Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Relatório de Estágio

Wild Animals Clinical Medicine and Surgery – Botulism in Gulls

Carolina Ramalho Rosado Gusmão

Orientador(es) | Professora Doutora Catarina Falcão Trigoso Vieira Branco Lavrador

Laura Suárez Regalado

Timothy Miles Partridge, BVSc, MRCVS

Évora 2019

Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Relatório de Estágio

Wild Animals Clinical Medicine and Surgery – Botulism in Gulls

Carolina Ramalho Rosado Gusmão

Orientador(es) | Professora Doutora Catarina Falcão Trigoso Vieira Branco Lavrador

Laura Suárez Regalado

Timothy Miles Partridge, BVSc, MRCVS

Évora 2019

O relatório de estágio foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

- Presidente | Rita Payan Carreira (Universidade de Évora)
- Vogal-arguente | Elsa Maria Leclerc Duarte (Universidade de Évora)
- Vogal-orientador | Catarina Falcão Trigoso Vieira Branco Lavrador (Direção Geral de Alimentação e Veterinária)

Évora 2019

“If you can dream it, you can do it”

- Walt Disney

Dedico-a a ti Mãe, por me ajudares a sonhar.

Acknowledgements

I would like to show my appreciation and gratitude and thank the people who have been part of my journey and helped me achieve my goal:

To my supervisor Professora Doutora Catarina Lavrador, for all the advice given, for supporting my decisions, for answering all my questions and doubts, and for the useful critiques that helped me grow and make me proud of this report.

To my GREFA supervisor, Laura Suarez Regalado, for all the teachings, trust and patience during my externship, for your amazing friendship and support and for making me laugh when my Spanish was hard to understand.

To my supervisor at Vale Wildlife Hospital & Rehabilitation Centre, Tim Partridge, for all the knowledge, patience and support shown, for being so insightful in veterinary knowledge, helping me with my English and boosting my confidence.

To professor Joana Reis, for the unexpected friendship, support and guidance, which were invaluable and truly special.

To the Erasmus + Programme, for the grant that financially supported my stay during my externship at GREFA and Vale Wildlife Hospital & Rehabilitation Centre.

To GREFA's volunteers and workers for becoming my family while I was in Spain. A special thank you to the veterinarians Irene, Virginia, Bárbara and Fernando, for teaching me and being the amazing people that you are. Also, to the volunteers that became my friends: Andrea Fernandez, Cris, Toño, Eli, Emma, Arantxa and Dani.

To Vale Wildlife Hospital & Rehabilitation Centre's volunteers and workers for being so welcoming. A special thank you to Jeff, for his patience and help provided. To Kat, for trusting in me and answering my questions. To Lydia, for letting me shadow her and learn with her, and for boosting my confidence when I had doubts. To my housemate and friend, Anna, for all the laughs and companionship.

To all my university friends that made the last six years unforgettable. A special thank you to my girls Cletts, Vali, Pimby, Frazão and Inês Maria, for all the wonderful moments we shared, for the support in times of crisis and exams, for putting up with me and being truthful when I needed it, there are no words to describe how special our friendship is to me.

To my hometown friends, all of us chose different paths but we kept supporting each other. A huge thank you to Leo, one of my long-time friends and one of the people I know I can count on whenever I need, for your unwavering support and friendship.

To my friends who I also consider my family, Carla, Dantas and Luisete, for supporting me despite the physical distance between us and for being my safe harbour when I needed.

To my family, because as the proverb says "it takes a village…" and you have helped me become who I am today.

Lastly, but most importantly, to my Mother because she is my pillar of strength. For your unconditional love and support. For making me believe in myself when it was hard to do so. For allowing me to dream and achieve my goal. For everything.

Abstract

This report was developed in the context of the Integrated Master's Degree in Veterinary Medicine carried out by the author and was based in her curricular externship at two wildlife hospitals/rehabilitation centres, one located in Spain and the other in the United Kingdom.

There are two main parts to this report. The first one includes a description of the activities performed by the author during the externship and data about the animals admitted at both centres. The second part includes a monograph about Botulism in Gulls and a case report of a Lesser Black-backed Gull (*Larus fuscus*) with botulism. Botulism is one of the most important diseases in wild birds worldwide. Outbreaks of this disease can have high mortality rates and a large impact on wildlife populations.

Keywords: avian botulism; botulism outbreaks; gulls; wildlife medicine; wildlife rehabilitation.

Resumo

Clínica e Cirurgia de Animais Selvagens - Botulismo em Gaivotas

Este relatório foi desenvolvido no âmbito do Mestrado Integrado em Medicina Veterinária e foi baseado no estágio curricular realizado pela autora em dois hospitais/centros de recuperação de animais selvagens, um localizado em Espanha e o outro no Reino Unido.

Este relatório está dividido em duas partes principais. A primeira inclui uma descrição das atividades desenvolvidas pela autora durante o seu estágio e dados sobre os animais admitidos nos dois centros. A segunda parte inclui uma monografia sobre Botulismo em Gaivotas e um caso clínico de uma Gaivota-d 'asa-escura (*Larus fuscus*) com botulismo. O botulismo é uma das doenças mais importantes do mundo em aves selvagens. Surtos desta doença podem ter elevadas taxas de mortalidade e um grande impacto nas populações de animais selvagens.

