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FACULDADE DE MEDICINA VETERINÁRIA

CLINICAL APPROACH TO RESPIRATORY MUCORMYCOSIS IN A BOTTLENOSE DOLPHIN (*Tursiops truncatus*) CALF UNDER HUMAN CARE

GONÇALO NOGUEIRA MARQUES

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2019

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Resumo

Maneio médico de mucormicose respiratória numa cria de golfinho-roaz (*Tursiops truncatus)* em contexto zoológico

A epidemiologia das infeções fúngicas com expressão na clínica humana e veterinária tem mudado ao longo do tempo, com várias espécies de fungos a emergir como agentes de infeção. Em golfinhos, vários espécies de fungos estão descritos como agentes etiológicos de infeções invasivas, incluindo fungos filamentosos, leveduras e fungos dimórficos.

Considerando os vários órgãos envolvidos em micoses descritas em golfinhos, o sistema respiratório é dos sistemas mais afetados. Efetivamente, vários fatores parecem contribuir para uma maior susceptibilidade destes animais a infeções respiratórias, entre elas as de etiologia fúngica. A nível anatómico, existem várias adaptações evolutivas respiratórias, que apesar de permitir aos golfinhos serem dos poucos mamíferos que estão completamente adaptados à vida exclusivamente aquática, contribuem para uma maior taxa de doença respiratória. Apesar de poucos estudos terem sido desenvolvidos neste aspeto, vários outros fatores podem ser considerados de risco e explicar a epidemiologia de infeções fúngicas em golfinhos. Estes incluem imunossupressão, tratamentos antimicrobianos prévios ou tratamentos imunossupressores, stress, tratamentos desinfetantes de água, fatores genéticos e exposição a partículas.

A mucormicose é um exemplo de infeção fúngica invasiva, associada a uma elevada taxa de mortalidade. Nas últimas décadas, verificou-se um aumento da incidência desta infeção, tanto em humanos como em golfinhos, representando atualmente a terceira maior causa de micoses invasivas em humanos.

Fungos pertencentes à ordem Mucorales (mucormicetes) são o agente etiológico desta infeção, considerados fungos filamentosos e oportunistas, com distribuição ubiquitária, cuja via de transmissão é principalmente através da inalação. O desenvolvimento da infeção ocorre essencialmente em casos de imunossupressão ou devido à inalação de uma grande carga de esporos. A germinação dos esporos gera hifas, que por sua vez têm uma grande capacidade angioinvasiva e consequentemente de disseminação. Apesar de vários sistemas de órgãos serem descritos como possíveis locais de invasão por mucormicetes, a mucormicose respiratória é uma das apresentações mais descritas em golfinhos. Esta infeção fúngica é comumente associada à distorção anatómica da traqueia e brônquios, com estenose das vias respiratórias devido à fibrose e inflamação local dos tecidos. A nível pulmonar, nódulos e zonas de consolidação e liquefação são frequentemente descritos.

A mucormicose pode ser uma doença rapidamente fatal e como tal é essencial um diagnóstico precoce de modo a iniciar atempadamente o tratamento, evitar a disseminação da infeção e melhorar o prognóstico. No entanto, a sintomatologia da mucormicose é inespecífica e/ou inexistente, o que dificulta o seu diagnóstico. O tratamento consiste não apenas no uso de antifúngicos mas também na excisão cirúrgica de todos os tecidos necrosados, quando possível, eliminação de fatores predisponentes e uso de tratamentos adjuvantes. Tendo em conta a heterogenidade desta infeção, o maneio médico requer um tratamento individualizado, considerando os vários aspetos específicos, e de pouco consenso científico, a nível microbiológico e farmacológico.

Nesta dissertação é apresentado um raro caso clínico referente a uma cria de golfinho-roaz (*Tursiops truncatus*) com um ano de idade, nascido no Zoomarine em junho de 2017. Esta cria apresentava uma história clínica que incluía episódios recorrentes de leucocitose e ocasionais sinais clínicos de etiologia respiratória. Ao longo do primeiro ano de idade foram instituídos vários tratamentos com antimicrobianos. Apesar de se verificar constantemente a melhoria dos resultados hematológicos após tratamento, recidivas eram comuns. Durante este período não foi determinada nenhuma etiologia.

Antes de completar um ano de idade, como parte do programa de medicina preventiva instituído, uma análise microscópica de fezes permitiu a visualização de estruturas fúngicas, juntamente com sinais de inflamação. A amostra fecal foi enviada para cultura fúngica que permitiu o isolamento de um mucormicete. Através de PCR e sequenciação, foi possível identificar *Cunninghamella bertholletiae* como o agente etiológico. Durante o maneio médico desta cria vários estudos imagiológicos foram feitos, que incluíram radiografia torácica, ecografia torácica e abdominal, broncoscopias e gastroscopias. As radiografias torácicas demonstraram a presença de um ligeiro padrão broncoalveolar nos ápices pulmonares e uma primeira broncoscopia permitiu visualizar múltiplas lesões esbranquiçadas e irregulares, difusamente distribuídas pelas mucosas traqueal e brônquica.

Tendo em conta os resultados do teste de sensibilidade a antifúngicos e o maneio médico nos poucos casos clínicos bem-sucedidos de golfinhos com mucormicose respiratória, o tratamento antifúngico consistiu na administração de cápsulas gastrorresistentes de posaconazol e nebulizações com anfotericina B lipossómica. Tratamentos adjuvantes à terapia antifúngica incluíram bromexina, suplementação vitamínica (vitamina C e complexo vitamínico B), proteção hepática com silimarina, probióticos (Antibiophilus® e Multi-Probiotic®), Imuno-2865™ e ozonoterapia (cápsulas orais e instilação de ozono médico por via retal). O

acompanhamento do caso clínico foi feito com base em dados hematológicos e bioquímicos, análises microscópicas/cultura de fezes e exsudado respiratório e broncoscopias.

O tratamento com posaconazol foi descontinuado após 95 dias de terapia, tendo em conta os resultados constantemente negativos na cultura e observação microscópica de amostras fecais e exsudado respiratório. O acompanhamento endoscópico permitiu também verificar uma clara melhoria das lesões macroscópicas, previamente identificadas. Quarenta e oito dias após descontinuação do tratamento com posaconazol, uma broncoscopia de seguimento permitiu observar lesões punctiformes esbranquiçadas a nível da submucosa traqueal. Dez dias depois, os mesmos achados fúngicos foram microscopicamente observados em amostras de fezes e exsudado respiratório. O tratamento antifúngico foi recomeçado e mantido até ao final do período considerado nesta dissertação. Apesar de não ter existido uma completa resolução clínica até ao fim deste período, a infeção fúngica considerou-se controlada tendo em conta os resultados endoscópicos e a ausência de quaisquer sinais clínicos. Os achados fúngicos previamente mencionados foram recorrentes nas amostras fecais e respiratórias avaliadas até ao fim do período considerado, além dos achados em termos de patologia clínica, incluindo leucocitose.

Tendo em conta a falta de estudos farmacocinéticos e por forma a avaliar objetivamente o tratamento antifúngico com posaconazol, um estudo retrospetivo foi feito, avaliando as concentrações séricas do fármaco ao longo do caso clínico. Com os resultados obtidos nesta monitorização, foi descartada a hipótese de não terem sido atingidas concentrações séricas terapêuticas como causa da recidiva da infeção. No entanto, é de realçar que os mucormicetes podem alojar-se a nível da cartilagem traqueal, local onde os azóis não conseguem atingir concentrações terapêuticas devido à sua lipofilicidade. Como tal, a cartilagem traqueal pode ser um local de reservatório fúngico, o que justifica eventuais recidivas.

Com este caso clínico mostrou-se que em crias ainda no processo de aprendizagem de comportamentos médicos voluntários, a intervenção médica, tanto preventiva como reativa, pode ser dificultada. A colaboração voluntária dos animais sob cuidado humano nos vários procedimentos médico-veterinários mostra ser uma arma imprescindível, especialmente tendo em conta a aplicação de um robusto programa de medicina preventiva.

Não existem quaisquer estudos de revisão relativos ao maneio médico de mucormicose respiratória em golfinhos e a escassa informação disponível é conseguida essencialmente através de apresentações em conferências de especialidade. Como tal, apesar de apenas ser considerado um caso clínico, os dados apresentados podem ser de extrema importância para outros veterinários em contexto zoológico ou de reabilitação.

Apesar do prognóstico reservado, a mucormicose respiratória é uma infeção cujo maneio é possível, com uma abordagem multidisciplinar, investimento financeiro e recursos humanos. É essencial o estabelecimento de um plano apertado de seguimento clínico, tendo em conta as prováveis recidivas da infeção. Apesar da micologia clínica ter frequentemente pouca expressão na prática clínica, médicos-veterinários devem ter conhecimento básico referente à epidemiologia, patogenicidade e métodos de diagnóstico e tratamento adequados, de forma a enfrentar de forma mais rápida e eficaz esta e outras infeções fúngicas invasivas.

Palavras-chave: *Tursiops truncatus*, micose, Mucorales, diagnóstico, agente antifúngico

Abstract

Clinical approach to respiratory mucormycosis in a bottlenose dolphin (*Tursiops truncatus*) calf under human care

Several fungi are described to cause invasive infections in dolphins, the respiratory system being a common site of involvement. Mucormycosis is considered one of the most devastating fungal infections in dolphins, associated with an elevated mortality rate, where hyphae are capable of invading blood vessels, producing tissue infarction and necrosis.

A one-year-old male bottlenose dolphin *(Tursiops truncatus)* calf presented with a history of recurrent episodes of leukocytosis and occasional respiratory signs. During a routine faecal examination, a myriad of hyphae were found. Fungal culture revealed a mucormycete isolation, the aetiologic agent of mucormycosis. Molecular studies allowed to identify *Cunninghamella bertholletiae*. Thoracic radiographs showed the presence of a bronchoalveolar pattern on both the right and left lung apexes. A bronchoscopy was performed, which revealed multiple whitish lesions, diffusely distributed on the tracheal and bronchial submucosa. The antifungal therapy prescribed was a combination of posaconazole and aerosolized liposomal amphotericin B. Adjunctive therapies included bromhexine, vitamin C, vitamin B complex, probiotics, silymarin, Imuno-2865™ and ozone therapy. Follow-ups were conducted with haematology and blood biochemistry, faecal and sputum culture and direct microscopy, and bronchoscopies. There was a good overall response to treatment and antifungal therapy was discontinued. However, the infection relapsed and posaconazole therapy was restarted. Serum concentrations of posaconazole were retrospectively evaluated and the set of results did not appear to show subtherapeutic concentrations as a plausible explanation for the relapse.

Although complete clinical resolution was not obtained during the timeframe considered, this case corroborates the idea that medical management of mucormycosis is possible, especially with a prompt diagnosis and treatment as well as a tight follow-up protocol. As described in the literature, mucormycosis treatment may take several years and relapses are common.

Keywords: *Tursiops truncatus*, mycoses, Mucorales, diagnosis, antifungal agents

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List of Abbreviations and Symbols

€ – Euro
ºC – Degrees Celsius
% – Percentage
® – Registered
™ – Trademark
µm – Micrometre

ALP – Alkaline phosphatase
ALT – Alanine aminotransferase
AmB – Amphotericin B
AMMPA – Alliance for Marine Mammal Parks and Aquariums
AST – Aspartate transaminase
AUC – Area under-the-curve
BDG – (1-3)-β-d-glucan
BID – Twice daily (*bis in die*)
CBC – Complete Blood Count
Ch – Charrière
Cm – Centimetre
CT – Computed tomography
DGAV – Direcção Geral de Agricultura e Veterinária
d – Day
dL – Decilitre
DNA – Deoxyribonucleic acid
DRT – Delayed-Release tablet
e.g. – for example (*exempli gratia)*
EAAM – European Association for Aquatic Mammals
EAZA – European Association of Zoos and Aquariums
ECMM – European Confederation of Medical Mycology
ECV – Epidemiologic cut-off value
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme-linked immunosorbent assay
ESCMID – European Society of Clinical Microbiology and Infectious Diseases
ESR – Erythrocyte sedimentation rate
FMV-Ulisboa – Faculty of Veterinary Medicine - University of Lisbon
G – Gauge
GMS – Grocott's methenamine silver
h – Hour
HE – Hematoxylin and eosin
i.e. – in other words (*id est)*
IFI – Invasive fungal infection
IM – Intramuscular
INIAV – Instituto Nacional de Investigação Agrária e Veterinária
$ISCO_3$ – International Scientific Committee of Ozone Therapy
ITS – Internal transcribed spacer
IUCN – International Union for Conservation of Nature
IV – Intravenous
IVf – Intravenous formulation
Kg – Kilogram
L – Litre
m – Metre
MALDI-TOF – Matrix-assisted laser desorption/ionization

MHC – Major histocompatibility complex
MIC – Minimum inhibitory concentration
mL – Millilitre
mm – Millimetre
MM – Mucormycosis
NMB – New methylene blue
NmL – Normalized mililiter
OS – Oral suspension
PAS – Periodic acid–Schiff
PCR – Polymerase Chain Reaction
PCZ – Posaconazole
PMN – Polymorphonuclear leukocyte
PO – Oral administration (*per os*)
RBC – Red blood cell
RFLP – Restriction fragment length polymorphism
Rpm – Rotations per minute
rRNA – Ribosomal ribonucleic acid
s – Second
SC – Subcutaneous
SID – Once a day (s*emel in die)*
spp. – Species
TDM – Therapeutic drug monitoring
TID – Thrice daily (*ter in die*)
U – Units
UPLC-MS/MS – Ultra performance liquid chromatography - tandem mass spectrometery
US – Ultrasonography
vs – Versus
WBC – White blood cell

1. TRAINING PERIOD ACTIVITIES

This dissertation was developed during the 6th year of an Integrated Masters in Veterinary Medicine from the Faculty of Veterinary Medicine, Lisbon University (FMV–ULisboa). The author's curricular placement was in Zoomarine – Mundo Aquático S.A., Portugal, for a six-month externship that began on the 2nd of November 2018 and was completed on the 30th of April 2019, summing a total of approximately 980 hours. This training was under the supervision of Dr Carla Anne Flanagan, Zoomarine's veterinarian and zoological director and the co-supervision of Professor Maria Teresa da Costa Mendes Vítor Villa de Brito (FMV–ULisboa).

Zoomarine is a zoological park established in 1991, located in Guia, Albufeira. Staying true to the motto "Together We Protect" and with a vast zoological collection (figure 1), Zoomarine's philosophy is to contribute to more effective wildlife protection, as well as to increase the welfare of animals under human care. Zoomarine has several zoological areas. Dolphins are housed in three different areas and pinnipeds are maintained in another pool complex. Avian species are divided into two different groups and areas: the tropical birds and the birds of prey. There is also a walkthrough habitat "Américas", which includes several species of birds and reptiles, the "Oceanus" aquarium and other habitats for reptiles.

Figure 1 - Variety of species of the zoological collection of Zoomarine – examples (Original).
a pacific blue tang fish (*Paracanthurus hepatus*) and ocellaris clownfish (*Amphiprion ocellaris*); b burrowing owl (*Athene cunicularia*); c scarlet ibis (*Eudocimus ruber*); d red-eared slyder (*Trachemys scripta*); e grey seal (*Halichoerus grypus*); f bottlenose dolphin (*Tursiops truncatus*).

The park has a rehabilitation centre – “Porto d’Abrigo” – dedicated to the rescue, rehabilitation and release of marine and other aquatic animals back into the wild. Moreover, Zoomarine has a Veterinary Hospital, with a team of three veterinarians and two veterinary nurses.

During his training, the author followed and actively participated in all the activities performed by the veterinary staff:

- Laboratory exams, processing blood samples for haematology and biochemistry analysis from bottlenose dolphins (*Tursiops truncatus*), pinnipeds [harbour seal (*Phoca vitulina)*, grey seal (*Halichoerus grypus),* California sea lion *(Zalophus californianus)* and South African fur seal (*Arctocephalus pusillus pusillus*)] and several reptiles, tropical bird species, aquatic birds and birds of prey. This included the erythrocyte and leukocyte total counts performed manually with a Neubauer chamber as well as performing blood smears and observing them under the microscope for evaluation and differential leukocyte count. The author also performed urinalysis and macroscopic and microscopic evaluations of sputum, gastric fluid, faeces and milk of *T. truncatus* as well as avian droppings analysis through faecal smears and floats;
- Attending numerous clinical cases regarding several medical specialities including ophthalmology in marine mammals and fish, gastroenterology in marine mammals and birds, reproduction in elasmobranchs and dermatology in marine mammals as well as birds, reptiles and fish;
- Several medical procedures in:

- *T. truncatus*: physical exam (figure 2), blood collection, drug administration through intramuscular (IM) injections, rectal probing for faecal collection and medical ozone administration, nutritional support through a stomach probe and vaccination against *Erysipelothrix rhusiopathiae*;
- Pinnipeds: drug administration through IM and subcutaneous (SC) injections;
- Birds: blood collection, physical exams under inhalation anaesthesia, choana, crop and cloacal swabs, fluid and drug administration through IM, SC injections and crop tube probes;
- Reptiles: blood collection, intracoelomic fluid injections and drug administration through an IM route and stomach probes;

- Diagnostic imaging, performing radiographs on several birds and freshwater turtles, assisting on ultrasound examinations of common eagle rays (*Myliobatis aquila*), *T. truncatus* and pinnipeds as well as endoscopic procedures in *T. truncatus* (bronchoscopy, gastroscopy, colonoscopy and hysteroscopy);

- Surgery techniques, performing penectomies in red-eared sliders (*Trachemys scrypta*) (figure 3), assisting in a member amputation and a surgical oesophagostomy tube placement in a mediterranean pond turtle (*Mauremys leprosa*) and assisting in a scarlet ibis (*Eudocimus ruber*) and sulphur-crested cockatoo (*Cacatua galerita*) orthopaedic surgeries;
- Hospitalization duties assuring and preparing all the animals' meals and medication as well as all the cleaning and disinfection procedures;
- Necropsy procedures, conducting the necropsies of several fishes, including elasmobranchs, several birds and freshwater turtles;
- Medical training behaviours, assisting on the ultrasound, blood collection and IM injections training of *T. truncatus* and pinnipeds;
- Miscellaneous activities, with visits to the different animals' departments and following the routine activities of the trainers, including the water quality laboratory. The author also had the opportunity to attend and act as a volunteer at the 2019 Annual Symposium of the EAAM (European Association for Aquatic Mammals), held by Zoomarine.

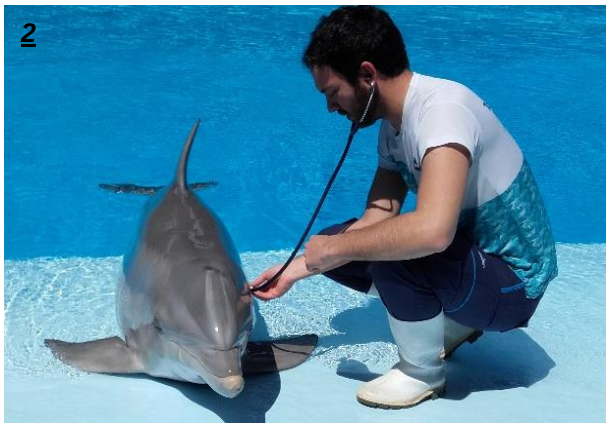


Figure 2 - Thoracic auscultation of a bottlenose dolphin (Original).
Figure 3 - Local administration of lidocaine previous to a penectomy surgery on a *Trachemys scrypta* (Original).

2. INTRODUCTION

The landscape of fungal infections in dolphins has markedly changed over the last decades. A recent survey regarding mycoses reports on marine mammals since 1997 demonstrated the isolation of more than twenty-three species of fungi across nine species of cetaceans (Reidarson et al. 2018).

Fungi described causing invasive fungal infections (IFIs) in cetaceans include moulds such as several mucoralean species, *Aspergillus* spp. and *Fusarium* spp. and dimorphic fungi (e.g. *Candida* spp., *Cryptococcus* spp., *Histoplasma capsulatum*, *Coccidioides immitis)* (Reidarson et al. 2018). Other fungal agents include *Paracoccidioides brasilensis*, a fungus that leads to a characteristic presentation of elevated grey and white plaques in the skin (Vilela and Mendoza 2018).

Pneumonia (of different aetiologies) is one of the most common diseases among captive and free-ranging dolphins largely because of the anatomical evolutionary modifications of the upper respiratory tract (Venn-Watson et al. 2012). Pulmonary fungal infections are reported to be the most common mycoses in dolphins (Reidarson et al. 2018), the most devastating caused by mucormycetes and *Aspergillus* spp. (figure 4), with an overall poor outcome and elevated mortality. *Candida* spp. are one of the most commonly found fungi in sputum samples of dolphins and considered part of the normal microbiota (Reidarson et al. 2018). Nevertheless, overgrowth may happen and antifungal treatment may be difficult due to the characteristical biofilm formation and to several antifungal resistances (Andes 2019).

