameter (mm)		Based on the Head		Based on Thorax	
	Biparietal	**Thoracic**	Days before birth	**PDD**	Days before birth	**PDD**
06/08/2018	14,4	18,2	293	**25/05/2019**	286	**18/05/2019**
14/08/2018	18,25	18,7	283	**24/05/2019**	285	**25/05/2019**
19/08/2018	20,7	20,8	278	**23/05/2019**	280	**26/05/2019**
28/08/2018	23,4	23,25	271	**26/05/2019**	275	**30/05/2019**
03/09/2018	27,4	27,6	262	**22/05/2019**	267	**27/05/2019**
11/09/2018	29,3333	29,6667	257	**25/05/2019**	262	**31/05/2019**
17/09/2018	33	33,8333	248	**23/05/2019**	254	**28/05/2019**
24/09/2018	25,5	36,2	266	**17/06/2019**	249	**30/05/2019**
01/10/2018	42,5		226	**14/05/2019**		
15/10/2018	43,2		224	**26/05/2019**		
22/10/2018	46,7	48,4	216	**25/05/2019**	224	**02/06/2019**
29/10/2018	48,3	51,4	212	**28/05/2019**	218	**03/06/2019**
05/11/2018	53,3	53,1	200	**23/05/2019**	214	**07/06/2019**
12/11/2018	53,9	56,0333	198	**29/05/2019**	208	**08/06/2019**
19/11/2018	61,65	61,3667	180	**18/05/2019**	197	**04/06/2019**
26/11/2018	64,7667		173	**17/05/2019**		
03/12/2018	67,2667	66,05	167	**18/05/2019**	188	**08/06/2019**
10/12/2018		73,6			172	**31/05/2019**
19/12/2018	75,8	72,1	146	**14/05/2019**	175	**12/06/2019**
30/12/2018	69,1	82,75	162	**10/06/2019**	154	**01/06/2019**
07/01/2019	86,9	93	120	**06/05/2019**	133	**19/05/2019**
14/01/2019		105,6			107	**30/04/2019**
PDD				***24/05/2019***		***29/05/2019***
SD				9 days		9 days
REAL DELIVERY DATE			*25th of May 2019*			

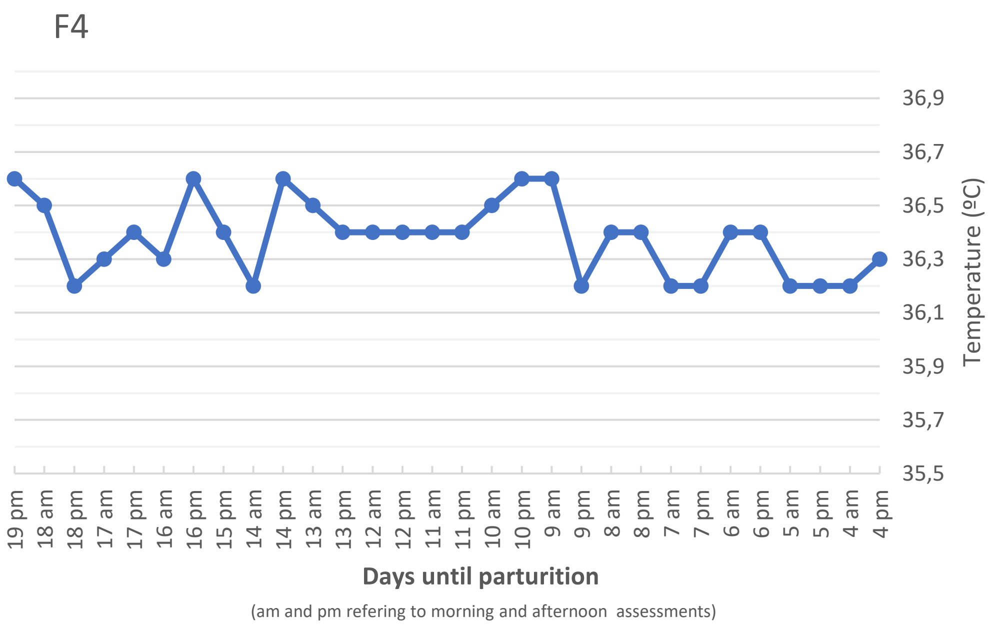
Annex 3- Mean diference and standard deviation between the actual and the predicted delivery date from the biparietal and thoracic diameters for each scanning period (≠ x̄).