Palavras-chave: botulismo aviário; surtos de botulismo; gaivotas; medicina de animais selvagens; recuperação de animais selvagens.

Table of Contents

List of Tables

List of Figures

List of Abbreviations, Acronyms and Symbols

GREFA - *Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat* (Rehabilitation Group of Native Fauna and its Habitat)

RSPCA - Royal Society for the Prevention of Cruelty to Animals

PO - Orally

SC - Subcutaneously

IV - Intravenously

IO - Intraosseously

VD - Ventrodorsal

LL - Laterolateral

DV - Dorsoventral

CrCa - Craniocaudal

AVMA - American Veterinary Medical Association

H - Heavy

L - Light

GI - Gastrointestinal

Ach - Acetylcholine

SNARE - Soluble n-ethylmaleimide sensitive factor attachment protein receptor

SNAP-25 - Synaptosomal-associated protein of 25kDa

VAMP - Vesicle-associated membrane protein

MBA - Mouse bioassay

ELISA - Enzyme-linked immunosorbent assay

PCR - Polymerase chain reaction

I. Introduction

Wildlife hospitals and rehabilitation centres have the main objective of treating and rehabilitating injured and orphaned wild animals and releasing them back into the wild. Most wild animals become injured due to human activity. These centres work to abate the impact that humans have on wildlife and the environment.

A veterinary surgeon should make an animal's health and welfare their priority. For wildlife this means making sure that they are fit to survive and thrive in the wild. When treating wild animals, it should also be taken into consideration that these belong to a complex ecosystem. In this ecosystem they interact, even if indirectly, with members of their species, other wildlife species, domestic animals, humans and the environment [1]. This is especially important when releasing an animal, since it will cause change in the ecosystem that will be released into. That is why there is habitually an effort to release a wild animal where it was found, so that it can occupy its previous place in the ecosystem and restore its balance.

Besides taking into account wildlife health and welfare, these centres are also significant for human health. Wild animals can be important carriers and transmitters of diseases, particularly zoonoses (for example, mange). Wildlife hospitals and rehabilitation centres are paramount in controlling and treating zoonoses, and when these pose a threat to human health, they can alert authorities and prevent diseases from spreading.

This report stems from the author's interest in treating and rehabilitating wildlife. The author opted to do her externship at two wildlife hospitals and rehabilitation centres, one in Spain and the other in the United Kingdom. In this report, a description of the activities performed by the author is included as well as data about the animals admitted at both centres. A monograph about Botulism in Gulls and a case report of a Lesser Black-backed Gull (*Larus fuscus*) with botulism are also included. Botulism is not a zoonotic disease, but it is a very important avian disease that can have high mortality rates and a large impact on wildlife populations.

II. Externship Report

1. Introduction of the locations of the externship

The author spent the first three months of her externship (from October first until the twenty-third of December 2018) at GREFA (*Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat* – Rehabilitation Group of Native Fauna and its Habitat). GREFA is a wildlife hospital and rehabilitation centre located in Majadahonda, a municipality of Madrid (Spain). It focuses on the rehabilitation, investigation and conservation of wildlife, as well as raising awareness and educating people about these animals. The centre is integrated in Monte del Pilar, an 804-hectare Mediterranean forest. The wildlife veterinary hospital and other support buildings occupy a small portion of the forest. The centre is divided into several departments: Hospital, Rehabilitation, Nursery, Captive Breeding and Education, as wells as a Rescue Team. This centre also collaborates in various projects in order to reintroduce species into the wild.

GREFA's hospital is equipped with two triage rooms, a reptiles' room, an operating theatre, an X-ray room, a laboratory, a post-mortem examination room and a passageway with various cages that can be adapted to each animal species as well as their species-specific injuries. The Rehabilitation department also has different cages for the admitted animals.

The remaining time of the author's externship (which corresponds to eight weeks between the first of April and the twenty-fourth of May 2019) was spent at Vale Wildlife Hospital & Rehabilitation Centre (henceforth referred to as Vale). Vale is located on a 12-hectare piece of land in Beckford, a small village in the county of Gloucestershire in the United Kingdom. The main purpose of the Hospital is treating and rehabilitating injured and orphaned wildlife. On site they have a veterinary hospital, several aviaries and enclosures for the animals, as well as a small gift shop and a reception where the public can leave injured animals that they have found with a member of the staff, who will then take it inside the hospital to be assessed.

Vale's hospital has a triage room and a treatment room, that also works as an operating room and an X-ray room. There are also three different wards for newly admitted animals which are split according to their species or injuries (bird room, brooder room and small mammals' room). It also has different outside enclosures that are designed to accommodate different species according to their specific needs.

Even though these centres are in different countries most of the wildlife is similar. There are however some exceptions, for example in Spain you can find vultures, but they usually do not fly to the United Kingdom. Both centres receive a large number of animals, in 2018, GREFA received a total of 6858 animals (including cadavers) and Vale received 6002 animals (including cadavers). As shown in Figure 1 and Figure 2, the variation of the number of animals admitted throughout the year is similar.