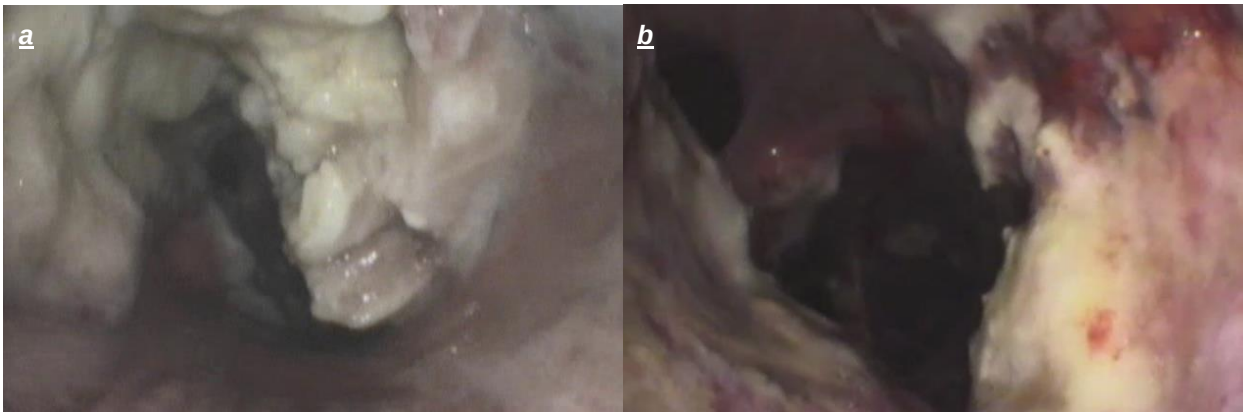


Figure 4 - Aspergillosis lesions in *Tursiops truncatus* - bronchoscopy (2017).
a proximal trachea b right accessory bronchus.

Mucormycosis (hereafter referred to as MM), formerly zygomycosis, is a general term for rare infections caused by the ubiquitous fungi from the order Mucorales. Clinical disease may

occur if the host's defences are lowered or if large numbers of spores are inhaled or ingested (Quinn et al. 2016).

Several clinical forms of MM are reported, all characterized by tissue necrosis due to the invasion of blood vessels and subsequent thrombosis. As this infection may be rapidly fatal, it is crucial to have timely diagnosis and perform an aggressive treatment (Edwards 2018).

Individual case reports have been documented in wild stranded and in managed dolphins, with common sites of infection including not only the respiratory system but also the skin and brain (Reidarson et al. 2018). Although MM is not considered a common infection, a greater susceptibility of dolphins is suggested (Leger et al. 2018). Historically, most of the dolphins diagnosed with MM have not survived despite treatment and the first case of a cetacean surviving this infection was only reported in 2004 (Townsend et al. 2006). Of the antifungal drugs used against MM in humans, posaconazole, an azole antifungal agent, appears to be the antifungal with better results in treating MM in dolphins (Walters et al. 2009).

A case of respiratory MM in a bottlenose dolphin calf is here presented. Considering the lack of published data on MM in non-human species, advances made in the understanding of MM in humans are frequently referenced throughout this dissertation in order to apply this knowledge in the management of MM in cetaceans.

2.1. *Tursiops truncatus*

Dolphins and whales (order Cetacea) are part of the small group of mammals that are fully adapted to life in water (Cozzi et al. 2017a).

Tursiops truncatus are members of the Delphinidae family and are geographically widespread, often found in coastal areas of temperate to tropical waters (Wells and Scott 2009). The International Union for Conservation of Nature (IUCN) has listed *T. truncatus* as Least Concern since 2008. This species is well-known for its well-documented social life and because it is the most commonly found species of cetaceans in zoological parks (Dold 2015).

Adult bottlenose dolphins weigh between 150 and 650 kg, with a length that ranges from 2-4 m. They have a streamlined body, possessing a fatty melon that together with their complex nasal anatomy is responsible for the ability to echolocate (figure 5) (Wells and Scott 2009; Cozzi et al. 2017a). The only way to discriminate between gender is through observation of the ventral region. Females have an almost continuous line between the genital and the anal slit, while the distance between the two is more pronounced in males. The genital slit closes the vulva in females and the penis in males. Furthermore, females have two grooves, which correspond to the external opening of the nipples (figure 6) (Cozzi et al. 2017a).

Females typically reach sexual and physical maturity before males, at an age between 5-13 years vs 9-14 years in males. They are spontaneous sporadic ovulators, ovulating repeatedly during a given season. The gestation period lasts for about 12.5 months. Although calves achieve most of their growth during the first 2 years, they are breastfed for a long period that can go up to 8 years (Wells and Scott 2009; Cozzi et al. 2017a).

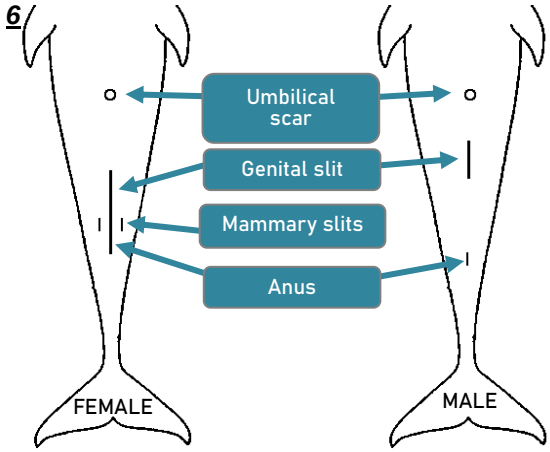


Figure 5 - Bottlenose dolphin (Original).
Figure 6 - Sexual dimorphism in *Tursiops truncatus* (ventral view – schematic). Adapted from The Marine Mammal Anatomy & Pathology Library, available at https://www.mmapl.ucsc. edu/basic-response/ gender-id/cetaceans

2.2. Management of dolphins in a zoological context

The European Association of Aquatic Mammals (EAAM) husbandry guidelines set minimum housing standards for dolphins kept in captivity, although compelling veterinary reasons may justify holding the animals temporarily in smaller enclosures (EAAM 2019). The importance of pool dimensions is one of the areas of welfare research, but surprisingly, few studies have been done on this subject (Brando et al. 2017).

Maintaining good water quality is crucial to ensure the health and the well-being of captive cetaceans (Meijer 2013). An adult dolphin discards around 1.45 kg of faeces and 4 L of urine daily and therefore a constant filtration and water quality controls are of paramount concern (Ridgway 1972 cited by Brando et al. 2017).

Pool water may be recycled through mechanical and biological filtration. Once gross organic material is removed, chemical treatment is accomplished with chlorine, ozone and/or ultraviolet radiation (EAAM 2019). Specific standards on water quality are available in guidelines from the EAAM, European Association of Zoos and Aquaria (EAZA) and Alliance of Marine Mammal Parks and Aquariums (AMMPA). Basic tested parameters should include, at least,

salinity, pH, chlorine levels (total, free and combined), nitrites, nitrates, ammonia and microbiological counts (e.g. coliforms, enterobacteria, *Candida* spp.).

Dolphins require a variety of fish of a quality suitable for human consumption, which is usually frozen and given freshly thawed. Fish is usually caught offshore and submitted to a deep-freezing process. Upon arrival to zoological institutions, a sample should be selected to be tested for quality criteria (Nollens et al. 2018). The freezing process causes a decrease in the concentration of some nutrients and therefore dolphins should be given a daily multivitamin supplementation to prevent nutrient deficiencies (Emilia et al. 2016).

Dolphins may mask signs of clinical illness appearing completely healthy and, on this basis, a routine preventive health programme is essential so that any slight indicators of disease can be immediately and thoroughly examined (Dold 2015). A robust preventive medicine programme should be a species-specific systematic plan for managing animal health through observation, communication with trainers and diagnostic monitoring (Nollens et al. 2018). Medical training is very common in marine mammal husbandry allowing more efficient preventive medical tasks and reactive care. The voluntary collaboration leads to less stress to the animals and also reduces the risk of humans and animals being hurt during handling (Lacave 2018). Training should be based on operant conditioning, with an emphasis on positive reinforcement (Brando 2010).

2.3. Anatomy of the respiratory system

There are several highly-specialized adaptations of the dolphins' respiratory system to life underwater, some of them still not completely understood (Piscitelli et al. 2013). These modifications are related to their diving behaviour, and include breath-hold diving and buoyancy control at varying depths, very effective oxygen uptake and also storage of limited air supply for foraging, echolocation and vocalization (Reidenberg and Laitman 2008; Piscitelli et al. 2013).

2.3.1. Blowhole and air sacs

Anatomical adaptations of the respiratory tract include the migration of the nostrils from the tip of the rostrum to the top of the head, where there is an external opening called the blowhole (Reidenberg and Laitman 2008; Cozzi et al. 2017b). The blowhole is not fixed in an open position and air is only admitted into the upper respiratory tract when the animal voluntarily opens the nasal plugs (Cozzi et al. 2017b). The blowhole is crescent-shaped and the paired nasal passages are merged for most of the length distal to the nasal septum (Berta et al. 2014).

Dolphins and other cetaceans have no paranasal sinuses since the walls of bone-enclosed air chambers would fracture during descent/ascent due to contracting/re-expanding air volumes. Flexible-walled ‘‘sinuses’’ (i.e. air sacs), attached to the airways, are a logical adaptation to diving (Reidenberg and Laitman 2008). Bottlenose dolphins have three types of air sacs: nasal (vestibular, nasofrontal and premaxillary), pterygoid and laryngeal (Mead 2011; Cozzi et al. 2017b).

2.3.2. Larynx

In dolphins, the larynx (i.e. goosebeak) is in a relatively rostral position compared to terrestrial mammals, bent at almost a 90-degree angle, to allow communication between the blowhole and the almost horizontal trachea (figure 7). The larynx is completely separated from the pharynx, hence avoiding any potential leakage of water from food into the respiratory system (Cozzi et al. 2017a). The epiglottal and arytenoid cartilages, composing the larynx, form a tubular structure that extends through a small opening in the roof of the pharynx into the almost vertical nasal passage (Reidenberg and Laitman 2008). A palatopharyngeus sphincter muscle keeps the goosebeak sealed, locking it into an intranarial position and thus waterproofing the laryngeal beak with the airways. Food in the oesophagus can pass either side of the larynx (Reidenberg and Laitman 2008; Cozzi et al. 2017b).

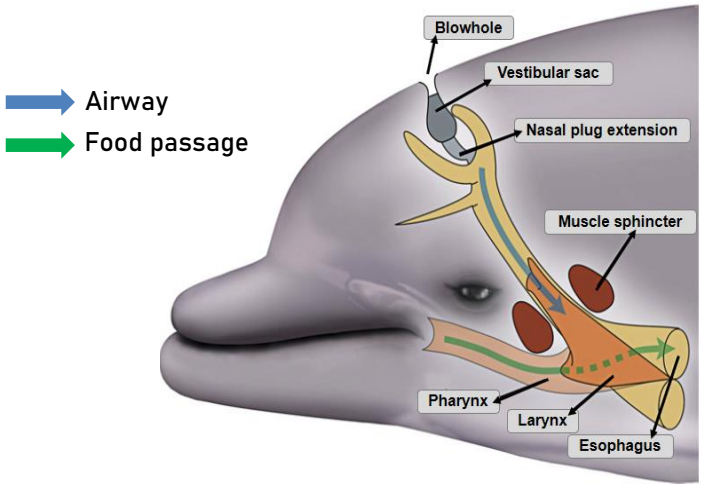


Figure 7 - Dolphin upper airways and laryngeal communication with the oesophagus (schematic). Adapted from Varela et al. (2007).

2.3.3. Trachea and bronchi

The trachea of dolphins is rather short but relatively large in diameter. It has a more rigid structure compared to terrestrial mammals and is composed of circular cartilaginous rings irregularly anastomosed and devoided of the dorsal open ends with, consequently, no *musculus*

trachealis (Cozzi et al. 2017b). One of the most distinctive features is the presence of blood-filled venous *lacunae* in the tracheal submucosa (figure 8) (Ballarin et al. 2018). These structures may contribute to the restoration of the tracheal lumen during ascent, by filling with blood and helping the trachea to return to its original shape, given that it flattens during descent (Cozzi et al. 2017b). The trachea ends with the main bronchi. The cranial aspect of the right lung receives the *bronchus trachealis*, an accessory right bronchus (figure 9) (Moore et al. 2014).

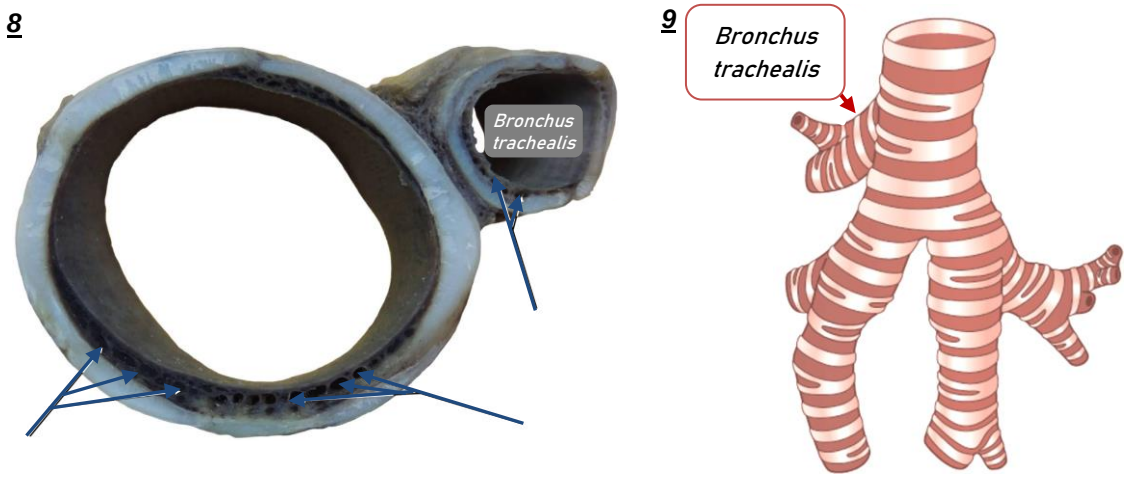


Figure 8 - Section of the trachea and *bronchus trachealis* of *Tursiops truncatus*. Arrows: vascular *lacunae*. Adapted from Huggenberger et al. (2018).
Figure 9 - Right accessory bronchus (arrow) – schematic. Adapted from Moore et al. (2014).

2.3.4. Lungs, pleura and lymph nodes

The dolphins' lungs are unlobed, oblong to pyramidal, with only a deep incisura of the apex. Dolphins, as short-duration divers, have a greater lung mass (2.7%) compared to terrestrial mammals (1%) (Piscitelli et al. 2013; Cozzi et al. 2017b). A characteristic feature in the terminal part of the dolphin bronchial tree system is the presence of myoelastic sphincters and cartilage in bronchioles of less than 2 mm wide. In deep dives, the dolphin lungs tend to collapse due to the external pressure and this bronchiole architecture is essential in maintaining the lungs collapsed during diving. In addition, they maintain air pressure within the upper respiratory tract where it is needed for sound emission (Cozzi et al. 2017b).

The pleura consists of dense fibroelastic tissue containing numerous blood and lymphatic vessels, connective tissue and smooth muscle (Piscitelli et al. 2013).

Three groups of lymph nodes are associated with the respiratory tract in *T. truncatus*: the hilar nodes, the diaphragmatic node mass, and the bilateral marginal nodes, the last two groups serving as the primary lymphatic drainage of the lungs (Martony et al. 2017).

3. LITERATURE REVIEW ON MUCORMYCOSIS

3.1. Aetiology

According to the fungi taxonomic information available in a recent compilation by Wijayawardene et al. (2018), a total of fifty-six genera and 254 species are included in the order Mucorales. Twenty-five species have been identified as the aetiologic agent of MM in humans (Hassan and Voigt 2019). In dolphins, Mucorales fungi from seven different genera have been isolated in clinical infections: *Apophysomyces* spp., *Cunninghamella* spp., *Lichtheimia* spp., *Mucor* spp., *Rhizomucor* spp., *Rhizopus* spp. and *Saksenaea* spp. (Reidarson et al. 2018).

Moulds from the order Mucorales (mucormycetes) are filamentous and are characterized by wide coenocytic (i.e. aseptate), or sparsely septate, hyphae, with a variable angle of branching (Antachopoulos et al. 2015). Root-like rhizoids are produced by several genera and promote anchorage to the substrate (Markey et al. 2013).

Mucormycetes reproduce both sexually and asexually (figure 10). Specialized hyphae, called sporangiophores, usually end in the sac-like sporangium, in which the asexual spores (sporangiospores) are formed (Samanta 2015). When the sporangiospores mature, the wall of the sporangium ruptures, releasing the spores (Markey et al. 2013). Regarding sexual reproduction, most of the mucormycetes are heterothallic, producing thick-walled zygospores, which do not play any direct role in the pathogenesis of MM (Antachopoulos et al. 2015).

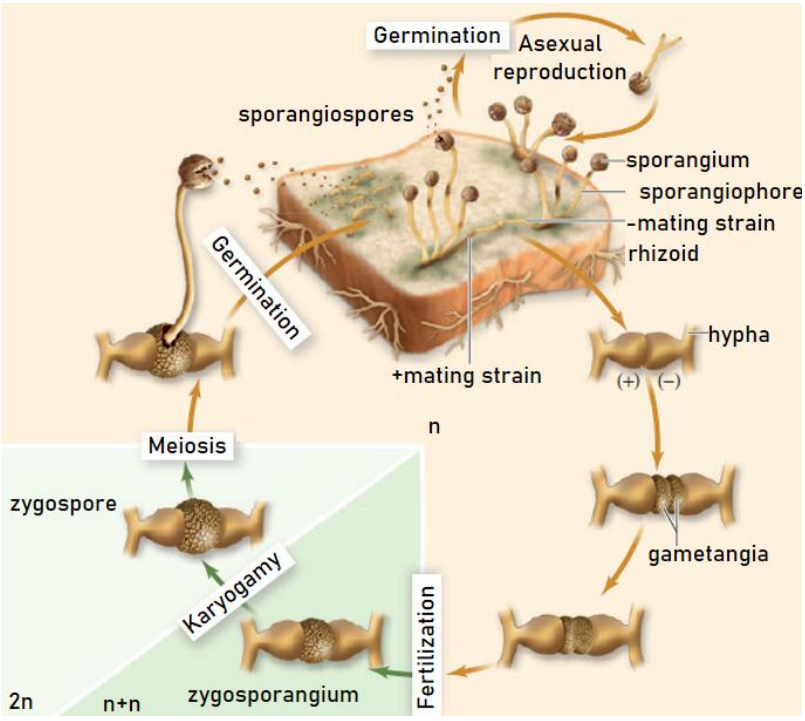


Figure 10 - Life cycle of a mucormycete - *Rhizopus stolonifera*. Adapted from Willey et al. (2017).

3.2. Epidemiology

In recent decades, many fungi have emerged as major causes of disease in both humans and marine mammals, probably triggered by opportunity and due to changes in host factors and/or fungal novelties (Reidarson et al. 2018; Hoog et al. 2018). The development of less toxic antifungal agents has contributed to the expansion of the use of these drugs. However, empirical and/or repeated or long-term therapies may have led iatrogenically to the emergence of resistances and this could undoubtedly have affected the landscape of fungal infections (Kanafani and Perfect 2007; Perlin et al. 2017). Another intriguing potential source of antifungal resistance is from the application of antifungal agents in agriculture (Perlin et al. 2017).

Most of the Mucorales fungi are cosmopolitan and widespread in the environment, usually found in soil, compost, animal faeces, agriculture debris, or other organic matter (Markey et al. 2013; Hassan and Voigt 2019). A change in the epidemiology of MM in humans has been observed, with a rise in incidence. This may be due to the growth of the number of immunocompromised patients and/or increased use of immunosuppressive therapies (Francis et al. 2017; Skiada et al. 2018). Another important factor is the selection pressure from antifungal prophylaxis with agents without activity against mucormycetes. It is also possible that the rise in incidence is related to increased awareness and a more thorough diagnostic approach, rather than a true increase in the number of cases (Francis et al. 2017).

As a group, mucormycetes represent the third most common cause of IFIs in humans (Kriengkauykiat et al. 2011). According to the Leading International Fungal Education portal (2019), the annual prevalence of MM is around 910,000 cases globally. The exact burden is not known, as it is not a reportable disease. Mortality rates of MM in humans declined in recent years but they remain high overall, between 76-96% (Hassan and Voigt 2019; Prakash and Chakrabarti 2019).