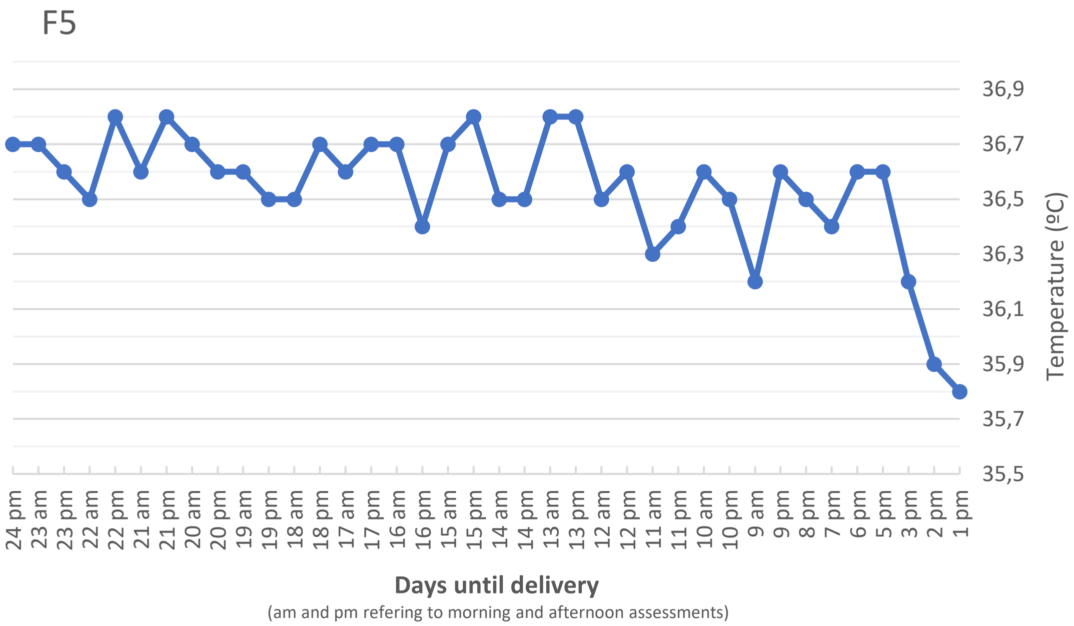
	Scanning period (months)	*≠ Real delivery date - Predicted delivery date*					**≠ x̄**	**SD**
		F1	F2	F3	F4	F5		
Biparietal diameter	<4	9	14	2	11	1	**7.4**	5.08
	4 to 8	12	11	6	14	1	**8.8**	4.71
	>8	16	17	10	11		**13.5**	3.04
Thoracic diameter	<4	9	9	1	10	1	**6.0**	4.10
	4 to 8	18	17	28	22	6	**18.2**	7.22
	>8	39	6	16	14		**18.8**	12.28

Annex 4- Temperature variation graphs of the five *T. aduncus* females in the last days of pregnancy.




F3
Temperature (ºC)
36,9
36,7
36,5
36,3
36,1
35,9
35,7
35,5
Days until delivery
(am and pm refering to morning and afternoon assessments)


F4
Temperature (ºC)
36,9
36,7
36,5
36,3
36,1
35,9
35,7
35,5
Days until parturition
(am and pm refering to morning and afternoon assessments)



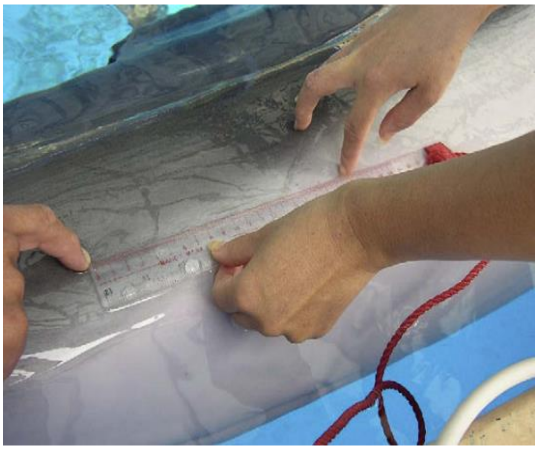
Appendix 1 – Image of a *T. aduncus* in a belly-up position presenting the typical speckling on the genital and belly area. Source: Kryszczyk and Mann (2011).

Appendix 2- Image of the diffuse epitheliochorial placenta of a *T. aduncus* female after delivery. The four-vessel umbilical cord is visible. Source: Original. Dolphin Bay, Atlantis the Palm, Dubai, 2019.

Appendix 3- Presentation of the dolphin for reproductive ultrasound assessment.

- Ultrasound of the testis in a *T. aduncus* male on top. Source: Yuen et al. (2009).
- Position of the probe in an ultrasound of the ovaries in a *T. truncatus* female on bottom. Source: Saviano, 2013.

Appendix 4 - Indirect measurement of the testis in a Male *T. aduncus*. Source: yuen et al. (2009).



Appendix 5- Photograph during the parturition of *T. truncatus* female. Tail-first delivery. Terasawa et al. (2021).

Appendix 6- Relationship between total length of adult bottlenose dolphin and estimated mean annual surface temperature. Image adapted from Ross and Cockcroft (1990) in The bottlenose dolphin.