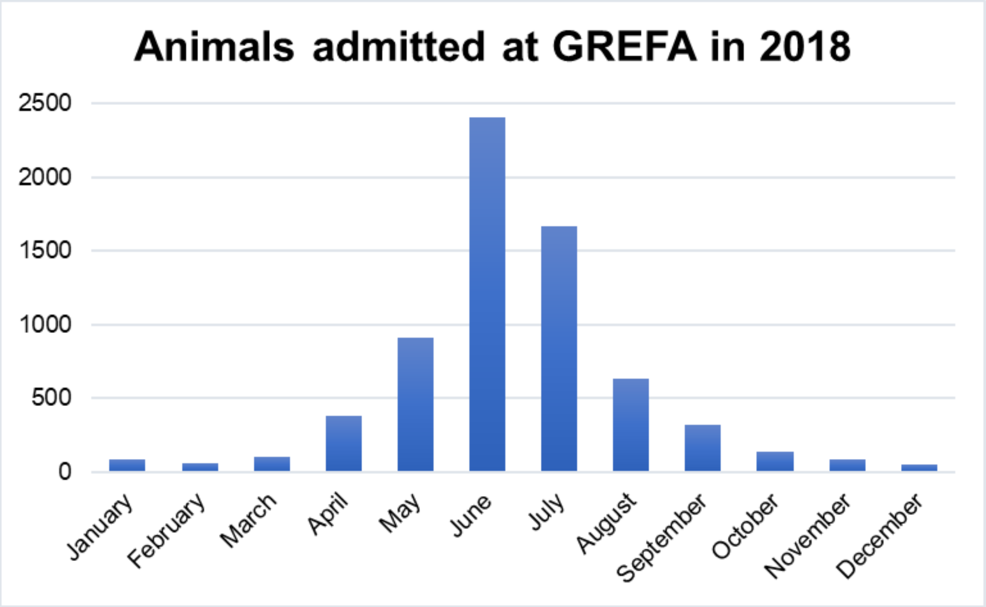

Figure 1 – Monthly distribution of animals admitted at GREFA in 2018 (n=6858).

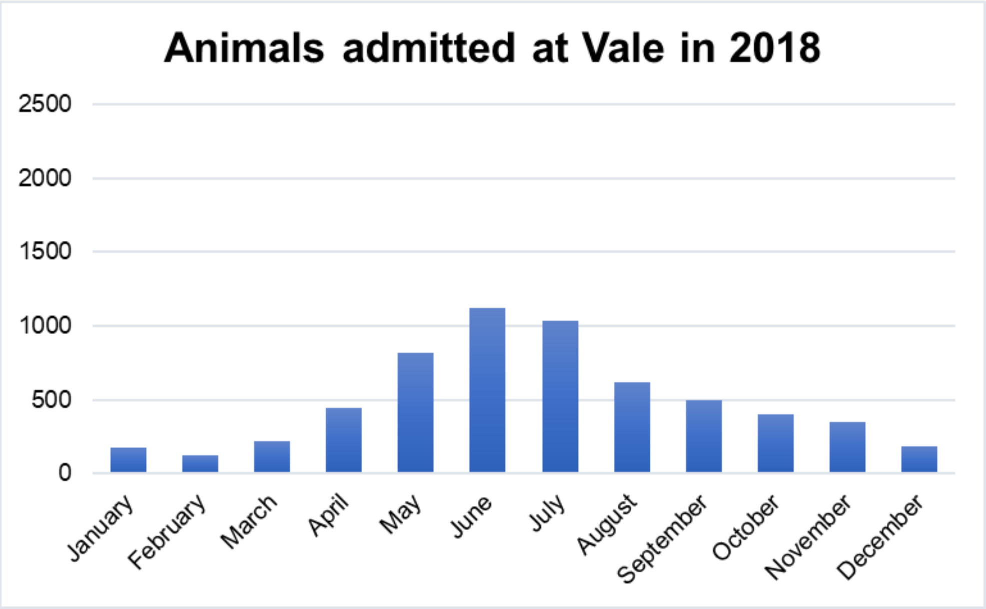

Figure 2 – Monthly distribution of animals admitted at Vale in 2018 (n=6002).

According to data from both centres, the variation in the number of admitted animals throughout the year, shown in the figures above, is repeated every year. The author observed this variation by seeing less animals at GREFA than at Vale, even though she spent more time at the former than at the latter. This happens because more animals are progressively admitted when Spring starts (around March/April), due to migrating birds and their mating season. When Autumn starts (around September/October) less animals are admitted because they start migrating to warmer countries and most species are no longer reproducing.

2. Externship at GREFA

Every day, before work started, a briefing was held to review what animals were in treatment and to discuss their diagnosis and treatment, as well as planning any samples that needed to be collected. After this, each of the veterinarians was paired with a group of volunteers and each of the groups was assigned one of the following tasks:

1. Daily medication and feeding of the animals in treatment;
2. Admission and triage of new animals;
3. Assessment of the animals kept at the centre, these could either be in the rehabilitation department, in captive breeding or irrecoverable animals.

During the author's externship at GREFA a total of 227 animals were admitted into the centre. Of these, 16 were already dead, meaning that 211 animals were treated during this period. As shown in Figure 3, the class of *Aves* was the one represented with a higher number of animals admitted, followed by *Mammalia*, *Reptilia* and *Amphibia*.