Recent studies have been developed regarding the epidemiology of MM, but only human cases have been taken into consideration. A recent survey has suggested a rise in the incidence of MM in cetaceans (Reidarson et al. 2018) but further studies need to be conducted.

3.2.1. Susceptibility to infection

There is a high prevalence of respiratory disease in both managed and wild dolphins worldwide (Martony et al. 2017). Although underlying causes need further investigation, many theories can help justify why dolphins are so susceptible to respiratory disease, in particular MM (figure 11).

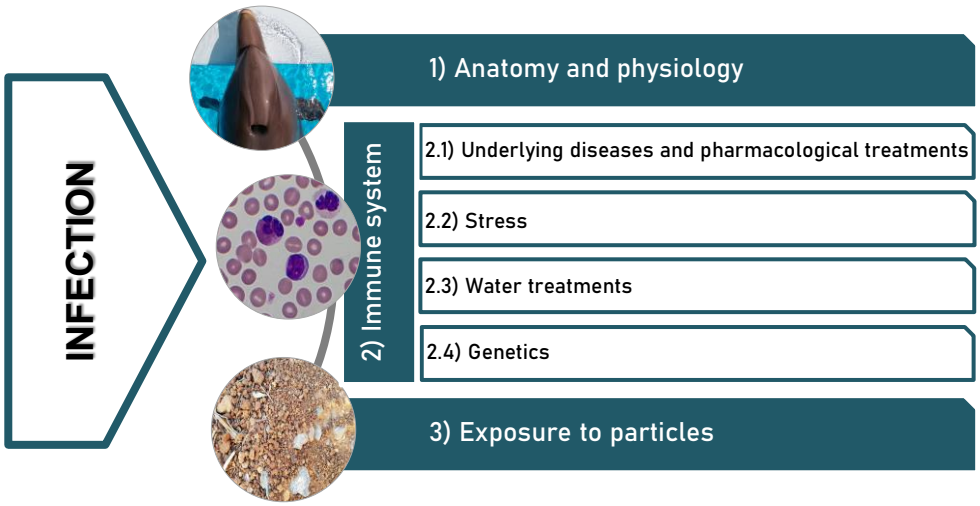


Figure 11 - Schematic representation of the susceptibility factors associated with respiratory mucormycosis in dolphins (Original).

3.2.1.1. Anatomy and physiology of the dolphin's respiratory system

As previously discussed, dolphins have undergone a series of evolutionary modifications, some of which contributing to the increased risk of respiratory disease (Reidenberg and Laitman 2008). The respiratory anatomy of dolphins enables the rapid exchange of large volumes of air during the brief encounters with the surface, with little frictional resistance to airflow, and hence allowing pathogens to be easily introduced into the lower respiratory tract (Venn-Watson et al. 2012). Additionally, dolphins lack protective hairs and turbinates to filter air (Reidenberg and Laitman 2008).

While in terrestrial mammals only about 10-15% of air is exchanged per breath, consisting mainly of air in the upper airways, in dolphins the exchanging capacity of air in the lungs is around 75–90%, which undoubtedly enables deep lung exposure to airborne threats (Irving et al. 1941 and Olsen et al. 1969 cited by Venn-Watson et al. 2012). Furthermore, the respiratory flow-rates of the gas exchanged can be very fast (130 and 30 L s^{-1} during expiration and inspiration, respectively) with a breath completed in 0.3 s (Fahlman et al. 2015).

3.2.1.2. Immune system

Unlike humans, in whom MM mainly threatens immunocompromised patients (Hassan and Voigt 2019), most MM cases in dolphins occurred in apparently immunocompetent individuals (Reidarson et al. 2018). This may, however, not be completely accurate, since basal immunity testing is still not frequently performed in marine mammal medicine (Le-Bert et al. 2017).

3.2.1.2.1. Underlying diseases and pharmacological treatments

In humans, MM is commonly found in patients suffering from the consequences of diseases such as diabetes mellitus, iron overload, haematological malignancy, solid organ tumours, autoimmune disorders, bone marrow or solid organ transplantation or prolonged corticosteroid or cyclosporine therapies (Hassan and Voigt 2019).

In most of the reported cases in managed dolphins, animals were considered clinically healthy before presentation with mucormycotic infection (Staggs et al. 2012; Reidarson et al. 2018). Concerning drug usage, immunomodulating drugs such as corticosteroids cannot be discarded as a contributory factor in the susceptibility to the disease (Reidarson et al. 2018). Furthermore, a greater use of broadspectrum antibiotics may disrupt the normal microbiota and lead to opportunistic mycoses (Martins et al. 2002; Reidarson et al. 2018). As in humans, some institutions have reported an increase in the incidence of MM coinciding with the use of voriconazole (Reidarson et al. 2018).

In wild individuals, immunosuppressive viruses, such as morbillivirus, are associated with generalized lymphoid depletion and are presumably contributing factors to the development of MM and other IFIs (Beineke et al. 2009; Reidarson et al. 2018).

3.2.1.2.2. Stress

The chronic response of stress has numerous impacts on the cardiovascular, neurologic, reproductive and immune systems, with innate and adaptive immunity exhibiting abnormal responses (Kozlowski 2012). Specifically, the immune dysfunction and hyperglycaemic state induced by glucocorticoids account for the predilection for IFIs including MM (Atkinson and Dierauf 2018).

Factors described in the previous subsection can be considered stressors along with poor husbandry, disgenesic conditions of the artificial habitat and restraint (Martins et al. 2002). In a free-ranging perspective, most likely stressors that can contribute to an impaired immune response are anthropogenic influences (noise, pollution, fisheries interactions), high parasitic loads, presence of predators, malnutrition and low body condition (Atkinson et al. 2015; Atkinson and Dierauf 2018).

3.2.1.2.3. Water treatments

In captive settings, integral reproduction of the conditions found in natural ecosystems is quite impossible even with recurrent monitoring of the water quality (Martins et al. 2002). The irritating action of disinfectants, such as chlorine, on the mucosa of the upper respiratory tract

may influence local and systemic immunity. In addition, the use of disinfectants has been associated with chronic allergic and other respiratory effects in humans, and while biological mechanisms contributing to these are unknown, there might be a modulation of the immune system (Vlaanderen et al. 2017).

According to the so-called “hygiene theory”, the lack of frequent exposure and stimulation of the immune system to potential pathogens due to the continuous presence of disinfectants may also facilitate abnormal immune-mediated responses of the host and hence the infection by opportunistic pathogens. Additionally, the continuous use of these disinfectants may lead to modifications of the normal marine microbiome, facilitating the selection and concentration of highly resistant microorganisms (Reidarson et al. 2018).

3.2.1.2.4. Genetics

Defence against infection is also shaped by the contributions of intrinsic factors such as the genetic constitution of an individual (Acevedo-Whitehouse and Bowen 2018). There are several genes encoding products central to the immune system, some of them exhibiting high levels of polymorphism. One of these genetic regions is the major histocompatibility complex (MHC), which has a key role in initiating an immune response and has been used to assess genetic diversity in free-ranging populations. MHC polymorphism has been associated with several factors important for population viability, including response to pathogens. Theoretically, populations with low polymorphism may have increased susceptibility to infectious diseases (Acevedo-Whitehouse and Bowen 2018; Manlik et al. 2019).

Factors shaping genetic diversity in dolphins include population size and social structure, exploitation history, inbreeding and environmental factors (Vachon et al. 2018).

3.2.1.3. Exposure to particles

Given the known widespread distribution of mucormycetes and the fact that infection is predominantly acquired by inhalation of sporangiospores, it is fair to recognize a wider contact to these pathogens in sites where the exposure to aerosolized soil particles is greater (Venn-Watson et al. 2012; Prakash et al. 2019). The proximity to dust from surrounding sites may be an important aspect, taking into consideration its role as an irritating agent of mucosae and the possibility of exposure to mucormycetes. This fact is crucial when considering construction projects in areas where many of these fungi thrive (Venn-Watson et al. 2012). Also, a major dirt upheaval, sometimes linked to natural disasters, have been associated with increased rates of infection in managed dolphins (Staggs et al. 2012).

3.3. Pathogenesis

3.3.1. Transmission and mucormycete-host interface

Mucormycetes are considered opportunistic fungi, which under certain conditions act as pathogens. Being a non-transmissible disease, only those who have been exposed to mucormycetes spores in the environment can be infected (Fisher 2018).

The most common portal of entry for mucormycetes is the respiratory tract, through inhalation of sporangiospores. Spores may also infect individuals through the gastrointestinal tract or sites of skin breakdown (Farmakiotis and Kontoyiannis 2016). A range of local factors is then responsible for spore germination, such as pH and nutrient availability (Roilides et al. 2012).

Once spores have overcome the non-specific barriers (skin or mucosal surfaces), innate immune effectors such as mononuclear cells and polymorphonuclear leukocytes (PMNs) are responsible for damaging spores and preventing germination (Roilides et al. 2012). Macrophages have the capacity to suppress resting spore germination but they are unable to kill them. Contrarily, swollen spores and hyphae are susceptible to damage by these cells. PMNs do not exert activity against resting spores but can damage swollen spores and hyphae (figure 12) (Ghuman and Voelz 2017).

Having crossed the endothelium layer, mucormycetes enter the bloodstream (angioinvasion). Herein, platelets may be able to suppress germination and damage hyphae (Ghuman and Voelz 2017). The inability to supress germination before the onset of filamentous growth leads to vessel thrombosis and necrosis (Ibrahim et al. 2012).

There is limited evidence for a major role of the adaptive immune system against MM (Ibrahim and Voelz 2017).

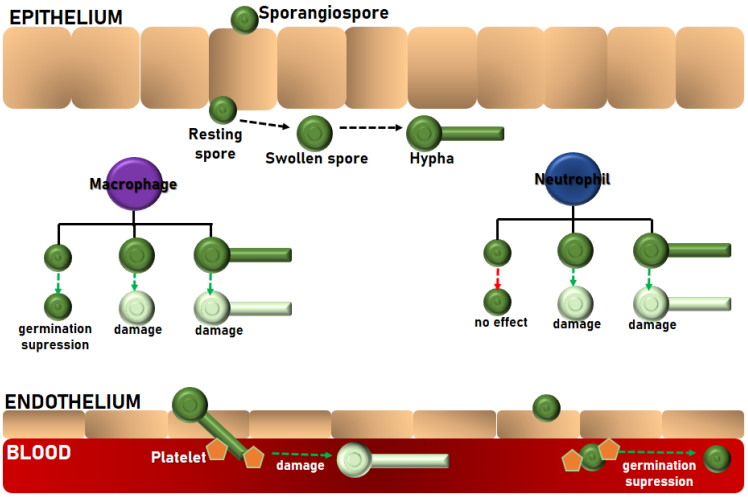


Figure 12 - Activity of the innate response effectors against mucormycetes (schematic). Adapted from Ghuman and Voelz (2017).

3.3.2. Virulence traits of mucormycetes

General fungal virulence can be attributed to a myriad of factors, including the adaptation to the environmental conditions of the host tissues and physiological stressors (temperature, hypoxia and osmolarity) (Fisher 2018). Highlights of specific virulence traits of some mucormycetes are featured in table 1.

Table 1 – Virulence traits of mucormycetes. Adapted from Ibrahim and Voelz (2017).

VIRULENCE TRAITS	FUNCTION
Activation of EGRF (epidermal growth factor) and PDGF (platelet-derived growth factor) signalling	Invasion of the epithelium
Fungal ligand-protein CotH	Invasion of the endothelium
Iron permease and ferrioxamine receptors	Acquisition of host iron
Toxins	Host cell damage
Spore size	Faster germination
Calcineurin pathway	Dimorphic transition to hyphae

3.3.3. Macroscopic lesions

MM is an infection that can affect several organ systems, with an acute or chronic progression. Based on the anatomical site of involvement, MM in humans is classified into rhino-orbito-cerebral, pulmonary, cutaneous, gastrointestinal, renal and disseminated forms (Prakash and Chakrabarti 2019). The most common presentations in dolphins appear to be pulmonary and cerebral, followed by the cutaneous and disseminated forms. In this chapter, only the pulmonary presentation is reviewed, commonly referred to as respiratory MM since the trachea and the bronchi are frequently involved (Reidarson et al. 2018).

Mucormycetes may invade and distort components of the trachea and bronchi, such as the cartilaginous rings, resulting in a significant reduction of the luminal diameter. Extensive mural fibrosis and inflammation may lead to an almost complete occlusion (Kinsel and Briggs 2005; Delaney et al. 2012). Fistulous tracts, multiple variably-sized granulomas, haemorrhages and multifocal to regional ulceration with friable, adherent plaques are all features of the trachea and bronchi described in MM cases in cetaceans (Robeck and Dalton 2002; Kinsel and Briggs 2005; Abdo et al. 2012; Delaney et al. 2012).

Lungs have been described as uniformly dark red or brown, with a firm texture (Robeck and Dalton 2002; Kinsel and Briggs 2005). Small, white, firm nodules have been observed throughout the lungs, as well as multiple areas of consolidation or liquefaction on lung section (Robeck and Dalton 2002; Abdo et al. 2012; Delaney et al. 2012).

Adherence of the lungs to the pleura may exist (Robeck and Dalton 2002). Raised foci have been found in the visceral pleura (Kinsel and Briggs 2005). Additionally, multiple white or yellowish plaques have been described in the thoracic wall and on the diaphragm (Robeck and Dalton 2002). Bronchial lymph nodes have been found markedly enlarged and/or oedematous (Kinsel and Briggs 2005; Abdo et al. 2012).

3.4. Diagnostic approach to respiratory mucormycosis

The recommendations presented in this dissertation regarding diagnosis and treatment are according to the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) and European Confederation of Medical Mycology (ECMM) Joint Clinical Guidelines (Cornely et al. 2013), as well as recent updates in managing MM in humans and dolphins.

As infections caused by mucormycetes may be rapidly fatal, early diagnosis is crucial to promptly initiate treatment and avoid tissue invasion (Walsh et al. 2012).

3.4.1. History, clinical signs and physical examination

A thorough history of the animal should consider factors such as a history of chronic fungal disease, treatments with broadspectrum antibiotics, voriconazole or glucocorticoids, immunosuppression and other susceptibility factors previously described (Delaney et al. 2012; García-Párraga et al. 2016).

Development of minimal to no clinical signs is expected in dolphins with MM (Townsend, Staggs, et al. 2012; Reidarson et al. 2018). When present, clinical signs are non-specific and commonly limited to hyporexia or anorexia (Dalton and McBain 1993; Staggs and Townsend 2006; Delaney et al. 2012; Staggs et al. 2012; Reidarson et al. 2018). Although anorexia may be the first indication of disease, in some cases food intake was described as normal and lack of appetite was only registered days after other initial presenting signs (Robeck and Dalton 2002). Behavioural changes, including lethargy, are also commonly found (Dalton and McBain 1993). Respiratory clinical signs may include harsh respiratory sounds, respiratory wheezes, abnormal goose-honking sounds, tachypnea, progressive dyspnoea and reduced exercise capacity (Robeck and Dalton 2002; Kinsel and Briggs 2005; Brudek-Wells et al. 2011; Delaney et al. 2012; García-Párraga et al. 2016).

Direct visualization of the nasal passages may provide insight into granulomatous plaques (Brudek-Wells et al. 2011). Auscultation may be a useful tool but has significant limitations because of the thick blubber layer, the rapid respiratory cycle and the loud transmitted sounds than can obliterate subtle rales (Nollens et al. 2018).

3.4.2. Clinical pathology

In the initial stages of MM, laboratory findings are usually unremarkable. Moreover, occasional haematological and biochemical changes are indistinguishable from bacterial, viral or other fungal infections (Reidarson et al. 2018). The parameters that have proven to be most helpful as indicators of inflammatory disease in cetaceans, with abnormal values described in MM cases, are the total white blood cell (WBC) and differential counts, serum fibrinogen, erythrocyte sedimentation rate (ESR), serum iron and alkaline phosphatase (ALP). The latter is also considered a reliable prognostic indicator in cetaceans (Nollens et al. 2018).

Most mucormycotic cases in the literature describe a mild leukocytosis, although both normal and extremely elevated counts may be seen (Robeck and Dalton 2002; Kinsel and Briggs 2005; Brudek-Wells et al. 2011; Wells et al. 2012; Clauss et al. 2014). Differential WBC counts may reveal neutrophilia or more rarely monocytosis (Robeck and Dalton 2002). A high fibrinogen concentration has been described as well as a rapidly increasing ESR and low serum iron values (Robeck and Dalton 2002; Brudek-Wells et al. 2011; Abdo et al. 2012; Townsend, Newton, et al. 2012; Wells et al. 2012; García-Párraga et al. 2016). Lastly, decreasing values of ALP have been noted (Abdo et al. 2012; García-Párraga et al. 2016).

3.4.3. Conventional laboratory methods

Direct microscopy, histopathology and culture are considered the cornerstones of diagnosing MM (Skiada et al. 2018). Adequate specimens for diagnosis of respiratory MM include sputum samples, bronchial lavages and biopsies of lesions. Despite the angioinvasive capacity of mucormycetes, blood cultures are of no benefit (Lass-Flörl 2009).

3.4.3.1. Direct microscopy

Direct microscopy of clinical specimens may allow a rapid presumptive diagnosis of MM, especially if using optical brighteners, such as Blankophor and Calcofluor-white (Cornely et al. 2013). Visualization of mucormycetes may be problematic due to the difficulty in extracting fungal elements from invaded tissues. Presence of mucormycetes must be considered significant, even with negative culture results, especially in the presence of inflammatory cells (Lass-Flörl 2009). Direct microscopy may permit differential diagnosis from other mycoses such as candidiasis and aspergillosis, the latter of utmost importance, as antifungal therapy may differ, whereas underlying factors and clinical presentation are often alike (Skiada et al. 2018). Morphological traits and differences between mucormycetes, *Aspergillus* spp. and *Candida* spp. are reviewed in table 2.

Table 2 – Main morphological characteristics of filamentous fungi: mucormycetes, *Aspergillus* spp. and *Candida* spp. (Ribes et al. 2000; Severo et al. 2010; Samanta 2015).

Characteristic	Mucorales	*Aspergillus* spp.	*Candida* spp.
Other forms	Rare	-	Yeast and pseudohyphae
Hyphae width	Wide (6-25 µm)	Narrow (3-6 µm)	Narrow (2-3 µm)
Hyphae branching	Random	Regular, acute angle	Random
Hyphae septa	Uncommon	Common	Common
Blastoconidia	Absent	Absent	Present
Sporulation	Absent	May be present	Absent

3.4.3.2. Histopathology

Histopathological examination of tissue specimens allows the identification of hyphae and distinguishes the presence of the fungus as a pathogen from a culture contaminant (Skiada et al. 2018). Mucormycete hyphae may become gnarled in the tissue sections, which may difficult identification (Lass-Flörl 2009). Tissue histopathology is usually dominated by a neutrophilic or granulomatous inflammation, along with infarcts and angioinvasion (Skiada et al. 2018). Stains of fixed tissues include haematoxylin and eosin (HE), Gomori methenamine-silver (GMS) or periodic acid-Schiff (PAS) (Lass-Flörl 2009).

3.4.3.3. Culture

Culture of specimens is strongly recommended although the sensitivity is not optimal (Cornely et al. 2013). Even when hyphae are seen in histopathology, cultures are only positive in 50% of the cases (Walsh et al. 2012).

All mucormycetes grow rapidly (3 to 7 days) on most fungal culture media, such as Sabouraud agar and potato dextrose agar, incubated at 25 ºC to 30 ºC. Growth at 37 ºC could add clarification to the diagnosis and could be considered a pathogenicity marker (Lass-Flörl 2009).

Mucormycetes colonies are described as a woolly mycelium, which grows to fill the entire plate, often raising the lid (“lid lifters”) (Antachopoulos 2015). According to the isolate, surface colouration may vary (white, brown, grey or black) (Lass-Flörl 2009). The reproductive structures and hyphae morphology features are also essential for differentiation of genera and species (Quinn et al. 2016).

It is important to note that these fungi are easily airborne and growing of a mucormycete from a respiratory sample may not always be a cause of concern (Morris et al. 2011).

3.4.4. Susceptibility testing

Although not necessarily a diagnostic tool, susceptibility testing usually comes as a follow up to a positive fungal culture. Clinical breakpoints have not been defined for mucormycetes due to limited data in the correlation between minimum inhibitory concentration (MIC) values and clinical outcome (Espinel-ingroff et al. 2015; Espinel-ingroff and Turnidge 2016). MIC values should then be compared to epidemiologic cut-off values (ECVs) to distinguish the wild type population (without known mechanisms of resistance) and the non-wild type population (with mechanisms of resistance) that are present in the MIC distribution of a species and agent combination (Lockhart et al. 2017).

Mucormycetes have shown distinct antifungal susceptibility profiles (Alastruey-izquierdo et al. 2009). Overall, amphotericin B, posaconazole and isavuconazole are the only agents available with *in vitro* activity against these fungi. Itraconazole, ravuconazole and terbinafine have shown some activity against certain strains (Alastruey-izquierdo et al. 2009).

3.4.5. Serological assays

Unlike Aspergillus galactomannan index and 1,3-β-D-glucan (BDG) assays, commonly used for the diagnosis of aspergillosis, there are no standardized serological assays for identification of mucormycetes (Dadwal and Kontoyiannis 2018). Since BDG is a common component of the cell wall of a wide variety of fungi but not of mucormycetes, these tests may play an important role in eliminating differential diagnosis (Cornely et al. 2013).

An experimental enzyme-linked immunosorbent assay (ELISA) for serum antibodies was developed for the early detection of *Apophysomyces* spp. infections in cetaceans. This test seems to be quite sensitive and has potential to diagnose occult MM caused by this agent and to provide monitoring for treatment and recurrence of infection (Barger et al. 2012; Wells et al. 2012).

3.4.6. Molecular-based methods

Molecular-based diagnostic assays are not widely used in the diagnosis of MM since they lack thorough clinical evaluation. Some of the molecular assays already tested include conventional polymerase chain reaction (PCR) and DNA sequencing, melt curve analysis of PCR products, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and restriction fragment length polymorphism analyses (RFLP) (Dadwal and Kontoyiannis 2018).

The majority of the molecular assays target either the internal transcribed spacer (ITS) or the 18S rRNA genes (Millon et al. 2019). Although panfungal primers are commonly used, experimental Mucorales-specific PCRs have been developed (Bernal-Martinez et al. 2013).

3.4.7. Imaging modalities

Imaging studies can be non-specific, but aid in the evaluation of the severity and extension of respiratory infections. Although only computed tomography (CT) is included in the ESCMID/ECMM Guidelines as part of the approach to MM, other imaging modalities are here reviewed, since they have been used in the diagnosis of MM in dolphins.

3.4.7.1. Thoracic radiography

Thoracic radiographs in mucormycotic cases may reveal no abnormalities (Brudek-Wells et al. 2011). In a reported case of a *T. truncatus*, initial radiographs were indeed unremarkable. However, follow-up radiographs revealed an increased bronchial pattern and two nodules in the left cranial lung field. After beginning antifungal therapy, follow-up radiographs again revealed no abnormalities, although sputum cultures remained positive (Clauss et al. 2014). Other descriptions include an area of increased density in the laryngeal region (poorly defined abscess), and an intratracheal mass (figure 13) (Kinsel and Briggs 2005; García-Párraga et al. 2016).

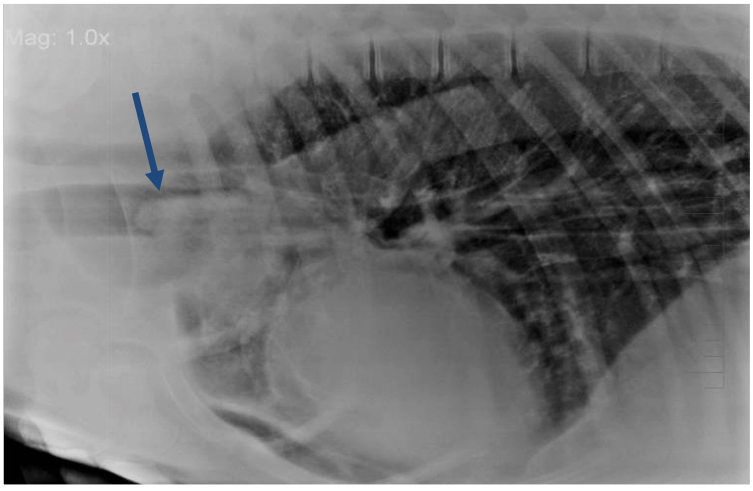


Figure 13 – Left-right lateral thoracic radiograph of a *Tursiops truncatus* showing a mucormycete-origin intratracheal mass (arrow). Courtesy of Dr Daniel García-Párraga, Oceanogràfic de Valencia.

3.4.7.2. Thoracic ultrasonography

On thoracic ultrasonography (US), only the periphery of the lungs is possible to evaluate due to the reverberation artefact at the surface of normal air-filled lungs (Saviano 2013). The

possibility to evaluate deeper structures may indicate the presence of pathological processes in the peripheral lung (Martony et al. 2017).

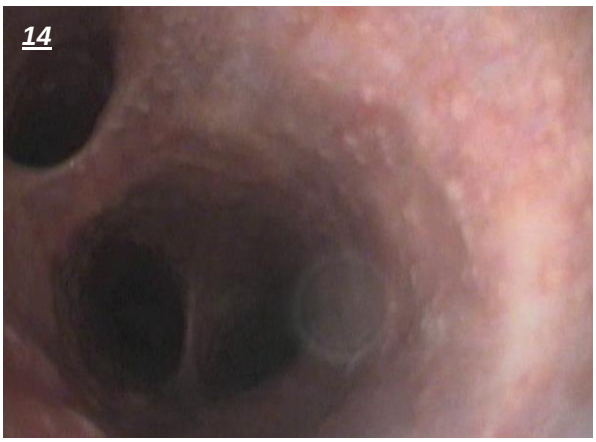
It is important to emphasize that a normal US pulmonary examination does not rule out MM, since lesions may exist deep within the pulmonary parenchyma (Robeck and Dalton 2002; Dennison and Saviano 2018). Of the lung-associated lymph nodes, the bilateral marginal nodes are of particular interest since they provide lymphatic drainage to the lungs and are superficial enough to allow US evaluation (Martony et al. 2017).

Even though thoracic US has been used as part of the medical management of MM, few descriptions exist in the literature. Abnormal findings comprise of an “irregular pleura” (Reidarson et al. 2018) and an “anechoic cavitation of the lung in the right thorax” (Robeck and Dalton 2002).

3.4.7.3. Respiratory endoscopy

Bronchoscopy using flexible instrumentation is considered an essential tool as part of a set of diagnostic procedures used in respiratory disease in cetaceans (Bonn and Dover 2018). There are reports describing individual cases of dolphins with suspicion of respiratory disease where bronchoscopy with additional biopsy sampling and/or washings was performed enabling a mucormycete isolation (García-Párraga et al. 2016; Reidarson et al. 2018).

Macroscopic lesions found with bronchoscopies include a “moderate exudate extending into the left bronchus to at least the third generation airways” (Clauss et al. 2014), multiple small white nodules in the trachea and bronchi (figure 14), and an “mucus-fibrinous sessile mass (…) occluding most of the lumen around the carina” (figure 15) (García-Párraga et al. 2016).



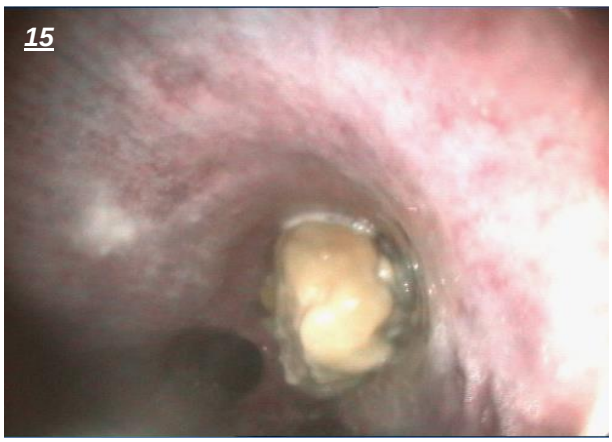


Figure 14 - Endoscopic image of a *Tursiops truncatus* with multiple nodules in the trachea and bronchi (*Lichtheimia corymbifera*) (2016).
Figure 15 - Bronchoscopy on a *Tursiops truncatus* with an intratracheal mass (*Rhizopus microsporus).* Courtesy of Dr Daniel García-Párraga, Oceanogràfic de Valencia.

3.4.7.4. Computed tomography

Computed tomography has been used in cases of MM to help evaluate the extension of respiratory damage (figure 16) (Clauss et al. 2014; García-Párraga et al. 2016). As an example, a CT of the thorax of a *T. truncatus* with MM revealed a multifocal interstitial pattern with occasional nodules (Clauss et al. 2014).

Considering the common use of this imaging technique when approaching MM in humans, radiological patterns described are important to revise. Sequential morphologic changes include the reversed halo sign, nodule or mass with halo sign and, finally, central necrosis and air-crescent sign (Pilmis et al. 2019). Pleural effusion, thick walled cavities, lymphadenopathy and pneumothorax are also described (Prakash and Chakrabarti 2019). All of these findings are however non-specific.

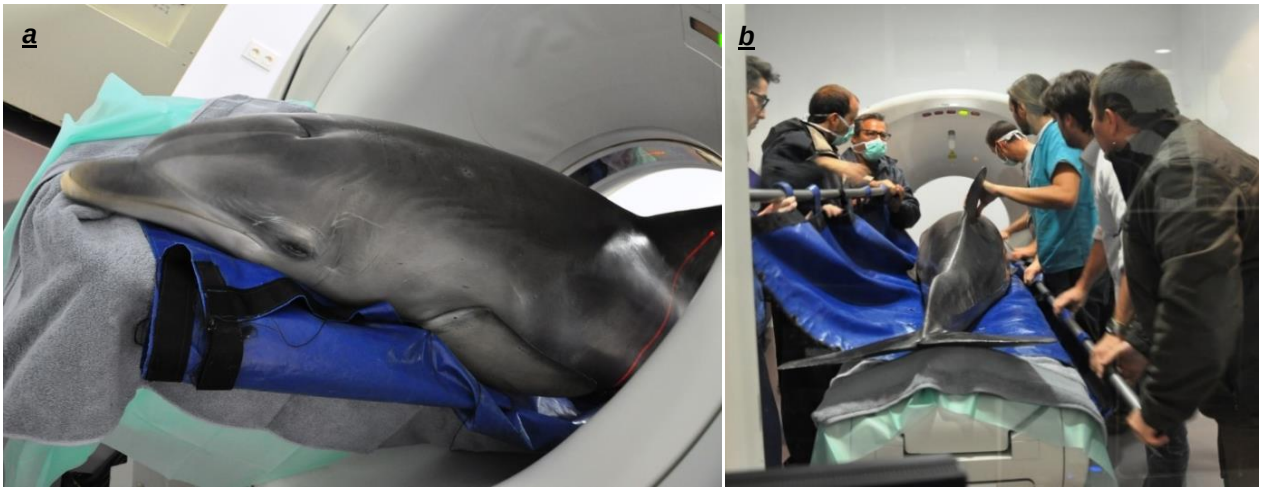


Figure 16 - Computed tomography procedure of a *Tursiops truncatus* with respiratory mucormycosis. Courtesy of Dr Daniel García-Párraga, Oceanogràfic de Valencia.

3.5. Treatment approach to respiratory mucormycosis

Successful management of MM in humans is based on a multimodal approach, with correction of underlying predisposing factors, early antifungal therapy, surgical debridement of all infected tissues, and use of adjunctive therapies. There is no standard duration of treatment for MM and it should be evaluated on an individual basis. Despite this, it is strongly recommended to continue antifungal therapy until resolution of all clinical, laboratory and imaging signs as well as a permanent reversal of risk factors (Cornely et al. 2013).

3.5.1. Antifungal therapy

3.5.1.1. Amphotericin B

Amphotericin B (AmB) is a polyene antifungal agent available in a conventional formulation, which is a complex with deoxycholate, and newer formulations that are lipid-based complexes. These lipid formulations can be administered at higher doses to produce greater efficacy with less toxicity (Davis and Maxwell 2018). They include a colloidal dispersion, a lipid complex and a liposomal complex.

The major action of AmB is to bind to ergosterol of the fungi cell membrane, making it more permeable, resulting in leakage of cell electrolytes and death. It also seems to have an important role in macrophage activation (Lewis and Fothergill 2015).

AmB is usually fungistatic but may be fungicidal in high concentrations or against very susceptible organisms, including certain mucormycetes (Alastruey-izquierdo et al. 2009; Lewis and Fothergill 2015). Some commonly used drugs that may interact with AmB include azole antifungal agents and aminoglycosides (Davis and Maxwell 2018).

There is scarce information concerning the pharmacokinetics of AmB, especially in veterinary medicine. This drug is registered for use in humans only and it lacks an oral formulation because it is poorly absorbed from the gastrointestinal tract and therefore must be given intravenously (IV), locally, or intrathecally (Lewis and Fothergill 2015).

The most common adverse effect associated with AmB therapy is nephrotoxicity. AmB binds to cholesterol in the renal tubular cells, which results in electrolyte leakage and tubular acidosis. Renal vasoconstriction and impaired acid excretion may also contribute to the nephrotoxicity. Other adverse effects observed in animals include phlebitis, fever, nausea, and vomiting (Davis and Maxwell 2018). In dolphins, only nephrotoxicity seems to have been reported (Townsend et al. 1996; Townsend, Newton et al. 2012).

Liposomal AmB is the drug of choice as first-line therapy for MM in humans, given IV (Cornely et al. 2013). In order to achieve a greater systemic distribution to the lungs, higher doses of AmB are needed and this may place individuals at increased risk for adverse drug reactions. An aerosolized formulation may allow a lower dose to be used while maintaining high lung concentrations (Mihara et al. 2013).

The regimen most used in dolphins concerns the use of nebulized AmB (20 mg total dose BID – twice a day - with distilled water) (Reidarson et al. 2018). Other reports regarding the use of this antifungal drug intravenously are scarce (Townsend et al. 1996; Townsend, Newton et al. 2012).

3.5.1.2. Posaconazole

Posaconazole (PCZ) is an azole antifungal drug, available as an oral suspension (OS), delayed-release tablets (DRTs) and an intravenous formulation (IVf). DRTs prevent the release of the drug in the low pH environment of the stomach, losing their structural integrity when cut, crushed or chewed (Guarascio and Slain 2015). There are no PCZ veterinary formulations.

Like all azoles, PCZ exerts its effect on the fungi cell membrane by inhibiting ergosterol synthesis, through inhibition of the cytochrome P-450 dependent enzyme, sterol 14α-demethylase (Davis and Maxwell 2018). PCZ exhibits organism-dependent fungicidal activity and may be considered fungicidal against some mucormycetes (Lewis and Fothergill 2015). Interactions with drug metabolism are found when PCZ is administered concurrently with CYP3A4 inhibitors or inducers (Davis and Maxwell 2018).

PCZ has an overall safe profile, causing little or no nephrotoxicity. Adverse effects reported in humans include nausea, vomiting, diarrhoea and a mild asymptomatic hepatic enzyme level elevation (Edwards 2018). There are no described adverse effects in cetaceans, at least in published articles.

PCZ is mainly recommended for salvage treatments of MM, which may be necessary because of the refractoriness of disease and/or intolerance towards previous antifungal therapy. Few data exist regarding the use of PCZ as first-line therapy (Pilmis et al. 2019). Although no pharmacokinetic studies have been developed in dolphins, PCZ (5 mg/kg PO – *per os* - BID of the OS) seems to be the drug of choice when dealing with MM, given the overall good clinical results (Reidarson et al. 2018).

3.5.1.3. Isavuconazole

Isavuconazole is the newest of the triazoles and there are no reports concerning its usage in cetaceans. It is available in oral and intravenous formulations (Seyedmousavi 2018). Isavuconazole has been suggested as an alternative to AmB in first-line treatment of MM or even in salvage treatments (Cornely et al. 2013).

Isavuconazole seems to show several advantages to other azoles, including linear pharmacokinetics, less toxicity, less drug interactions and an improved oral bioavailability (Seyedmousavi 2018).

3.5.1.4. Other antifungal drugs and drug combinations

There is a lack of clinical data of managing MM with other agents or a combination of different antifungal agents. Although drug combinations have become an increasingly common

practice, small retrospective studies cannot serve as the basis for introducing combination therapy as the standard of care (Spellberg et al. 2012).

Data regarding the efficacy of AmB and PCZ combination are contradictory. Some *in vitro* studies have shown synergy for this combination but *in vivo* studies of MM showed no benefit (Kyvernitakis et al. 2016). Moreover, azole-induced depletion of fungal cell membrane ergosterol seems to result in fewer sites on which AmB can bind. Many clinicians do recommend, based on the slower onset of action of azole antifungals, initial therapy with AmB, followed by longer therapy with an azole therapeutic protocol (Cornely et al. 2013).

Echinocandin agents such as caspofungin lack *in vitro* activity against mucormycetes but some studies suggest that combination therapy with AmB may improve survival (Cornely et al. 2013). In addition, as previously stated, itraconazole, ravuconazole and terbinafine seem to show some *in vitro* activity against certain strains of mucormycetes. Currently, there is no evidence-based recommendation for the usage of any of these antifungal agents *per se* or in combination with AmB, PCZ or isavuconazole in the treatment of MM.

Interestingly, some successful cases of dolphins with MM describe the use of voriconazole and/or itraconazole, with or without terbinafine combined. Combination of fluconazole, terbinafine and nebulized voriconazole has also shown positive results. The combination of AmB and PCZ has been described, in some cases with terbinafine (Reidarson et al. 2018).

3.5.1.5. Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is a valuable addition to antifungal therapy. TDM in humans is generally indicated for triazoles and the nucleotide flucytosine (Ashbee et al. 2014). In dolphins, TDM may also be an important adjunct to the routine administration of other agents, such as AmB, given the lack of pharmacokinetic studies.

TDM may increase the probability of a successful outcome, prevent drug-related toxicity and potentially prevent the emergence of antifungal drug resistance. This is especially relevant in clinical circumstances such as significant pharmacokinetic variability, when changing pharmacokinetics, when using interacting drugs or in poor prognosis diseases (Ashbee et al. 2014).

Given the role of PCZ as the drug of choice for treatment of MM in dolphins, a specific review of the TDM of this drug is needed. In contrast to the OS, the newer formulations (DRTs, IVf) are expected to result in more stable concentrations and within the therapeutic range, hence the non-interchangeability of DRT and OS formulations (Guarascio and Slain 2015).

As with other azoles, the parameter that is best associated with clinical success is the total exposure, measured by the area under the curve in relation to the MIC (AUC/MIC ratio) (Goodwin and Drew 2008). Little consensus exists regarding the PCZ concentration target although some *in vitro* studies suggest maintaining serum concentrations above 1.0 µg/mL (Jang et al. 2009; Ashbee et al. 2014; Lenczuk et al. 2018). This does not however specifically incorporate the MIC of the invading pathogen and PCZ is shown to have a variable *in vitro* activity against mucormycetes. In a study of 131 clinical isolates, the MICs of PCZ for various Mucorales species varied between 1.0 and 8.0 µg/mL (Dannaoui et al. 2003).

Given the lack of clinical breakpoints and the likely impossibility of AUC/MIC monitoring, clinicians may need to focus on the MIC value of the mucormycete isolated, integrate what is known about pharmacokinetics and pharmacodynamics of PCZ and consult literature concerning the efficacy of PCZ in similar clinical circumstances (Skiada et al. 2018).

Serum concentrations of PCZ have been measured in dolphins with MM receiving therapy. Results available in the literature where a 5 mg/kg BID dosage of the OS was used are reviewed in table 3.

Table 3 – Serum levels of posaconazole in dolphins with mucormycosis.

Species	Serum levels (mg/L) d= days after beginning PCZ therapy	Reference
Lagenorhynchus obliquidens	2.24 (1d), 4.26 (134d), 5.09 (252d)	Walters et al. 2009
T. truncatus	5.25 (49d), 1.49 (7 days after discontinuing)	Walters et al. 2009
T. truncatus	1.19-5.15	Wells et al. 2012
T. truncatus	~4.0-5,9 / >6.0 when associated to fish oil	García-Párraga et al. 2016

3.5.2. Surgical debridement

Surgical debridement of mucormycotic lesions has to be extensive, involving all necrotic and surrounding tissue and repeated surgical procedures may be necessary (Pilmis et al. 2019). In dolphins, only endoscopic procedures have been performed. A case report regarding a bottlenose dolphin with an almost completely obstructive tracheal mucormycete-caused mass was removed under sedation and local anaesthesia, using an endoscopic nitrogen cryosurgery probe and cauterizing with an argon plasma unit (García-Párraga et al. 2016). Another report concerns the use of balloon bronchoplasty in a *T. truncatus* with airway stenosis. In this case, however, no diagnosis was obtained, although fungal infection was suspected (Renner et al. 2014).

3.5.3. Adjunctive therapies

Several pilot studies have been developed regarding the use of new weapons against MM in humans, most of them not yet proven to be clinically meaningful.