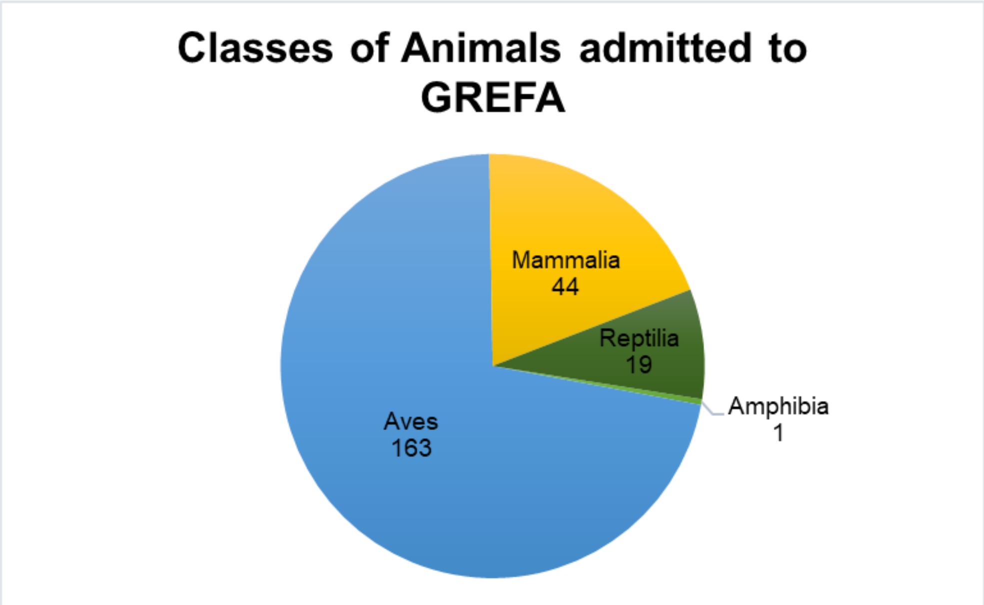

Figure 3 – Distribution of the absolute frequencies of the animals admitted at GREFA during the training period (n=227).

From Table 1 we can conclude that the admitted animals corresponded to 59 different species, which consisted of 44 species of *Aves*, nine of *Mammalia*, five of *Reptilia* and one of *Amphibia*. The species of *Aves* with a higher number of admissions was the Wood Pigeon (*Columba palumbus*), with 16 specimens. In the *Mammalia* class the highest number of admissions belonged to the Rabbit (*Oryctolagus cuniculus*), in the *Reptilia* class it was the Mediterranean Pond Turtle (*Mauremys leprosa*) and finally in the *Amphibia* class it was the Iberian Green Frog (*Pelophylax perezi*).

There are also some non-native species on this table, like Feral Pigeons (*Columba livia*) and Chinese Pond Turtle (*Mauremys reevesii*), that were admitted by mistake, as this centre is not allowed to treat these species. This is because they can pose a threat to the native species, therefore these were sent to other centres that can treat them.

Table 1 – Absolute frequencies of animals admitted of each species at GREFA, and corresponding common names and classes.

Scientific Name	Common Name	Class	Animals Admitted
Columba palumbus	Wood Pigeon	*Aves*	16
Larus fuscus	Lesser Black-backed Gull	*Aves*	14
Oryctolagus cuniculus	Rabbit	*Mammalia*	13
Passer domesticus	House Sparrow	*Aves*	13
Mauremys leprosa	Mediterranean Pond Turtle	*Reptilia*	12
Streptopelia decaocto	Collared Dove	*Aves*	12
Erinaceus europaeus	Hedgehog	*Mammalia*	11
Apus apus	Swift	*Aves*	8
Bubo bubo	Eagle Owl	*Aves*	7
Falco tinnunculus	Common Kestrel	*Aves*	7
Accipiter nisus	Sparrowhawk	*Aves*	6
Capreolus capreolus	Roe Deer	*Mammalia*	6
Gyps fulvus	Griffon Vulture	*Aves*	6
Pica pica	Magpie	*Aves*	5
Ardea cinerea	Grey Heron	*Aves*	4
Buteo buteo	Common Buzzard	*Aves*	4
Ciconia ciconia	White Stork	*Aves*	4
Mus musculus	House Mouse	*Mammalia*	4