Considering the iron overload as a risk factor, the use of iron chelators without xenosiderophore activity (e.g. deferipone, deferasirox) has been discussed as an adjunctive therapy (Prakash and Chakrabarti 2019). Additionally, hyperbaric oxygen treatment has been reported in small numbers of human patients, where a high oxygen pressure is thought to improve neutrophil function, inhibit the growth of mucormycetes and improve wound healing. (Cornely et al. 2013)

Other adjunctive therapies include the investigational drug VT-1161, which seems to have an inhibitor selective activity against the fungal sterol 14α-demethylase, and lovastatin, a statin drug that appears to inhibit the growth of some mucormycetes (Cornely et al. 2013). Protective immunity may be achieved by correcting immune deficiencies or inhibiting virulence strategies employed by mucormycetes (Sipsas et al. 2018). Adjunctive cytokine therapies, colony-stimulating factors, interferon-gamma and WBC transfusions have all been used to enhance immune responses (Skiada et al. 2018).

3.5.3.1. Ozone therapy

Ozone therapy is a complementary therapeutic technique based on the use of an oxygen-ozone mixture (International Scientific Committee of Ozone Therapy - $ISCO_3$ 2015). Ozone therapy is intrinsically dependent on the antioxidant capacity of the individual, leading to the formation of free radicals, which are continuously produced during physiological conditions, stimulating this natural protection system (Bocci 2006; Zotti et al. 2008).

Medical ozone can be applied locally or parenterally and the various routes of application can be used alone or combined, in order to achieve a synergistic effect ($ISCO_3$ 2015). The ozone gas mixture can be used *per se* or associated with vehicles (e.g. blood, saline solution, water, oils). In the few reports of ozone therapy use in dolphins, there are both local and systemic routes of application described, the latter including oral (e.g. Ozolife Softgels®), rectal, IV and IM administrations (Infante 2016; Reidarson et al. 2018).

Some studies specifically studied the fungicidal properties of ozone against yeasts (e.g. *Candida albicans*) and moulds such as *Aspergillus* spp. (Zotti et al. 2008). There are no reports available in the literature concerning the systemic use of medical ozone in the management of MM in humans. In dolphins, ozone therapy has been described as an adjuvant therapy in some

MM cases, using an IV route (20 mg/kg, in six sessions) or using both IV and rectal administration (total of 3400 g, in twenty-four sessions) (Reidarson et al. 2018).

3.6. Antifungal prophylaxis and other preventive measures

Given the uncommon nature of this infection, primary antifungal prophylaxis for MM is not usually recommended (Cornely et al. 2013). In patients with previous mucormycotic infections, a secondary prophylaxis with PCZ may be deliberated, especially considering the possibility of relapse. An example is the consolidation therapy with PCZ after the theoretical resolution of infection with AmB (Kontoyiannis and Lewis 2016). Currently, there is no vaccine available for MM, although it may be a promising strategy against this and other IFIs.

As part of a preventive medical plan, a multi-diagnostic fungal surveillance programme for managed cetaceans has been proposed (Staggs 2017). It is of paramount concern to keep a thorough update on respiratory health, namely through microscopic evaluation of sputum samples, behavioural observation, fungal cultures and serology testing. Susceptibility factors previously described should be taken into account. Providing a balanced diet with nutritional supplements, social well-being and a stress-free environment is essential to keep animals with a functional immune system (Levin et al. 2018).

Given the wide distribution of sporangiospores in the environment, it may be difficult to avoid inhalation. Nevertheless, personnel must take measures to decrease exposure to any dust, aerosol, or poor air quality in general, which may lower the chances of not only developing MM but also respiratory disease as a whole (Walsh et al. 2002). Despite the increasing efficiency of some cleaning techniques, these may result in decreased air quality. For example, pressure washing easily aerosolizes debris and it is critical to not be used near cetaceans, as well as combustion engines and sprinkler systems that form spray or mist. Moreover, routinely changed filters should be used in air circulation systems and air exchange rates should be adequate to avoid the air becoming stale. Indoor environments must be monitored for air contaminants. Lastly, cleaning procedures should be reinforced during construction works or cleaning of air ducts (Walsh et al. 2002).

4. CLINICAL APPROACH TO RESPIRATORY MUCORMYCOSIS – A CASE REPORT

4.1. General considerations

For practical issues and to facilitate the reader's understanding, the description of the clinical case here reported considers a specific timeframe. This goes from the first diagnostic indication of a fungal aetiology (30th of May 2018 – day 1) until the last day of the author's curricular externship at Zoomarine – Mundo Aquático S.A. (30th of April 2019 – day 336). Past medical history is also included for a complete review of the medical case.

Taking into consideration the specificities of cetacean medicine and for the sake of simplicity, the conditions in which the diagnostic methods and treatment techniques took place are explained throughout the description of the clinical case.

A graphic overview of the evolution of the indicators of inflammatory disease during medical management is presented in the end of the subsection 4.2. (graphic 2). Furthermore, a schematic representation of the crucial points regarding the clinical approach of this case is available in annexe 1, along with a review on haematological and biochemical reference intervals of *T. truncatus* (annexe 2).

4.2. Clinical case

A male bottlenose dolphin calf was born at Zoomarine on the 13th of June 2017 (figure 17). This dolphin, as well as his mother and other six bottlenose dolphins, were housed at "Delfinário do Sam", an outdoor dolphinarium with five interconnected pools. One of the pools was a maternity pool, with a glass observation window and a lifting platform used when mother and/or calf needed to be handled (figure 18). Water was obtained through a catchment in sea waters and put through chlorine disinfection. Water quality was monitored and documented at least two to three times daily for basic chemical parameters and weekly for microbiological counts, in accordance with EAAM, EAZA and AMMPA guidelines.

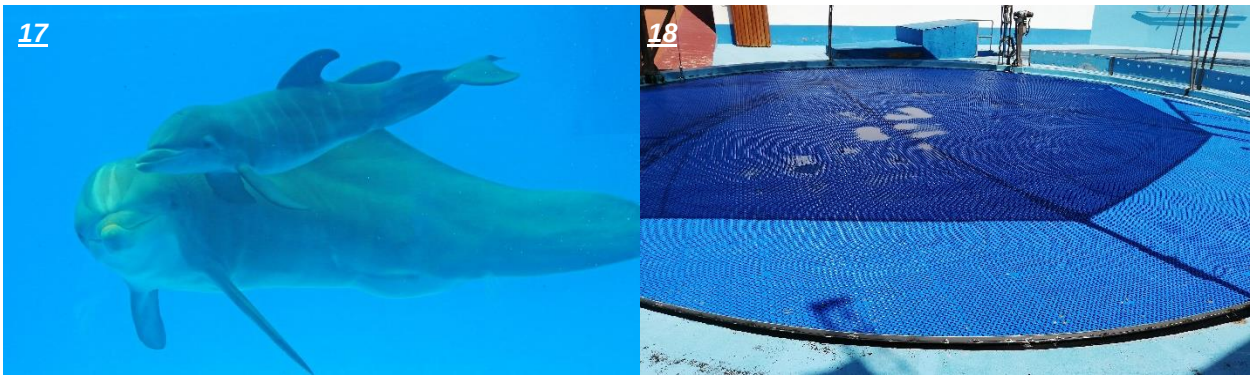


Figure 17 - Calf with his mother (a few days old). Courtesy of Zoomarine.
Figure 18 - Medical lifting platform (Original).

After birth, the calf showed excellent vitality, coordination and swimming pattern, with the first effective nursing occurring within 1 h after delivery. The established neonatal monitoring programme was implemented, that included respiratory rate, nursing and behaviour records. No abnormalities were noted.

As with other animals in the zoological collection of Zoomarine, a routine preventive medical programme was adopted in order to guarantee precise monitoring of the calf's health status. All components of this programme are usually performed with the voluntary collaboration of the animals, made possible by trained medical behaviours. However, as this animal was a calf and not yet trained for these behaviours, monitoring required involuntary procedures on the medical platform. Monthly medical procedures were performed, in which the calf was weighted and blood, faecal and sputum samples were collected. Additional procedures were performed when necessary, according to the calf's clinical status.

Blood samples were taken from the main superficial vessel of the tail fluke, either dorsally or ventrally, with a butterfly needle (21 or 23 G) coupled to vacutainer tubes (serum-separating tube, EDTA tube and citrate tube). Faeces were obtained through the introduction of a lubricated nasogastric catheter (10 to 12 Ch) into the anal slit. Sputum samples were collected by placing a container above the blowhole on expirations.

In the laboratory of the Veterinary Hospital of Zoomarine, complete blood count (CBC) and general biochemistry panel were performed, the latter through a VETSCAN® VS2 chemistry analyser. Both total WBC and RBC counts were done manually, in which a 20 µL and a 5 µL blood-filled pipettes were respectively inserted into Leuko-TIC® and Ery-TIC® tubes. Counting was performed with a Neubauer chamber. Evaluation of blood smears was performed after Diff-Quik staining. Haemoglobin levels were obtained through a haemoglobin analyzer (HemoCue®) and haematocrit after centrifugation of microhematocrit tubes (Centurion Scientific Ltd - Pro-Vet, 120 rpm, 5 minutes). Further analyses were requested to an external laboratory (AQUALAB - Laboratório Clínico e de Saúde Pública Lda.). These included fibrinogen concentrations, ESR and serum iron levels. Passive transfer of immunoglobulins was indirectly evaluated through protein electrophoresis and no failure of passive transfer seemed to exist. Endocrine bioindicators were also requested and used to evaluate stress response, with constantly unremarkable results. These included cortisol (values ranging from 0.2 to 1.39 µg/dL), triiodothyronine (2.11-2.21 ng/mL) and thyroxine (16.1-22.9 µg/dL).

Sputum and faecal samples were also evaluated in the house laboratory. Wet mounts were prepared by adding and mixing 10 µl of the sample with a drop of New Methylene Blue (NMB) stain on a glass slide, immediately before microscopic observation (100× and 400×).

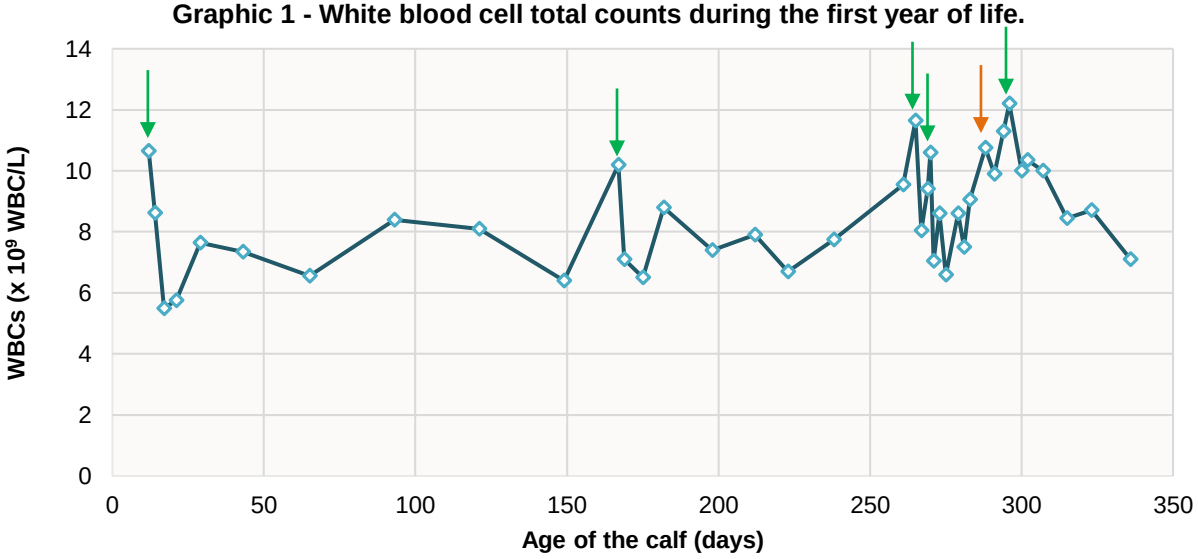
The calf's diet consisted mainly of his mother's milk (20% of fat content), although fish was gradually and consistently introduced from 4 months old, supplemented with AKWAVIT Regular mini® (1 tablet a day) or ½-1 tablet a day of AKWAVIT Dolphin® (Kasper Faunafood).

Over the first 12 months of the calf's life, CBCs revealed several leukocytosis episodes (graphic 1). Serum biochemistries abnormalities included occasionally increased fibrinogen concentrations and ESR and slight decreases in serum iron levels. Respiratory signs were sporadically reported, including hoarse and harsh breaths, cough episodes and a foul-smell from the blowhole.

Several different antibiotic treatments were given throughout this period (graphic 1). These included ceftriaxone (Fresenius Kabi), 20 mg/kg, IM, SID – once a day; enrofloxacin (Baytril®, Bayer), 5 mg/kg, IM, SID and 5 mg/kg, PO, BID; amoxicillin and clavulanic acid (Ratiopharm), 10 mg/kg, PO, BID.

Total WBC counts decreased after antibiotherapy, but given the recurrence of leukocytosis, a fungal aetiology was suspected and fluconazole (Generis), 2 mg/kg, PO, BID, was initiated. Occasional episodes of leukocytosis were maintained during antifungal therapy. During this period of 12 months, no clinical resolution was achieved and the aetiology was never truly identified.

Graphic 1 - White blood cell total counts during the first year of life.



Arrows mark the episodes of leukocytosis which led to antibiotic (green) or antifungal (orange) therapy.

On day 1 (30th of May 2018), as part of a monthly routine check-up, the faecal microscopic evaluation revealed the presence of several aseptate hyphae with sporangiola as well as numerous WBCs (figure 19). CBC showed a mild leukocytosis (10.0 x 10^9 WBC/L; graphic 2a). Sputum sample evaluation and physical examination were unremarkable. At this point, the calf was almost one year old and weighed 85.4 kg.

The faecal sample was maintained refrigerated (4 ºC) and sent to an external laboratory (INIAV - Instituto Nacional de Investigação Agrária e Veterinária) for culture. Follow-up cultures were always conducted in this laboratory.

While waiting for results, treatment was initiated with itraconazole (Generis), 2.5 mg/kg, PO, BID; silymarin (Legalon®, NeoFarmacêutica), 140 mg, PO, TID – three times a day; vitamin B complex (Becozyme® forte, Bayer), 1 tablet, PO, SID; Imuno-2865™ (Animal Necessity), 1 capsule, PO, BID, and probiotics (*Lactobacillus casei,* Antibiophilus®, Azevedos), 1 capsule, PO, SID. All medications were given inside small pieces of fish.

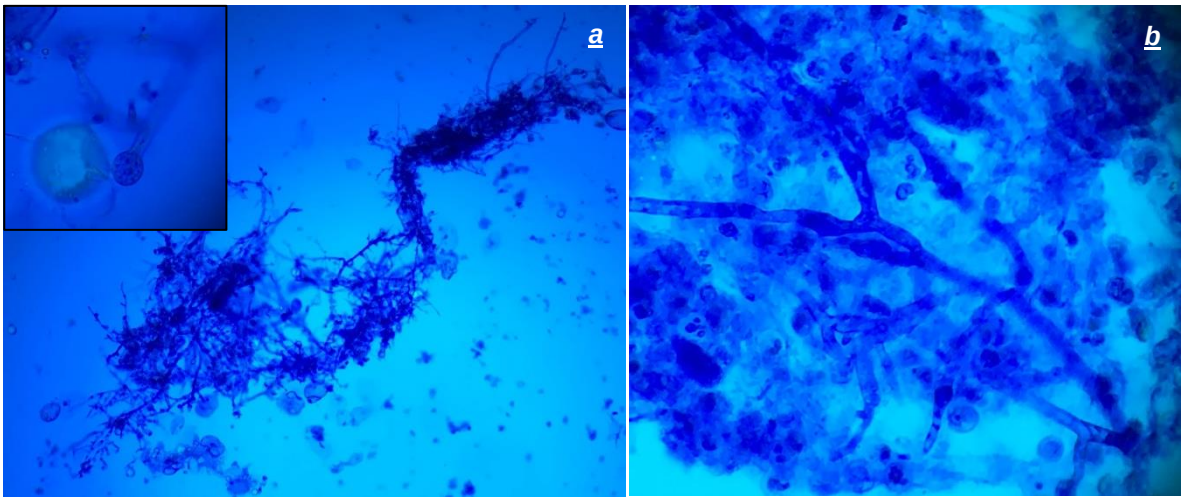


Figure 19 - Faecal sample – aseptate hyphae and leukocytes. Courtesy of Zoomarine.
a **NMB × 100. Insert: magnification showing sporangiola (NMB × 400).** ***b*** **NMB × 400.**

A more frequent protocol was initiated, with medical procedures two to three times a week that included weighing and blood, sputum and faeces collection. There were slight and transient improvements on the total WBC counts during the following month (graphic 2a). Microscopic evaluation of faeces continued to show hyphae while sputum samples remained unremarkable.

On day 20, faecal culture results showed the isolation of *Absidia* spp. (now *Lichtheimia* spp.), an agent of mucormycosis. Identification was based on the morphologic characteristics of the colonies, hyphae and spores (lid lifter white woolly mycelium, with branched

sporangiophores, conical apophyses, rhizoids, conical columellae and hyaline smooth-walled sporangiospores).

In order to identify the aetiologic agent to the species level and guide treatment, the same faecal sample was sent for molecular identification and susceptibility testing to Instituto Nacional de Saúde Doutor Ricardo Jorge. Results and consequent treatment changes are later described.

On day 23, abdominal ultrasound examination using portable ultrasound equipment (Esaote veterinary MyLab^{TM}Gamma) was performed involuntarily, and no abnormalities were seen.

Systemic ozone therapy was initiated on day 24 through rectal instillation (figure 20) and oral capsules (Ozolife Softgels®, Ozolife Group C.b), 1 capsule, PO, TID. Rectal ozone therapy sessions were initiated at a concentration of 6 µg/NmL (200mL) and gradually increased to 15 µg/NmL (200mL), in weekly sessions. Medical ozone was generated through an Ozonobaric P (Sedecal) machine (figure 21) or an O&L1.5RM machine, immediately before the rectal instillation.

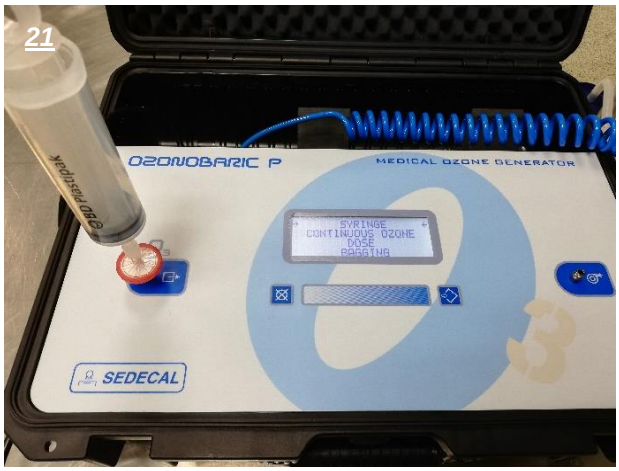


Figure 20 - Rectal ozone therapy. Courtesy of Zoomarine.
Figure 21 - Medical ozone generator (Ozonobaric P) (Original).

After harsh breaths reported by the trainers, thoracic radiographs (left-right lateral and dorsoventral views) were performed on day 29, with portable equipment (GIERTH HF300) at the poolside. The calf was placed in ventral recumbence for both a horizontal-beam lateral projection and a vertical beam dorsoventral projection. Radiographs showed a slight bronchoalveolar pattern of the left and right pulmonary apexes (figure 22).

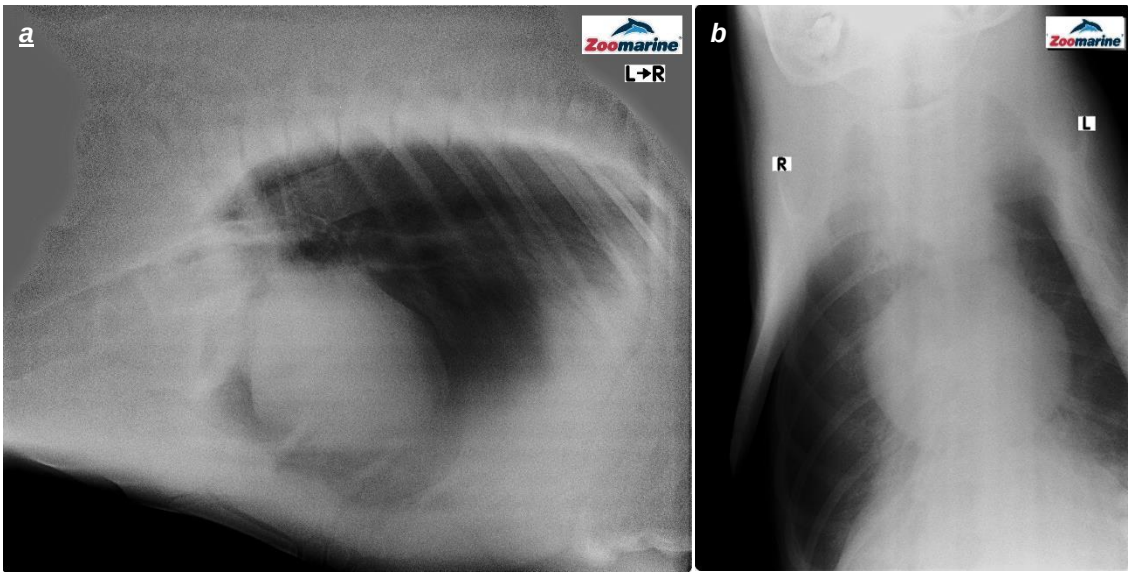


Figure 22 - Thoracic radiographs. Courtesy of Zoomarine.
a **Left-right lateral view** ***b*** **Dorsoventral view**

Simultaneously, probiotic medication was changed from Antibiophilus® to Multi-Probiotic® (Douglas), 2 capsules, PO, SID, which includes several strains of *Lactobacillus* spp., *Bifidobacterium* spp. and *Streptococcus* spp. Vitamin C supplementation (Vitamin C Retard®, Generis), 2 capsules, PO, SID, was also added.