Sus scrofa	Wild Boar	*Mammalia*	4
Sylvia atricapilla	Blackcap	*Aves*	4
Anas platyrhynchos	Mallard	*Aves*	3
Bubulcus ibis	Cattle Egret	*Aves*	3
Larus ridibundus	Black-headed Gull	*Aves*	3
Melopsicattus undulatus	Budgerigar	*Aves*	3
Sturnus unicolor	Spotless Starling	*Aves*	3
Testudo graeca	Greek Tortoise	*Reptilia*	3
Turdus merula	Blackbird	*Aves*	3
Vulpes vulpes	Red Fox	*Mammalia*	3
Accipiter gentilis	Goshawk	*Aves*	2
Athene noctua	Little Owl	*Aves*	2
Cervus elaphus	Red Deer	*Mammalia*	2
Circaetus gallicus	Short-toed Eagle	*Aves*	2
Dendrocopos major	Great Spotted Woodpecker	*Aves*	2
Ficedula hypoleuca	Pied Flycatcher	*Aves*	2
Mauremys reevesii	Chinese Pond Turtle	*Reptilia*	2
Milvus milvus	Red Kite	*Aves*	2
Parus/Periparus ater	Great Tit	*Aves*	2
Pipistrellus pipistrellus	Common Pipistrelle Bat	*Mammalia*	2
Scolopax rusticola	Woodcock	*Aves*	2
Strix aluco	Tawny Owl	*Aves*	2
Aegypius monachus	Black Vulture	*Aves*	1
Alauda arvensis	Skylark	*Aves*	1
Aquila/Hieraaetus pennatus	Booted Eagle	*Aves*	1
Burhinus oedicnemus	Stone Curlew	*Aves*	1
Caprimulgus europaeus	Nightjar	*Aves*	1
Carduelis cannabina	Linnet	*Aves*	1
Columba livia	Feral Pigeon	*Aves*	1
Delichon urbicum	House Martin	*Aves*	1
Emys orbicularis	European Pond Turtle	*Reptilia*	1
Loxia curvirostra	Common Crossbill	*Aves*	1

Motacilla alba	Pied Wagtail	*Aves*	1
Pelophylax perezi	Iberian Green Frog	*Amphibia*	1
Phoenicurus ochruros	Black Redstart	*Aves*	1
Phylloscopus trochilus	Willow Warbler	*Aves*	1
Picus viridis	Green Woodpecker	*Aves*	1
Prunella modularis	Dunnock	*Aves*	1
Tadarida teniotis	Free-tailed Bat	*Mammalia*	1
Tarentola mauritanica	Common Wall Gecko	*Reptilia*	1
Turdus philomelos	Song Thrush	*Aves*	1

When animals arrive at the hospital, they are brought in either by the GREFA Rescue Team, the police/forest guards or the general public. Upon arrival they are asked for any history relating to the animals in order to determine the reason for admission. When the cause of admission was unknown, one would be determined based on the injuries found while performing the physical examination or it would be considered unknown.

During the author's externship, 102 animals were admitted due to trauma, being this the most common cause of admission. The causes of admission for the animals treated at GREFA during this period are shown in Figure 4.

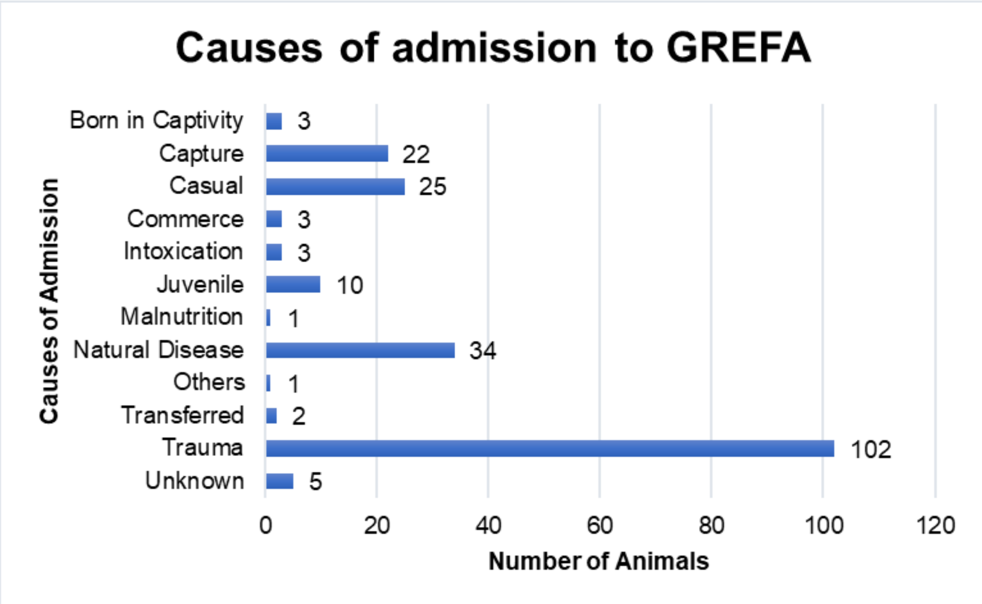

Figure 4 – Causes of admission and corresponding absolute frequencies of the animals admitted to GREFA (n=211).

As shown by the figure above there are several causes of admission for the animals that arrive at this centre. Each of the causes of admission is explained below:

- Born in captivity – animals that were born in captivity and later brought to the centre.
- Capture – animals that were intentionally caught by members of the public and kept by them; animals that got caught in traps, netting, etc.
- Casual – animals found outside their habitats, like private gardens, balconies, buildings, etc.
- Commerce – animals that were bought as pets.
- Intoxication – poisoned animals (for example, with lead).
- Juvenile – young animals that were orphaned, abandoned, fell from the nest or are fledglings.
- Malnutrition – animals extremely underweight and debilitated.
- Natural disease – animals affected by natural diseases, for example, botulism or myxomatosis.
- Others – cause of admission that doesn't fall into any of the other categories.
- Transferred – animals received from other rescue centres (that have no capacity to treat them).
- Trauma – animals that arrived with a suspicion of trauma (window strike, road traffic accident, animals shot, animal attacks, etc).
- Unknown – when no cause of admission can be identified.