A further three faecal samples were sent for culture isolation over the following two weeks. Two of them confirmed the previous isolation of *Lichtheimia* spp. In one sample no fungi were cultured. Bacterial culture was also performed with the isolation of *Escherichia coli* and *Morganella morganii*.

On day 42, the first bronchoscopy and gastroscopy were performed (figure 23). Endoscopy procedures were always done involuntarily, with the calf in sternal recumbence, at the poolside. Bronchoscopy was performed using a flexible bronchoscope (Storz 60714 PKS® video-endoscope with a 7.9 mm insertion tube diameter, a 2.8 mm instrument channel and a 140 cm working length) and gastroscopy using a flexible gastroschope (Storz 60914 PKS® video-endoscope with a 9.7 mm insertion tube diameter, a 2.8 mm instrument channel and a 140 cm working length). Sedation was accomplished with midazolam (Mylan) 0.045 mg/kg, slow rate IV, and reversion with flumazenil (Anexate®, Cheplapharm), 0,5 mg, IV. Doxapram (Dopram®, Eumedica), 1 mg/kg, was administered IV preventively before returning the calf to water.

Bronchoscopy images were compatible with a severe fungal infection, shown by multiple whitish lesions diffusely distributed on the tracheal and bronchial submucosa and mucosa (figure

24). A bronchial lavage was performed and a sample sent for fungal and bacterial culture. Results were negative. Cytology brushing was also performed and cytological evaluation was executed in another external laboratory (DNATech). This showed numerous epithelial cells, rare inflammatory cells (mainly neutrophils and macrophages) and sporadic hyphae.

Gastroscopy revealed no macroscopic abnormalities. The gastric fluid was collected from the first and second stomach compartments. Microscopic examination showed evidence of hyphae in the gastric fluid from the first stomach compartment.

Figure 23 - Bronchoscopy procedure. Courtesy of Zoomarine.

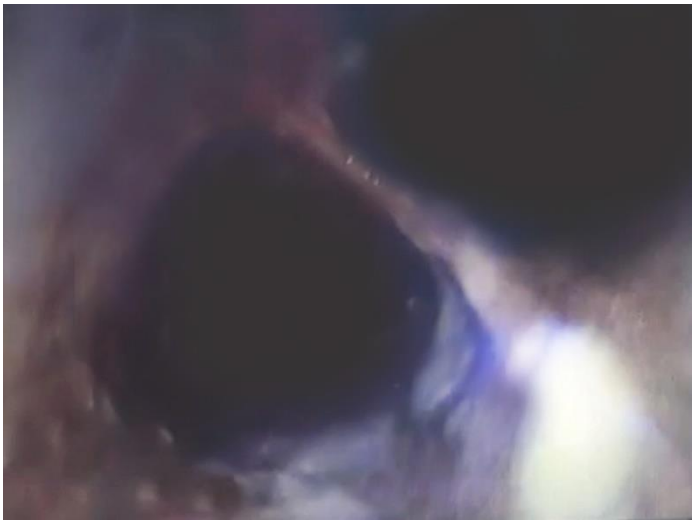


Figure 24 - First bronchoscopy (day 42). Whitish lesions distributed on the trachea and bronchi. Courtesy of Dr José Sampayo. ***Due to technical issues, only a photograph of the screen is available, thus the poor quality of the image.***

Only on day 45, results of the molecular identification through conventional PCR and sequencing of the faecal sample previously sent showed the identification of *Cunninghamella bertholletiae*[1]. Sensitivity testing was performed through the Epsilometer-test. MIC results were compared with ECVs of *Lichtheimia corymbifera*[2] given the lack of defined ECVs specific to *C. bertholletiae* (table 4).

1 Comparison of the sequence of the clinical isolate with the ITS region in GenBank and CBS database showed 99% and 99.37% homology to *C. bertholletiae*, respectively.

2 ECVs presented were calculated by Espinel-Ingroff et al. (2016) comprising ≥ 97.5% of the statistically modelled population as per Clinical & Laboratory Standards Institute criteria.

Table 4 – Susceptibility testing results (*Cunninghamella bertholletiae*) and epidemiological cutoff values of *Lichtheimia corymbifera*.

Antifungal drug	MIC (mg/L) for *C. bertholletiae*	ECV (mg/L) for *L. corymbifera*
Amphotericin B	>32.0	2
Voriconazole	>32.0	-
Itraconazole	1.0	-
Posaconazole	1.0	2

On day 50, aerosolized liposomal amphotericin B nebulizations (Ambisome®, Gilead), 20-25 mg, BID, were initiated, with an Air Project® (Pic Solution) machine connected to a mask device. Several mask devices were used, but technical issues were noted, especially given the long time needed to assure an effective nebulization procedure (10 to 15 minutes). An Equine Haler® inhalation mask appeared to show better results (figure 25). Nebulizations were initially performed voluntarily (figure 26), but involuntary procedures were needed to ensure the efficacy of nebulizations.

Itraconazole was discontinued after 40 days of therapy and posaconazole therapy with delayed-release tablets (Noxafil®, MSD), 5 mg/kg, BID, began on day 52. Bromhexine therapy was initiated (Bisolvon®, Boehringer Ingelheim), 1 tablet, PO, BID, given the reports of cough episodes and the presence of dense mucus in sputum samples.

On day 56, four days after beginning PCZ administration, nausea episodes and hyporexia were noted and medication compliance revealed to be difficult. Daily involuntary procedures began, which included oral hydrations through gastric probes with an electrolyte and vitamin solution (Duphalyte®, Zoetis), 5 mL/kg, SID, for four days. Since PCZ tablets must be taken intact, force-feeding with herring or capelin (with the tablets placed inside the fish) was also necessary.

On day 61, nine days after initiating PCZ therapy, the dosage regimen was changed to 10 mg/kg, SID. Immediate improvement was noted, including a significant increase in appetite and general behaviour.

Routine biochemistry analysis showed a slight increase of alanine aminotransferase (ALT) and aspartate transaminase (AST) after initiating PCZ therapy but they remained within reference intervals throughout treatment.

Figure 25 - Equipment used in nebulization procedures: Air Project® (*) and Equine Haler® inhalation mask (#) (Original).
Figure 26 - Nebulization: voluntary procedure. Courtesy of Zoomarine.

On day 63, another faecal culture was performed, with the isolation of *E. coli*, *M. morganii* and *Lichtheimia* spp. Simultaneously, the CBC showed leukocytosis (11.8 x 10^9 WBC/L – graphic 2a) and there was a decrease in serum iron (68 µg/dL – graphic 2d). No additional therapies were initiated and blood parameters continued to be thoroughly monitored twice to three days a week. Weight measurements started to reveal a stagnation or even slight decreases. Total WBC counts and serum iron levels remained outside the reference intervals during the following weeks (graphic 2a and 2d). Moreover, occasional increases in fibrinogen levels and ESR were noted (graphics 2b and 2c). ALP values started to decrease gradually over the following three months (graphic 2e).

Vitamin C supplementation was discontinued on day 65, after 36 days of therapy.

On day 99, a second bronchoscopy was performed. Given the therapy with PCZ, midazolam was used at half the dosage (0.023 mg/kg, instead of 0.045 mg/kg) because concomitant administration of PCZ increases benzodiazepines plasma concentrations and may potentiate hypnotic and sedative effects. Again, reversion was accomplished with flumazenil (Anexate®, Cheplapharm), 0,5 mg, IV, and Doxapram (Dopram®, Eumedica), 1 mg/kg, IV, was administered preventively. Lesions seen in the previous endoscopic exam were no longer observed, but a mildly hyperaemic tracheal mucosa was noted (figure 27).

Nebulizations with amphotericin B were stopped after 49 days of therapy. The frequency of check-ups was decreased to weekly procedures.

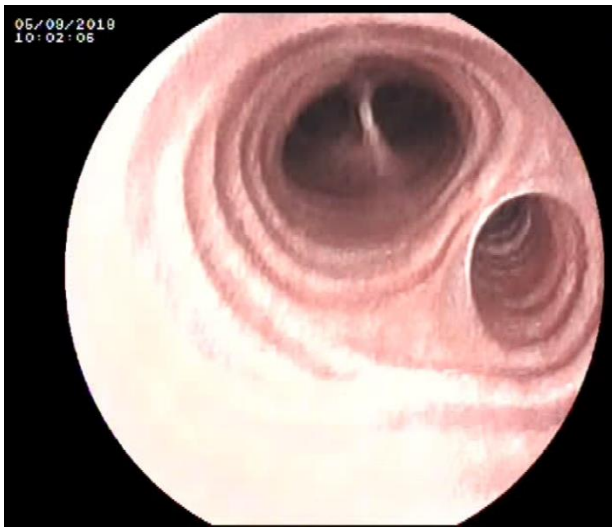


Figure 27 - Second bronchoscopy (day 99). Mildly hyperaemic mucosa. Courtesy of Dr José Sampayo.

Over the following thirteen weeks (from day 99 to day 194) significant improvements were seen, with progressive weight gain and negative results in both culture and microscopic evaluation of faeces and sputum.

PCZ, bromhexine and Imuno-2865^{TM} were discontinued on day 146 after 95, 103 and 141 days of therapy, respectively. Vitamin C supplementation was restarted on the same regimen. ALT and AST values showed a slight decrease after terminating PCZ therapy and ALP values started to increase gradually (graphic 2e). Serum iron levels also started to increase (graphic 2d).

On day 184, a third follow-up bronchoscopy was performed, with the same protocol used in the previous endoscopy procedure. Although the tracheal mucosa appeared healthy with no fungi plaques, whitish punctiform spots were identified on the tracheal submucosa, decreasing distally and almost disappearing on the primary bronchi (figure 28). These were thought to be calcified old fungi-originated lesions on the trachea wall.

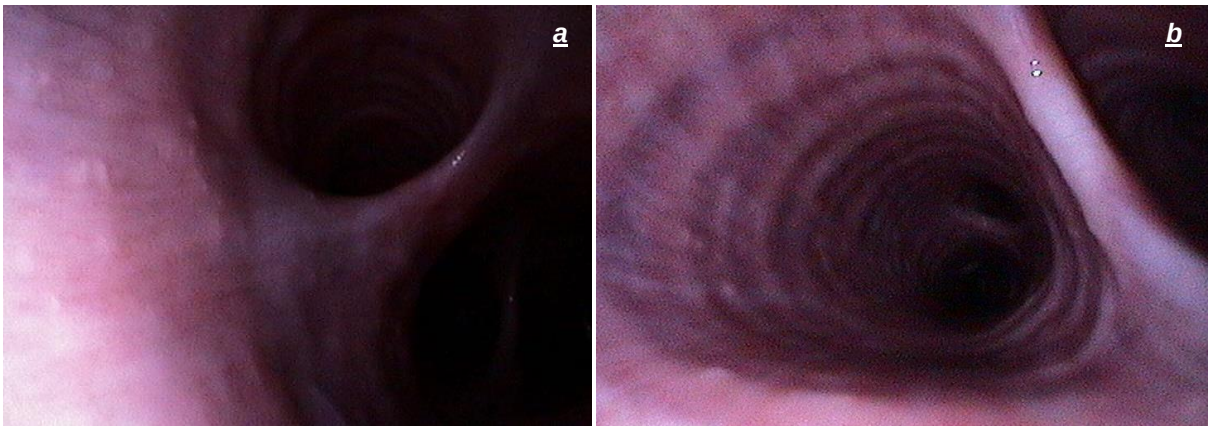


Figure 28 - Third bronchoscopy (day 184). Whitish spots on the tracheal and bronchial mucosa. Courtesy of Dr José Sampayo.

On day 194, 10 days after the bronchoscopy, a faecal microscopic evaluation revealed the presence of several hyphae, morphologically similar to the previous findings. For the first time, sputum samples also revealed fungal structures (figure 29), although culture results were negative. These findings were commonly found throughout the rest of the timeframe considered.

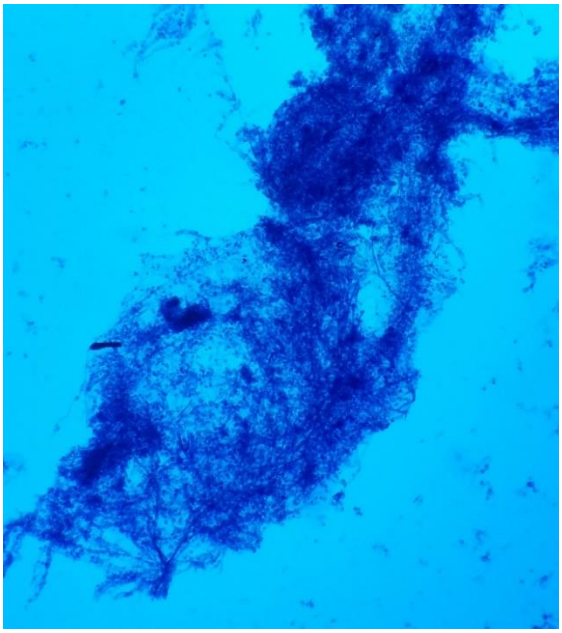


Figure 29 - Hyphae, leukocytes and mucus (sputum sample). NMB × 100 (Original).

Administration of PCZ was resumed at the same dosage (10 mg/kg, PO, SID) on day 202. Probiotic medication was discontinued after 179 days.

Leukocytosis was a common finding throughout the rest of the clinical case (graphic 2a), as were low values of serum iron (graphic 2d). ALP values showed a deep decrease (graphic

2e). ALT and AST values increased after reinitiating PCZ therapy but stayed within physiological ranges. The calf's appetite remained inconsistent with occasional episodes of nausea and hyporexia. Only a slight weight gain was registered throughout this period.

On day 274, a fourth follow-up bronchoscopy was performed, using the same protocol as the previous two bronchoscopy procedures. Although the trachea and bronchial mucosa were almost clear of lesions, some whitish spots were seen (figure 30). A second gastroscopy was also performed with no macroscopic abnormalities to report.

A thoracic ultrasound was possible to perform on day 285, given the visit of a specialist marine mammal veterinarian with experience in ultrasound examination in cetaceans. Ultrasound was performed involuntarily with the same portable equipment previously used. No abnormalities were seen on the pleura, peripheral lungs or marginal lymph nodes (figure 31).

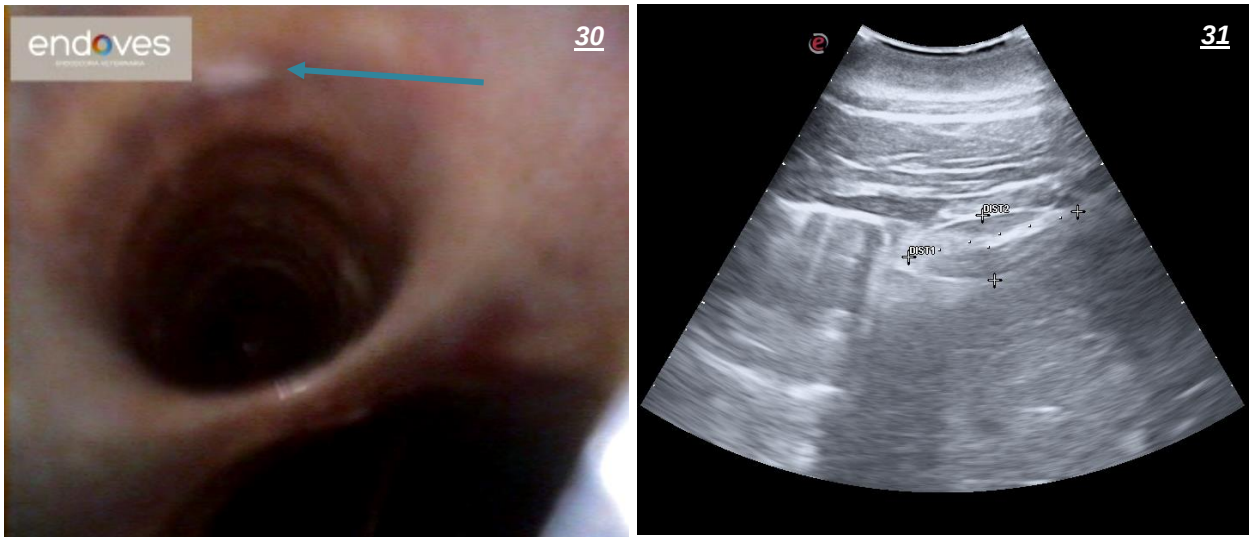


Figure 30 - Fourth bronchoscopy (day 274). Whitish spot on the bronchial mucosa (arrow). Courtesy of Dr José Sampayo.
Figure 31 - Left marginal lymph node (56.0 x 20.4 mm). Longitudinal view. Courtesy of Dr Pietro Saviano.

On day 287, sudden anorexia was reported and blood analysis revealed a severe neutrophilic (77.2%) leukocytosis (19.2 x 10^9 WBC/L), with the presence of reactive neutrophils on the blood smear. Fibrinogen reached values of 411 mg/dL and there was an increase in ESR (16 mm/h) together with a severe iron deficiency (32 µg/dL) and low ALP values (graphic 2).

As a bacterial infection was suspected, urine, faeces and sputum samples were collected and sent for culture. Urine was collected through catheterization using a sterile nasogastric catheter (8 Ch) and the other biological samples with the same methodology already described, adding previous disinfection of the blowhole and anal slit with chlorhexidine.

Immediate daily involuntary procedures were started, with the administration of ceftriaxone (Frasenius Kabi), 20mg/kg, IM off the midline and parallel to the dorsal fin, SID. Daily blood collections, gastric intubation for oral hydrations (figure 32 - Duphalyte®, Zoetis, 5mL/kg, PO, SID) and force-feeding to assure PCZ compliance were also performed for three days. Appetite increased by the third day.

Figure 32 - Oral hydration with Duphalyte® (Original).

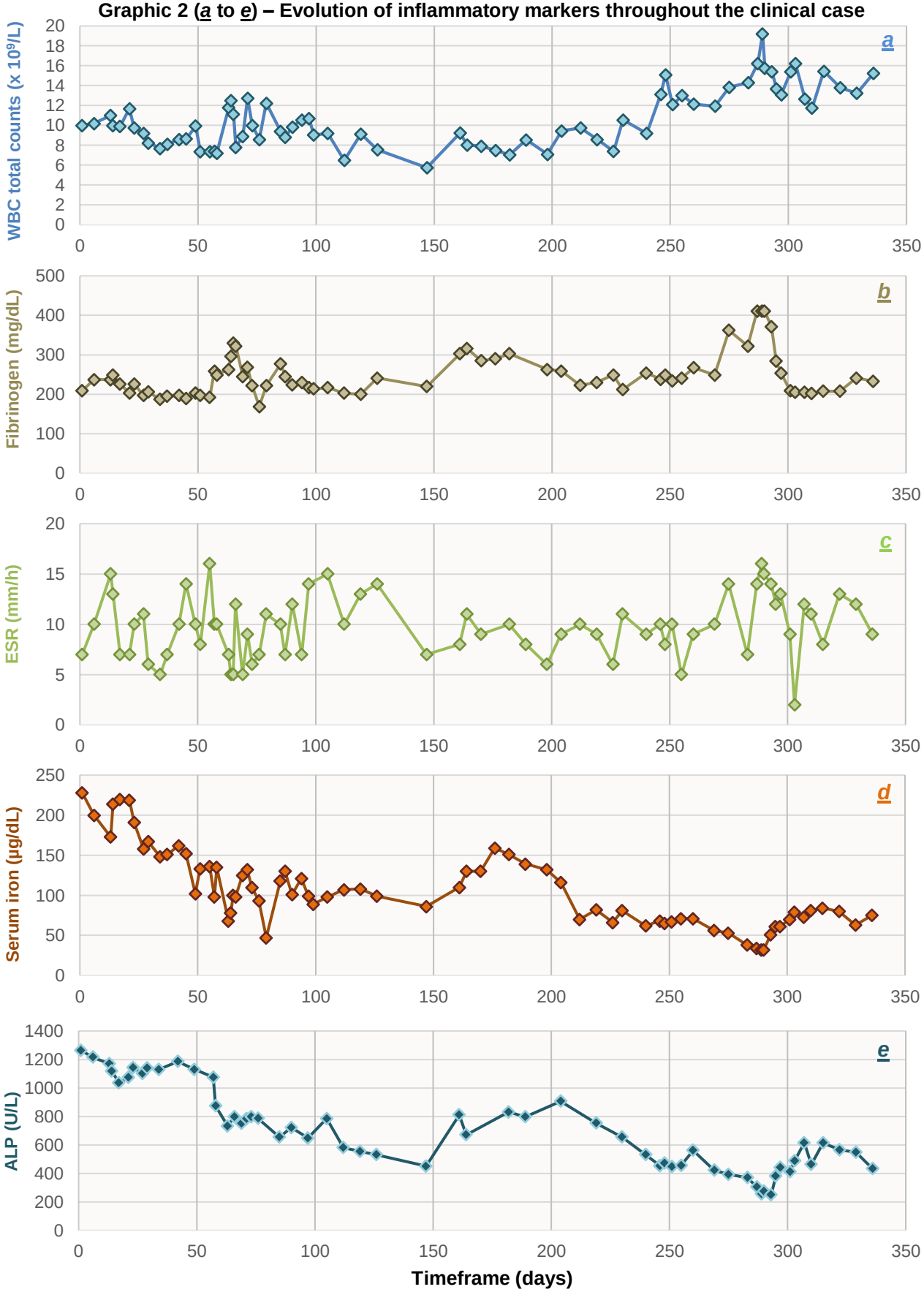
From the cultured samples collected on day 287, only *Staphylococcus aureus* was isolated from the sputum sample. Given the sensitivity testing, ceftriaxone therapy was maintained for a total of 15 days. Slight improvements were seen on haematological and biochemistries parameters throughout treatment (graphic 2), although AST values showed peak levels outside reference intervals (330 U/L). At the end of the antibiotherapy, leukocytosis was still present (15.4 x 10^9 WBC/L). There was a decrease in fibrinogen levels (209 mg/dL) as well as ESR (9 mm/h) and an increase in serum iron (70 µg/dL). Normal appetite was regained. A follow-up sputum culture was negative.

Leukocytosis episodes continued to occur until the end of April (day 336; graphic 2a). Overall, fibrinogen concentrations maintained inside the reference interval (graphic 2b), although serum iron levels were frequently below physiological values (graphic 2d). A slight increase in ALP values was noted (graphic 2e). Although sporadic episodes of nausea and hyporexia continued to be reported until the end of the considered timeframe, there was an overall

improvement in appetite and general behaviour. There were no reports of respiratory signs. Weight measurements revealed minor changes during the final month (final weight of 106 kg).

Treatment was maintained with PCZ, ozone therapy (rectally and Ozolife Softgels®), silymarin, vitamin B complex and vitamin C. Routine weekly/twice a week monitoring was maintained with blood analysis, faecal and sputum evaluation and body weight measurements.

Graphic 2 (*a* to *e*) – Evolution of inflammatory markers throughout the clinical case



4.2.1. Therapeutic drug concentrations – a retrospective study

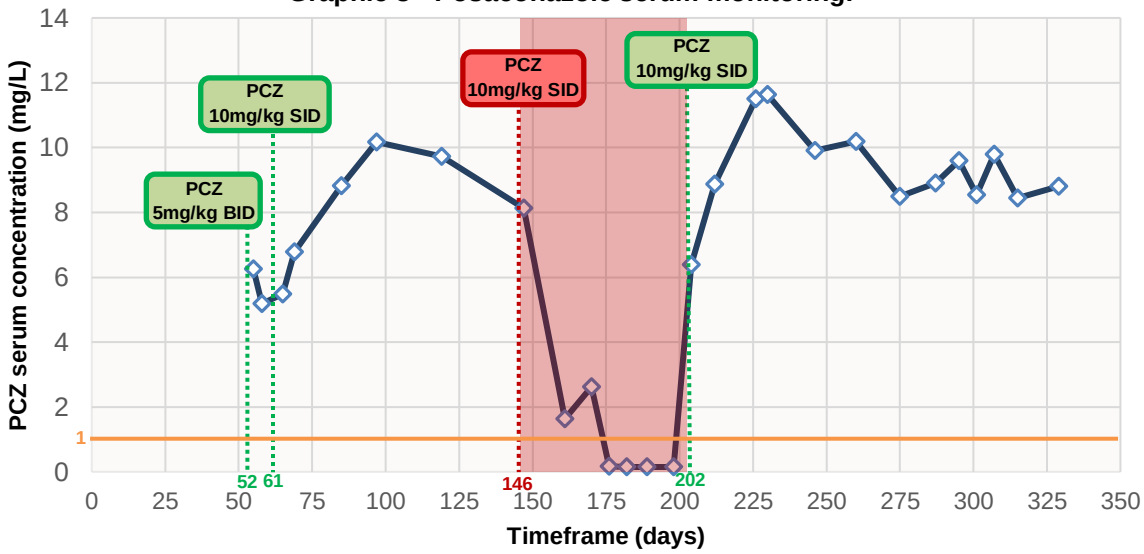
Given the subjective regimen of PCZ, the author retrospectively evaluated PCZ serum concentrations throughout treatment. Archived frozen serum samples were sent to an external laboratory (Klinisch farmaceutisch en toxicologisch lab – UMC Utrecht). PCZ concentrations were measured using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Results are summarized in graphic 3. During PCZ therapy, concentrations varied between 5.18 and 11.63 mg/L. The first two measurements were 3 and 6 days after initiating PCZ (5 mg/kg BID), reaching concentrations of 6.26 and 5.18 mg/L, respectively. Four days after changing the regimen of PCZ to 10 mg/kg SID, PCZ serum concentration was 5.48 mg/L and started to increase in the following measurements reaching levels of 10.17 mg/L.

After discontinuing PCZ therapy, concentrations decreased rapidly, reaching values of 1.64 mg/L 15 days after stopping PCZ therapy. A concentration peak was registered 24 days after stopping PCZ (2.62 mg/L). Values below 0.15 mg/L were achieved 30 days after discontinuing PCZ therapy.

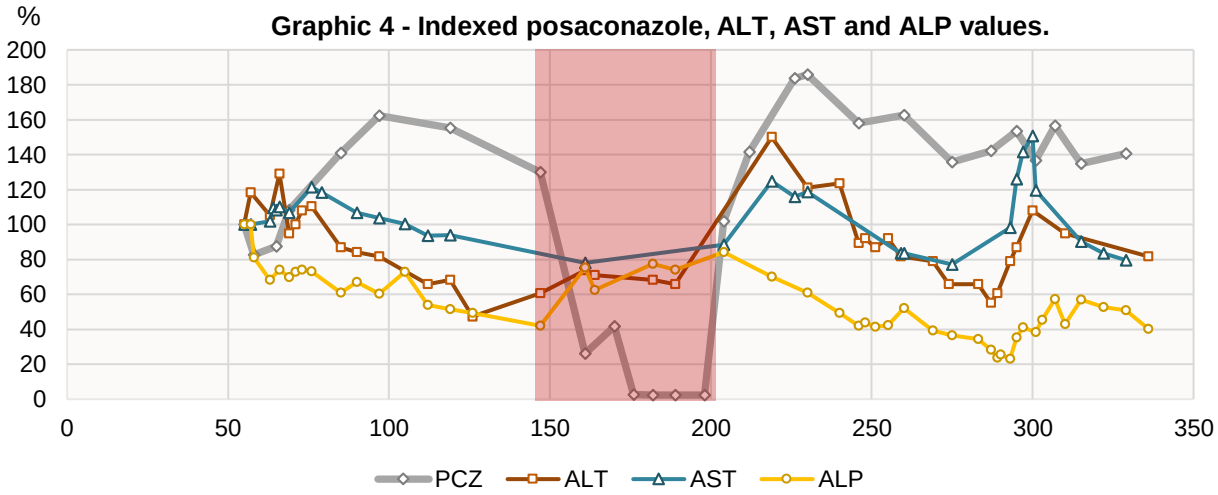
Two days after resuming PCZ, there was a serum concentration of 6.38 mg/L. During the following months until the end of the timeframe considered, serum concentrations were maintained between 8.5 and 11.63 mg/L.

Graphic 3 - Posaconazole serum monitoring.



Dotted green lines: PCZ was initiated or the regimen was changed. Dotted red line: PCZ was discontinued. Red area: period were PCZ therapy was discontinued. Orange line: MIC of the isolate.

Overall, as previously stated, hepatic biochemistries stayed inside physiological ranges and only slight increases were noted during antifungal therapy. Nonetheless, a graphic representation of the changes in ALT, AST, ALP and PCZ serum levels was made (graphic 4).



Graphic 4 - Indexed posaconazole, ALT, AST and ALP values.

Red area: period were PCZ therapy was discontinued.

4.3. Discussion

Respiratory mucormycosis is one of the most common presentations of this infection (Reidarson et al. 2018) and in this specific case, several susceptibility factors may be investigated when explaining the development of infection.

The placenta of dolphins is diffuse epitheliochorial and therefore immunity is transferred to the calf mainly through the colostrum (Cozzi et al. 2017a). Past medical history showed no failure of passive transfer of immunoglobulins. However, protein electrophoresis is too insensitive a method for investigating hypoglobulinaemia due to the failure of passive transfer and other immune assays should be considered when evaluating immunocompetence and measuring the presence and function of immune cells (Levin 2018; Skeldon 2018). In cetaceans, several assays have been studied to measure phagocytosis, respiratory burst, natural killer cell activity, lymphocyte proliferation, delayed-type hypersensitivity and immunophenotyping (Levin 2018).

No genetic studies were performed, but it is interesting to note that the mother of the calf has a history of chronic respiratory disease, and immunodepression associated with intrinsic factors might be suggested. In fact, offspring of the calf's grandmother has shown a variety of health issues. Furthermore, the use of antibiotics may be associated with a microbiota disruption (Reidarson et al. 2018) and the several continuous antimicrobial treatments during the first year

of the calf's life may have contributed to the mucormycete invasion. Even considering the effect of stress in suppressing the effectiveness of the immune response, which can lead to a greater susceptibility to disease (Kozlowski 2012), the results of the endocrine bioindicators of stress response used in cetaceans were unremarkable (Atkinson and Dierauf 2018). Water quality treatments were always done according to standard practice guidelines, but the hygiene theory suggested by some authors may have played a part in the infection by mucormycetes (Reidarson et al. 2018).

Little is known concerning the time of transmission and development of infection. A probable portal of entry was through inhalation of sporangiospores, in which exposure to dust particles should be considered. A higher airborne dust concentration may have been present during the first year of the calf's life since some occasional construction work occurred at Zoomarine. The leukocytosis episodes and occasional respiratory signs seen in the first twelve months after birth may indicate that the infection was already present. No respiratory disease was confirmed throughout this period but it is important to note that some MM cases in the literature describe a history of recurrent respiratory disease (García-Párraga et al. 2016).

Cunninghamella bertholletiae was the species identified through molecular assays and is the most frequently reported species of this genus in both humans and cetaceans with a mucoralean infection (Bragulat et al. 2017; Reidarson et al. 2018). *Cunninghamella* spp. have been shown to be more virulent compared to other Mucorales species, however, this does not explain why these mucormycetes have only been isolated in humans and cetaceans. A susceptibility issue may be suggested (Bragulat et al. 2017).

Similarly to other clinical cases, the diagnosis of respiratory MM was confirmed through culture and bronchoscopy, although direct microscopy revealed to be a valuable tool for early diagnosis and as part of a complete preventive medical programme (Clauss et al. 2014; García-Párraga et al. 2016; Bonn and Dover 2018).

As described in the literature, clinical signs, in this case, were also non-specific and occasional respiratory signs were inconsistent (Townsend and Staggs, et al. 2012). Lack of appetite and lethargy were not described during the onset of infection and these were mainly seen as adverse effects to antifungal therapy. Nonetheless, since there was no monitoring programme at that time to register and evaluate nursing behaviours, hyporexia cannot be completely discarded. There were no signs of central nervous system or other organs involvement, which might suggest a non-disseminated infection. Like in other MM cases, respiratory auscultation was unremarkable (Reidarson et al. 2018).

The results of the inflammatory markers evaluated were similar to those described in other reports of cetaceans with MM (Robeck and Dalton 2002; Brudek-Wells et al. 2011; Abdo et al. 2012; Clauss et al. 2014; García-Párraga et al. 2016; Reidarson et al. 2018). Total WBC counts fluctuated throughout the timeframe considered, and both normal and elevated values were seen (5.8 to 19.2 x 10^9 WBC/L), as described in the literature (Robeck and Dalton 2002; Brudek-Wells et al. 2011; Clauss et al. 2014). Even when the clinical status was considered stable, episodes of mild leukocytosis were registered. Fibrinogen confirmed to be an important inflammation marker, with peak elevations associated with a worsening clinical picture (day 65 – 330 mg/dL; day 287 – 411 mg/dL) (Robeck and Dalton 2002; Abdo et al. 2012; García-Párraga et al. 2016). Low serum iron levels were frequent, as expected, reaching values as low as 32 µg/dL (Brudek-Wells et al. 2011; Townsend, Newton, et al. 2012). This iron deficiency is explained by the sequestration of iron as a host defence mechanism (Nollens et al. 2018) and by the iron uptake by mucormycetes, one of its characteristic virulence traits (Ibrahim et al. 2012; Farmakiotis and Kontoyiannis 2016). A rapidly increasing ESR (Brudek-Wells et al. 2011; Townsend, Newton, et al. 2012; Wells et al. 2012) was not present in this case, where only occasional slight increases were observed (16 mm/h). Decreasing ALP was also noted and expected, being this a reliable indicator of inflammation and prognosis (Nollens et al. 2018). Similarly to other MM cases (Abdo et al. 2012; García-Párraga et al. 2016), fluctuations of ALP were according to the overall clinical situation, increasing during theoretical resolution of infection (reaching values of 906 U/L) and showing a deep decrease after relapse (reaching values as low as 249 U/L). An important note concerns the mild abnormalities in clinical pathology compared to the severe results on bronchoscopy.

Biological samples included sputum, faeces and gastric fluid samples, the latter not commonly evaluated given it would involve involuntary gastric intubation. Direct microscopic examination of these clinical specimens did not, however, allow identification of mucormycetes, which supports the idea that mycological expertise is needed when differentiating fungal species (Cornely et al. 2013). Moreover, no optical brighteners were used, making it difficult to visualize and differentiate structures. Despite the above, morphologic characteristics of the hyphae allowed to eliminate candidiasis as a possible diagnosis, and led to a more consistent and thorough diagnostic approach in order to identify the aetiologic agent.

In this case, the non-visualization of hyphae alone did not rule out infection since sometimes microscopic evaluation showed no signs of hyphae, even when other diagnostic tools (e.g. bronchoscopy) revealed that the infection was still present. These negative and inconsistent results on direct microscopy can be explained by the capacity of invasion of

mucormycetes and subsequent difficulty in expelling fragments of the fungi-invaded epithelium (Ibrahim and Voelz 2017). Nevertheless, this explanation is universal for all the clinical specimens and does not explain why, interestingly, visualization of hyphae was often possible in faeces but not in sputum samples, given that this is a case of respiratory MM.

Clarification on this last subject comes months after the initial diagnosis, specifically during relapse of the infection. A few weeks before confirmation of relapse, the calf started to perform forceful expirations ("chuffs") voluntarily for the first time, in order to collect routine sputum samples. A greater strength associated with the voluntary "chuffs" may explain why fragments of the mycelium were more easily expelled when compared to the samples obtained involuntarily (weaker "chuffs").

Besides, an explanation is needed concerning the reason why hyphae of respiratory origin were seen in faecal samples and why these should be included as adequate specimens for diagnosis of respiratory MM. The larynx of dolphins is completely separated from the pharynx and thus it would be expected that respiratory-origin material would not appear in samples from the gastrointestinal tract (Cozzi et al. 2017b). However, the waterproofing sealing given by a sphincter muscle might permit the passage of respiratory cells and others (e.g. hyphae) into the oesophagus, thus appearing in gastric and faecal samples (Sweeney et al. 1999; Reidenberg and Laitman 2008).

Culture of specimens allowed to identify a mucormycete to the genus level, although the species further identified by molecular methods (*C. bertholletiae*) did belong to a different genus of the one isolated. The same faecal sample was used for both culture and PCR. The isolation of a specimen different from the one identified by PCR and the fact that some follow-up culture results came back negative, even when infection was confirmed by direct microscopy, can be explained by the friability of mucormycetes and subsequent damage during tissue manipulation (Lass-Flörl 2009). Also, biological samples were maintained at refrigerated temperatures until culture, which might have led to the non-survival of fungi (Lass-Flörl 2009). Overall, this case supports the suboptimal sensitivity of culture of mucormycetes described in the literature (Cornely et al. 2013).

Even considering the identification of a different species, there is sparse evidence that identification of a mucormycete to the genus and/or species level could guide medical management (Cornely et al. 2013). Nonetheless, identification of *C. bertholletiae* did raise extra concern, given the overall higher virulence associated with this species (Petraitis et al. 2013).

The role of *in vitro* susceptibility testing for mucormycetes is not yet fully determined (Espinel-ingroff et al. 2015; Espinel-ingroff and Turnidge 2016). Not only are clinical breakpoints

not available for Mucorales species, but ECVs are also not available for *C. bertholletiae*, which represented another hurdle in the choice of antifungal agents. Moreover, given the prolonged period of antifungal therapy, repeated susceptibility testing should have been performed throughout treatment, since mucormycetes can rapidly become resistant (Reidarson et al. 2018).

Both histopathology examination and serological assays were not performed mainly due to the use of other complementary diagnostic methods (e.g. culture, PCR). Also, no serological tests were taken into consideration given the overall lack of methods for identification of mucormycetes (Dadwal and Kontoyiannis 2018). While serological testing seems to be an extremely important tool for monitoring recurrence of infection, ELISA tests previously used in cetaceans were specific to *Apophysomyces* spp. (Barger et al. 2012; Wells et al. 2012).

Concerning diagnostic imaging, although thoracic radiographs raised the suspicion of respiratory involvement, bronchoscopy revealed to be the most useful tool in both the initial diagnosis and follow-ups. Moreover, radiographs did not show any abnormalities on the trachea or bronchi, unlike some other case reports (Kinsel and Briggs 2005; García-Párraga et al. 2016). Thoracic ultrasound was only performed at a time where bronchoscopy images showed almost no abnormalities and the calf was on antifungal therapy, and results of thoracic ultrasound examination during the onset of infection would be interesting to analyse. Although CT was not used in this case, it could have been a useful tool considering its importance in confirming lung and surrounding tissue invasion, checking the dissemination of infection to other organs and for prognosis purposes (Clauss et al. 2014; García-Párraga et al. 2016). The lack of easy-access equipment, the stress associated with the need to transport the calf, the lack of central nervous system signs and the results on follow-up bronchoscopies were the main reasons to discard the use of CT.

The observation of macroscopic lesions, in this case, was possible through bronchoscopy, with images compatible with descriptions of severe respiratory fungal infections (Delaney et al. 2012; Bunskoek et al. 2017). It is important to note that differential diagnosis with other fungal infections (e.g. aspergillosis) only with bronchoscopy is not possible and laboratory confirmation is essential. Image interpretation was challenging given the lack of information on mucormycotic cases in cetaceans and the fact that fungal respiratory infections are quite rare, both in veterinary and human medicine. The lumen of the trachea and bronchi did not seem to be affected and no occlusion was detected, unlike descriptions of some other cases (Kinsel and Briggs 2005; Delaney et al. 2012). Gastroscopy was initially performed to rule out a concomitant infection of the gastric compartments. This showed that although dissemination of the agent to

other organs is possible (hyphae were found in gastric samples), development of infection is not mandatory.

Antifungal therapy began with itraconazole after identification of fungal structures on direct microscopy, given that culture results would not be immediately available. Posaconazole and amphotericin B therapy were perhaps initiated later than desired not only given the waiting period for culture results but also due to the special authorizations required for the use of these drugs. Requests made to Direção Geral de Alimentação e Veterinária (DGAV) were necessary for the use of AmB and PCZ. Both of these drugs are not licensed for veterinary use and moreover, AmB is a drug of exclusive hospital use (Despacho conjunto n.º 317/99 de 19 de março and Despacho n.º 730/98 de 24 de Setembro).