During the training period, 211 live animals were admitted to GREFA. Seventy nine of these had to be euthanised (37%), due to various reasons that will later be explained in this report; 44 died while receiving treatment (21%), 44 were still in treatment by the time the author finished her externship (21%), 36 were released back into the wild (17%) and eight were transferred to other rescues centres/wildlife hospitals (4%) (Figure 5). Animals are usually transferred to other rescues centres/wildlife hospitals because they cannot be released back into the wild and these rescues/hospitals can keep them for educational purposes or conducting research with these species.

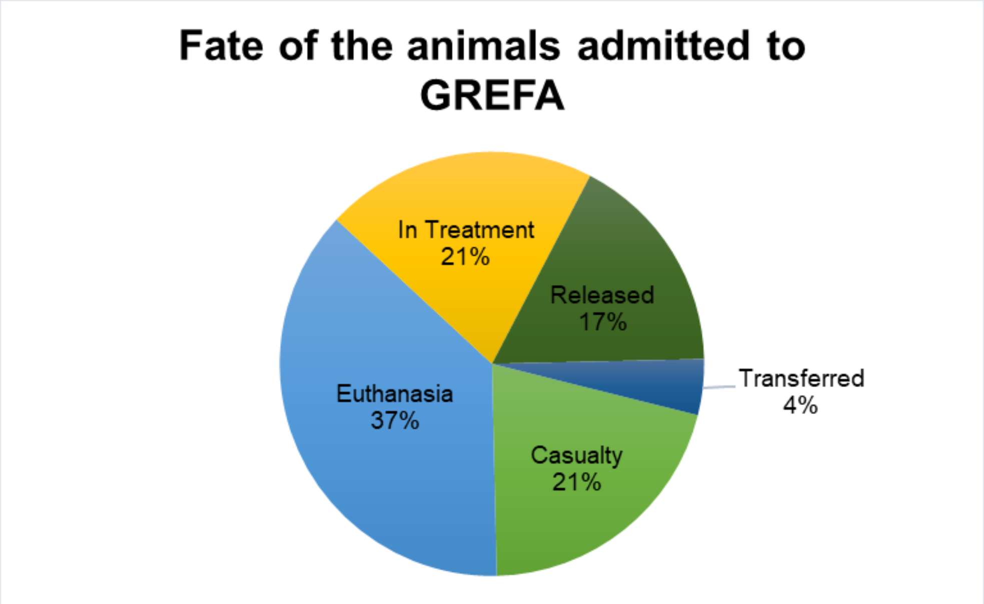

Figure 5 – Fate of the animals admitted to GREFA and respective relative frequencies.

While at GREFA the author was also able to attend lectures for the volunteers at this centre. These helped to reinforce what was being learnt and put into practice, and included:

- First aid for wild animals I and II;
- Post-mortem examination techniques for birds;
- Post-mortem examination techniques for reptiles;
- Parasitology techniques for wild animals;
- Power lines and their problems in relation to GREFA projects;
- Principles of haematology for birds;
- X-ray diagnosis for birds.

The activities developed and the knowledge obtained during the externship will be described in more depth in a later section of this report.

3. Externship at Vale Wildlife Hospital & Rehabilitation Centre

Every day, before starting work, the author would be assigned to one of the areas of the centre: bird room, brooder room, small mammals' room, hedgehog unit, triage room or outside enclosures. The author was also able to participate in some of the rescue calls to injured animals.

A total of 1163 animals were admitted during the author's externship at Vale. Besides these animals, the centre also gave remote assistance to 12 animals, giving advice on how to proceed with certain animals (mainly foxes with mange). The rescue team was also called to nine other animals, but these couldn't be found or caught. Twenty of the admitted animals were already dead on arrival, which means that 1143 were treated at this centre during this period. It is shown on Figure 6 that the class of *Aves* is the one represented by the highest number of animals admitted, followed by *Mammalia*, *Reptilia* and *Amphibia*.