Isavuconazole was not considered in this case, as it was never been used in cetaceans and PCZ was an available option with similar advantages.

The role of AmB, in this case, is uncertain, especially comparing the MIC results and the ECVs. Nevertheless, therapy was initiated given the reports of successful use in some clinical cases (Reidarson et al. 2018). Nebulization therapy was performed given the difficulty (i.e. long administrations) and adverse effects described with IV administrations (e.g. nephrotoxicity) (Townsend et al. 1996; Townsend, Newton et al. 2012). No adverse effects were observed, although technical issues showed that nebulization therapy might be a challenge, especially in younger animals and others that do not have specific medical training. Equipment specifically designed for dolphins has been developed and may eventually present a more effective alternative to equine nebulization systems (Kuo-chieh et al. 2013). The simultaneous use of PCZ is also questionable, given the reported contradictory interactions between the two drugs (Kyvernitakis et al. 2016). All the above were taken into consideration and led to the discontinuing of AmB therapy.

Since no literature support was available regarding the use of DRTs of PCZ in dolphins, the regimen used was the same reported for the oral suspension. Tablets were chosen over the suspension mainly because high volumes would be needed to be injected in small pieces of fish and compliance would be challenging. Moreover, in human studies, the absorption of the oral suspension was shown to be highly dependant on a high-fat diet and multiple doses would be needed to sustain higher mean concentrations over time (Lewis and Fothergil 2015). It is interesting to note the financial investment associated with DRTs therapy in this case, with a daily mean expense of 240€ regarding only the PCZ therapy.

After the observation of adverse effects associated to PCZ therapy, such as nausea, hyporexia and anorexia, the pros and cons of stopping this compound were considered.

However, it was recognised that there were no other alternatives for MM management, and PCZ therapy was continued with a change of regimen from 5 mg/kg BID to 10 mg/kg SID. This regimen change appeared to be a valuable and effective alternative to reduce adverse effects. Nausea has been described associated with PCZ therapy in humans but there is no published information on adverse reactions to this drug in cetaceans (Edwards 2018). Oral hydrations and force-feeding were of extreme importance in managing inappetence in this calf. However, these procedures were only performed when extremely necessary.

Hepatic evaluation thoroughout the clinical case showed only slight increases on transaminases and it is believed that clinical hepatotoxicity was not one of the adverse effects related to antifungal therapy. In humans, a mild hepatic enzyme level elevation associated with PCZ therapy is described (Edwards 2018). The AST values were outside reference intervals in only two measurements (days 297 and 300), which are thought to be non-related to PCZ therapy but related with muscle damage caused by IM injections of ceftriaxone.

The optimal frequency of monitoring serum concentrations throughout PCZ therapy is unknown and consideration of the clinical circumstances should guide the frequency of measurements (Ashbee et al. 2014). In this study, however, PCZ concentrations were not evaluated throughout treatment and were only retrospectively analysed. The choice of samples was according to the calf's clinical status in specific periods and also in order to obtain at least a concentration result for every two weeks of treatment.

Results showed higher serum concentrations (reaching 11.63 mg/L) compared to the successful reports of MM management in dolphins where PCZ concentrations were monitored (Walters et al. 2009; Wells et al. 2012; García-Párraga et al. 2016). In these cases, a concentration of 6 mg/L was barely reached. It is important to note, however, that in these reports a different formulation (OS vs DRT) and a different regimen were used (5 mg/kg BID vs 10 mg/kg SID).

In humans, DRTs have shown an improved bioavailability and pharmacokinetics (Lewis and Fothergil 2015). Although they can be administered regardless of meals, in this case, the ingestion of a fatty diet might have improved absorption of PCZ (Guarascio and Slain 2015). This is especially important when considering that dolphins' milk has a fat content of 10-36% (in this case 20%) and lactation was the primary source of nutrition of this calf (Silva 2014).

In humans, PCZ serum concentrations seem to reach a steady-state around 7 to 10 days after beginning therapy, increasing in the first week and plateau thereafter (Ashbee et al. 2014; Guarascio and Slain 2015). In this case, measurements 2, 3 and 6 days after initiating PCZ showed levels around 5.18-6.36 mg/L, and most results later obtained showed concentrations

slightly above 8 mg/L. This suggests that a steady-state in this calf was also not obtained during the first week of therapy.

Although during the first period of PCZ therapy, serum concentrations reached levels above 8 mg/L, a decrease was registered after initiating therapy (5.18 mg/L; day 58), which can be explained by a rapid distribution of the drug to fatty deposits, especially given its lipophilicity.

During PCZ discontinuation, a peak level (2.62 mg/L; day 170) was registered, which may be due to an analytical error. In addition, although there was no weight decrease at that time, there might have been a mobilization of fatty acids from triglyceride stores in adipose tissues, leading to an increase in serum concentrations.

Overall, after resuming PCZ therapy, there were slight changes in serum concentrations. These can be explained by small weight changes, occasional episodes of diarrhoea and possible variations in milk intake.

Although the AUC/MIC ratio is the parameter best associated to the clinical success of PCZ therapy, it was not possible to calculate the area under the curve. A pilot study suggests that a total drug serum AUC/MIC target of > 100 may be an appropriate benchmark for future pharmacodynamic studies and explorations of breakpoints in mucormycetes (Lewis et al. 2014). However, to measure this parameter, several blood collections (and involuntary procedures) would be necessary to get a set of results within a period of 24 h. For ethical reasons, this could not be taken into consideration. Given the above and as suggested by the literature, the results on posaconazole drug monitoring can be compared to the MIC of *C. bertholletiae* (1.0 mg/L), although this does not incorporate pharmacokinetic parameters. In this case, and only taking into consideration the steady-state, concentrations achieved were, for a long period, 8 to 10 times the MIC of the pathogen isolated, which may support the future use of DRTs of PCZ (10 mg/kg, PO, SID) in cases of MM in dolphins. Given the set of results, the dosage used could eventually have been reduced, which might have helped with the adverse effects described. However, clinical decisions on this subject are challenging given the known controversies of serum concentration targets (Martson et al. 2018).

While a surgical approach may be deliberated in mass-like lesions (García-Párraga et al. 2016), surgical debridement was not considered in this case given the site and the diffuse appearance of the lesions of the trachea and bronchi, intrinsically associated to the mucosa.

Adjunctive therapies considered in this case relate to the use of mucolytic drugs, Imuno-2865™, vitamin supplementation, liver protectors, probiotics and ozone therapy. The role of each of these therapies in the management of this case is unclear. There is a lack of available data concerning the use of adjunctive therapies in other MM cases in cetaceans, and therefore

comparisons with this case report are not feasible. Moreover, it is important to note the lack of pharmacological studies specific to cetaceans and thus the reason why some drugs in cetacean medicine, and in this case, are not used in a weight-based dosing strategy.

Bromhexine confirmed to be an effective tool, facilitating the release of mucous sputum and decreasing cough, given its mucolytic effect.

Imuno-2865™ is a naturally obtained hemicellulose and fatty acid mixture and is a commonly used product in marine mammals' medicine. By enhancing natural killer cell cytotoxicity, Imuno-2865™ helps promote and maintain a healthy immune response (Animal Necessity 2019), which might have an important role in MM management.

Some studies report that high concentrations of ascorbic acid seem to inhibit the growth of some fungi (Ojha et al. 2009). Vitamin C supplementation may be considered in mucormycotic infections, although no direct involvement of ascorbic acid in fighting MM has been described. Nonetheless, both vitamin C and B complex seem to contribute to the immune defence by supporting various cellular functions of the immune system, and their use in mucormycotic cases seems to be beneficial (Carr and Maggini 2017).

General management of MM in dolphins includes not only the correction of pathological states that may act as risk factors but also a thorough follow-up, especially regarding adverse responses to antifungal therapy. Given the hepatotoxicity commonly associated with the use of antifungal agents, the use of silymarin was considered (Reidarson et al. 2018). Moreover, although no specific studies regarding the interaction of silymarin and mucormycetes are available, this compound is known to exert antifungal effects on some fungi (Yun and Lee 2017).

Rectal ozone therapy sessions were initiated at low dosages and gradual increases were then applied. Given that not all patients respond equally to the small acute oxidative stress that is produced, it is crucial to start ozone treatments with low dosages ($ISCO_3$ 2015). Interestingly, this is the first successful reported case of MM treatment in cetaceans where ozone therapy was used. Objective and specific results are difficult to measure but several biological actions may have played an important role in the treatment management of this case. These include the pathogen inactivation capacity, regenerative effect, immunostimulation capacity and improvement of blood circulation and oxygen delivery. The latter two might have been crucial given the well-known capacity of mucormycetes to necrotize tissues and thus preventing immune cells and drug-delivery ($ISCO_3$ 2015).

Given the prolonged antifungal therapy and eventual antibiotic treatments associated with MM management, dysbiosis may develop and the use of prebiotic and/or probiotic medication may be considered. There are no prebiotic or probiotic medications specifically

developed for cetaceans. Faecal transfaunation procedures have been used in the management of MM (García-Párraga et al. 2016). In this clinical case, only preventive probiotic medication was used throughout treatment, with a change to Multi-Probiotic® given the greater mix of strains compared to Antibiophilus®. No dysbiosis appeared to exist.

Several theories may be suggested regarding the relapse of the infection. As previously discussed, given the overall high results of the PCZ monitoring, subtherapeutic serum concentrations do not seem a plausible cause of relapse. The short length of therapy compared to other cases seem to be an important factor to bear in mind. There are reported cases with treatment longer than one year (Townsend et al. 2006; Reidarson et al. 2018) and, in this case, the first period of PCZ therapy lasted only three months. Even considering the above, perhaps the main explanation for relapse resides in the capacity of mucormycetes to invade tracheal cartilage (mainly constituted by water), where PCZ may not reach given its high lipophilicity, independently of the overall good results in serum concentrations of PCZ (Kinsel and Briggs 2005). Although PCZ has been shown to have a high distribution in lung tissue (Felton et al. 2014), further studies are needed considering the capacity of this antifungal agent to achieve tracheal cartilage. Although there were no signs of infection in the diagnostic routine tools, including bronchoscopy, a reservoir of organisms might have been present in cartilage. Also, mucormycetes seem to be able to adapt an intracellular lifestyle within the innate immune effectors and they can also act as intracellular pathogens within granulomas, which may lead to the possibility of latent infections and disease reactivation (Ibrahim and Voelz 2017).

Concomitant bacterial isolation during this case were not always addressed, however, they were closely monitored. Firstly, it is important to note that knowledge regarding the association of microorganisms with marine mammals is still limited. Some studies have been developed in order to establish a baseline of microorganisms associated with bottlenose dolphins, investigating associations in microbiota between healthy and diseased specimens (Morris et al. 2011). Secondly, increased fungal growth is a common side effect of antibiotherapy and this should be taken into account when deciding to start antibiotics (Reidarson et al. 2018). Both *E. coli* and *M. morganii* were isolated in faecal samples, and, as in other species, they are believed to be part of a commensal relationship within the intestinal tracts of *T. truncatus* (Morris et al. 2011). In both isolation episodes, no gastrointestinal clinical signs were reported and occasional respiratory signs, as well as abnormalities on the CBC, were thought to be MM-associated. Antibiotherapy was performed on one occasion, where bronchoscopy follow-ups seemed to show a controlled fungal infection, although there was an abnormal set of results on

clinical pathology. In this case, *S. aureus* was isolated from a sputum sample, an agent commonly associated with respiratory infections in cetaceans (Tryland et al. 2018).

A particular approach was needed in the presented case since the animal was, in fact, a calf and therefore some specificities needed to be taken into consideration. These included the fact that this animal was still in the learning process of several medical behaviours, and therefore most medical tasks revealed to be problematical. In addition, since milk was an important component of the calf's diet, compliance with oral drug therapy needed to be considered. The calf was always accompanied by his mother and therefore when medical procedures were performed, not only the calf needed to be addressed while lifting the medical platform, but also his mother. This fact shows the required human resources capacity when considering involuntary medical procedures, where a team of at least six trainers are needed just to ensure an effective and secure containment of a 100 kg, one to two-year-old dolphin. Early fish introduction to the calf proved to be crucial to guarantee medication and thus avoid further involuntary procedures. Of course, precocious fish introduction is only to guarantee the calf interest for fish and milk feedings are not to be discarded. At the end of the period considered, the animal was in the learning process for voluntary blood collection, nebulization therapy and gastric and faecal sampling.

Overall, discussion of results is quite challenging given the lack of specifically described reported cases worldwide and the many controversies that still exist in the approach of MM in cetaceans and other species.

4.4. Conclusion

Although considered a rare infection in both humans and animals, mucormycosis is an emerging life-threatening fungal disease with a high mortality rate, despite the advent of newer antifungal agents. Even considering a greater susceptibility of dolphins and a wide distribution of mucormycetes, especially in soil and decay matter, they rarely cause disease. Further studies are needed when considering risk factors, both in free-ranging and in captive settings.

Historically, most dolphins diagnosed with MM have not survived. Over the past two decades, some cases with successful outcomes have been reported but diagnosis and treatment are still very challenging. This case report allowed to broaden the knowledge of MM management in dolphins, giving specific insights and analysing different diagnostic and treatment procedures used in a bottlenose dolphin. This case also explores the difficulties of medical management of MM in calves and the importance of voluntary medical behaviours in both preventive and reactive medical tasks. Additionally, invasive fungal infections are not

common diseases that a veterinarian must treat on a daily basis and a review on several important mycology related topics is of great importance when dealing with these infections, which includes notions on fungal structural and reproductive characteristics, epidemiology and pathogenesis.

Recognition and treatment at early stages do seem to contribute to a better outcome. However, nonspecific clinical manifestations, clinical pathology abnormalities and easily available diagnostic tools rarely permit differential diagnosis with other invasive fungal diseases, which may delay treatment. In this case, clinical resolution was not obtained up until the end of the timeframe considered. However, it is important to note that treatment of MM may take several years and relapses are common. This case clearly illustrates the importance of bronchoscopy in diagnosing respiratory disease in dolphins, together with conventional laboratory methods, such as direct microscopy and culture, and molecular assays. Additionally, this case supported the idea of PCZ as the drug of choice when dealing with MM. Drug concentrations should be measured throughout therapy, although the question of minimal serum-target values and specific dosages are yet to be answered.

Since only one individual was considered, conclusions from this clinical case may be biased. However, in the author's opinion, this case report emphasizes the pathogenic potential of MM in dolphins and the data presented might be crucial given the lack of information available. In fact, the lack of knowledge on this subject was one of the difficulties in developing this dissertation, as review studies regarding MM in cetaceans have not been developed. The access to abstracts and conferences presentations were an essential tool.

In order to optimize the clinical approach of MM, further studies on this matter and sharing knowledge between veterinary surgeons who have come across this infection are of utmost importance, given the numerous host-related, microbiological and pharmacological aspects that lead to highly individualized scenarios. Although some data presented in this case report has not been previously described in the literature (e.g. use of DRTs of PCZ in cetaceans), a lack of publication may be the reason.

Overall and in spite of the high mortality rate, MM may be a manageable disease, which requires a multidisciplinary approach, human resources and substantial financial investment. Lastly, follow-up of the patient to monitor overall health is essential.

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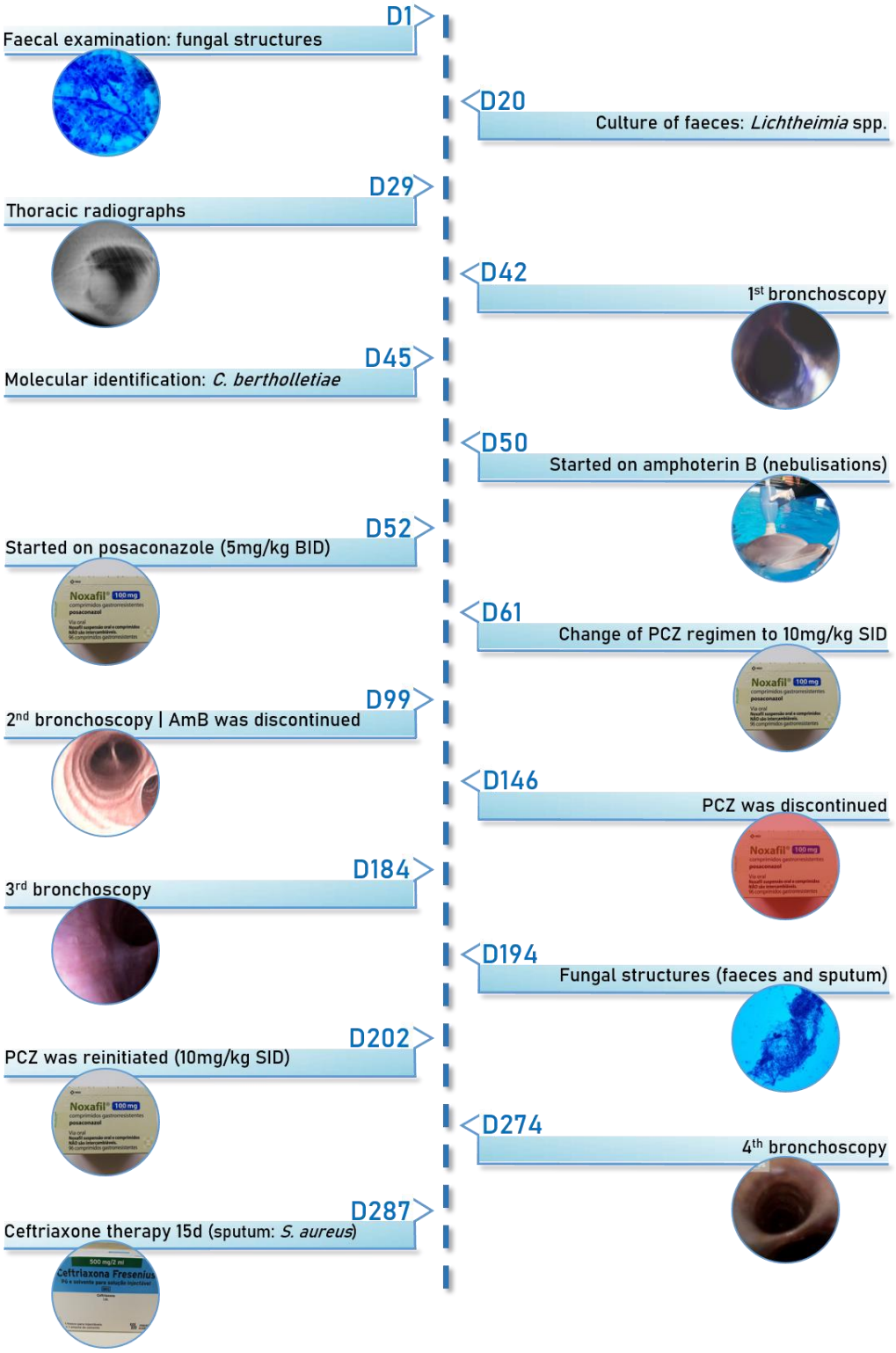
ANNEX 1 – TIMELINE WITH CRUCIAL POINTS OF MEDICAL MANAGEMENT (SCHEMATIC)



ANNEX 2 – REFERENCE HAEMATOLOGY AND SERUM CHEMISTRY RANGES - CAPTIVE *TURSIOPS TRUNCATUS*[3]

Parameter	Minimum	Maximum
RBC (10^6/mm^3)	3.00	3.74
Hb (g/dL)	13.5	15.5
HCT (%)	38	44
MCV (fL)	115	135
MCH (pg)	38	48
MCHC (g/dL)	34	36
Platelets (10^3/mm^3)	80	150
WBC x 10^9/L	5.0	9.0
Neutrophil (band)	0	
Neutrophil (mature)	3230	4850
Lymphocyte	840	1660
Monocyte	140	350
Eosinophil	530	1020
Basophil	0	
Total protein (g/dL)	6.0	7.8
Albumin (g/dL)	4.3	5.3
Globulin (g/dL)	1.3	2.5
Glucose (mg/dL)	90	170
BUN (mg/dL)	42	58
Creatinine (mg/dL)	1.0	2.0
Bilirubin (mg/dL)	0.1	0.2
Cholesterol (mg/dL)	150	260
ALP (U/L)	300	1300
ALT (U/L)	28	60
AST (U/L)	190	300
GGT (U/L)	30	50
CK (U/L)	100	250
LDH (U/L)	350	500
Calcium (mg/dL)	8.5	10.0
Phosphorus (mg/dL)	4.0	6.0
Sodium (mEq/L)	153	158
Potassium (mEq/L)	3.2	4.2
Chloride (mEq/L)	113	125
Iron (µg/dL)	120	340
Fibrinogen (mg/dL)	170	280
ESR (mm/h)	0	30

[3] Courtesy of SeaWorld (n = 38, sample = 1150)