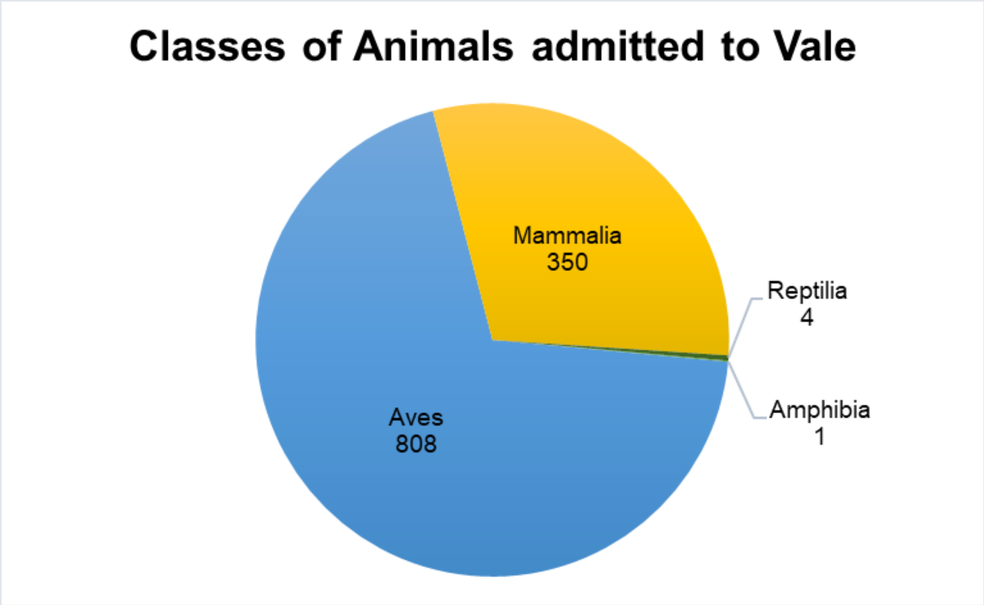

Figure 6 – Distribution of the absolute frequencies of the animals admitted at Vale during the training period (n=1163).

It is shown on Table 2 that the animals admitted at Vale belonged to 74 different species. This excludes the eggs that were admitted but had not hatched when the author finished her externship, so it was not always possible to know to which species they belonged. It also excludes the unidentified nestlings, that were too young to identify.

From this table it is also possible to see that 52 of these species were of the *Aves* class, 18 of the *Mammalia* class, three of the *Reptilia* class and one of the *Amphibia* class. The species of *Aves* with the highest number of admissions was the Mallard Duck (*Anas platyrhynchos*), with a total of 158 specimens admitted. For the remaining classes the species with the highest number of admissions were: the Hedgehog (*Erinaceus europeus*) for the *Mammalia* class, the Yellow-Bellied Slider (*Trachemis scripta scripta*) for the *Reptilia* class and the Toad (*Bufo bufo*) for the *Amphibia* class.

Some of the species shown in this table are domestics and unlike at GREFA, some of them can stay at this centre. For example, the Feral or Racing Pigeons (*Columba livia*) are considered wildlife at this centre and are treated like any other wild animal. The other domestic species, like the Ferret (*Mustela putorius furo)*, the Chicken (*Gallus gallus domesticus*), the Yellow-Bellied Slider (*Trachemys scripta scripta*), the Call Duck (*Anas platyrhynchos* *domesticus*), the Corn Snake (*Pantherophis guttatus*), the Royal Python (*Python regius*) and the Hybrid Duck, were only boarding at the centre until the RSPCA (Royal Society for the Prevention of Cruelty to Animals) could collect them back and put them in suitable housing. The RSPCA is an organization that also collects wild animals and sometimes asks Vale to hospitalise domestic animals when their own facilities are full at that time or are inadequate for them.

Table 2 – Absolute frequencies of animals admitted of each species at Vale, and corresponding common names and classes.

Scientific Name	Common Name	Class	Animals Admitted
Anas platyrhynchos	Mallard Duck	*Aves*	158
Erinaceus europaeus	Hedgehog	*Mammalia*	133
Columba palumbus	Wood Pigeon	*Aves*	97
Turdus merula	Blackbird	*Aves*	70
Erithacus rubecula	Robin	*Aves*	57
Vulpes vulpes	Fox	*Mammalia*	48
Oryctolagus cuniculus	Rabbit	*Mammalia*	42
Columba livia	Feral/Racing Pigeon	*Aves*	41
Sturnus vulgaris	Starling	*Aves*	36
Streptopelia orientalis	Collared Dove	*Aves*	33
Passer domesticus	Sparrow	*Aves*	32
Apodemus sylvaticus	Wood Mouse	*Mammalia*	31
Cyanistes caeruleus	Blue Tit	*Aves*	29
Sciurus carolinensis	Grey Squirrel	*Mammalia*	29
Prunella modularis	Dunnock	*Aves*	21
Parus major	Great Tit	*Aves*	20
Corvus corone	Crow	*Aves*	19
Pica pica	Magpie	*Aves*	17
Capreolus capreolus	Roe deer	*Mammalia*	16
Meles meles	Badger	*Mammalia*	16
Strix aluco	Tawny owl	*Aves*	16
Muntiacus reevesi	Muntjac deer	*Mammalia*	15
Corvus monedula	Jackdaw	*Aves*	13

Turdus philomelos	Song Thrush	*Aves*	13
Cygnus olor	Mute Swan	*Aves*	11
Phasianus colchicus	Pheasant	*Aves*	10
Aegithalos caudatus	Long-tailed Tit	*Aves*	9
Buteo buteo	Buzzard	*Aves*	9
Carduelis carduelis	Goldfinch	*Aves*	9
Larus fuscus	Lesser Black-Backed Gull	*Aves*	9
Branta canadensis	Canada Goose	*Aves*	7
Larus argentatus	Herring Gull	*Aves*	7
Troglodytes troglodytes	Wren	*Aves*	6
-	Unidentified Nestling	*Aves*	5
Aythya fuligula	Tufted Duck	*Aves*	5
Corvus frugilegus	Rook	*Aves*	5
Dama dama	Fallow Deer	*Mammalia*	5
Tadorna tadorna	Shelduck	*Aves*	5
Apus apus	Swift	*Aves*	3
Mustela putorius furo	Ferret	*Mammalia*	3
Pipistrellus pipistrellus	Pipistrelle Bat	*Mammalia*	3
Rattus norvegicus	Brown rat	*Mammalia*	3
-	Egg	*Aves*	2
Accipter nisus	Sparrowhawk	*Aves*	2
Alectoris rufa	Red-Legged Partridge	*Aves*	2
Anser anser	Greylag Goose	*Aves*	2
Dendrocopos major	Great-Spotted Woodpecker	*Aves*	2
Fringilla coelebs	Chaffinch	*Aves*	2
Gallinula chloropus	Moorhen	*Aves*	2
Gallus gallus domesticus	Chicken	*Aves*	2
Lepus europaeus	Hare	*Mammalia*	2
Trachemys scripta scripta	Yellow-Bellied Slider	*Reptilia*	2
Turdus viscivorus	Mistle Thrush	*Aves*	2
-	Hybrid Duck	*Aves*	1
Anas crecca	Common Teal	*Aves*	1

Anas platyrhynchos domesticus	Call Duck	*Aves*	1
Bufo bufo	Toad	*Amphibia*	1
Cairina moschata	Muscovey Duck	*Aves*	1
Carduelis cannabina	Linnet	*Aves*	1
Chloris chloris	Greenfinch	*Aves*	1
Corvus corax	Raven	*Aves*	1
Delichon urbicum	House Martin	*Aves*	1
Falco peregrinus	Peregrine Falcon	*Aves*	1
Lutra lutra	Otter	*Mammalia*	1
Microtus agrestis	Vole	*Mammalia*	1
Motacilla alba	Pied Wagtail	*Aves*	1
Motacilla cinerea	Grey Wagtail	*Aves*	1
Mustela erminea	Stoat	*Mammalia*	1
Myotis daubentonii	Daubentons Bat	*Mammalia*	1
Pantherophis guttatus	Corn Snake	*Reptilia*	1
Periparus ater	Coal Tit	*Aves*	1
Phylloscopus trochilus	Willow Warbler	*Aves*	1
Python regius	Royal Python	*Reptilia*	1
Rhinolophus hipposideros	Lesser Horseshoe Bat	*Mammalia*	1
Scolopax rusticola	Woodcock	*Aves*	1
Sylvia atricapilla	Blackcap	*Aves*	1

Similarly to what happens at GREFA, a cause of admission is determined when an animal is admitted to Vale, and this is also done using similar criteria.

During the author's externship, a total of 458 animals were admitted due to trauma, this also being, as mentioned before, the most common cause of admission at this centre.

The different causes of admission that are used at Vale are very similar to the ones used at GREFA, so these were grouped in the same way. The only exception is that at this centre they define a category called "Human Involvement", which includes cases where direct human action was the reason for the animals to be brought in, for example, nests that were disturbed or animals that were picked up with no injuries. The causes of admission of the animals treated at Vale during this period are shown in Figure 7.

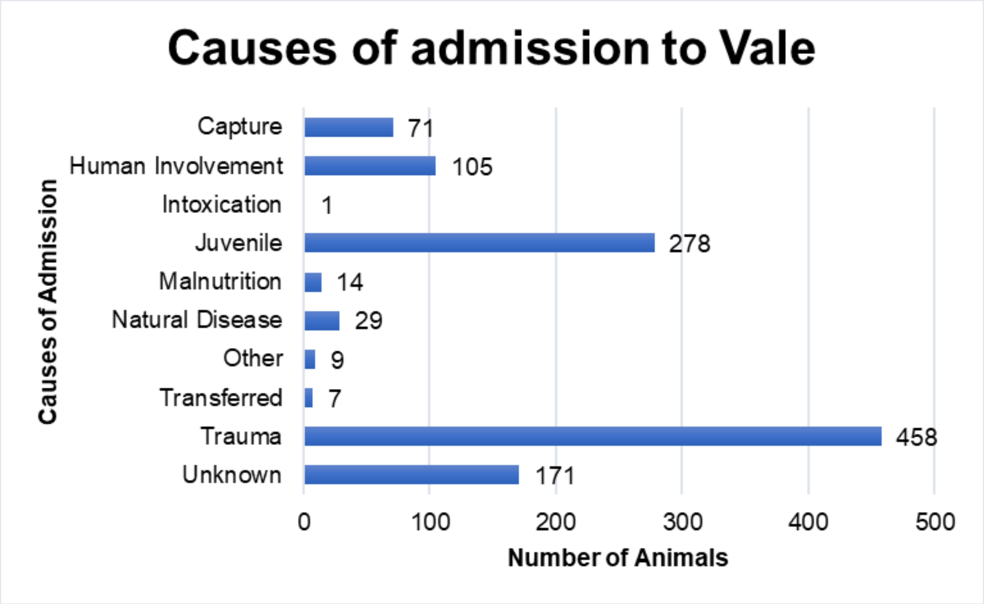

Figure 7 – Causes of admission and corresponding absolute frequencies of the animals admitted to Vale (n=1143).

A total of 1143 animals were treated at Vale during the training period. Euthanasia had to performed to 371 of these animals (32%), due to various reasons that will later be explained in this report. Also, 212 died while receiving treatment (19%), 381 were still in treatment by the time the author finished her externship (33%), 166 were released back into the wild (15%) and 13 were transferred to other rescues centres/wildlife hospitals, because these were better equipped to treat them (1%) (Figure 8).

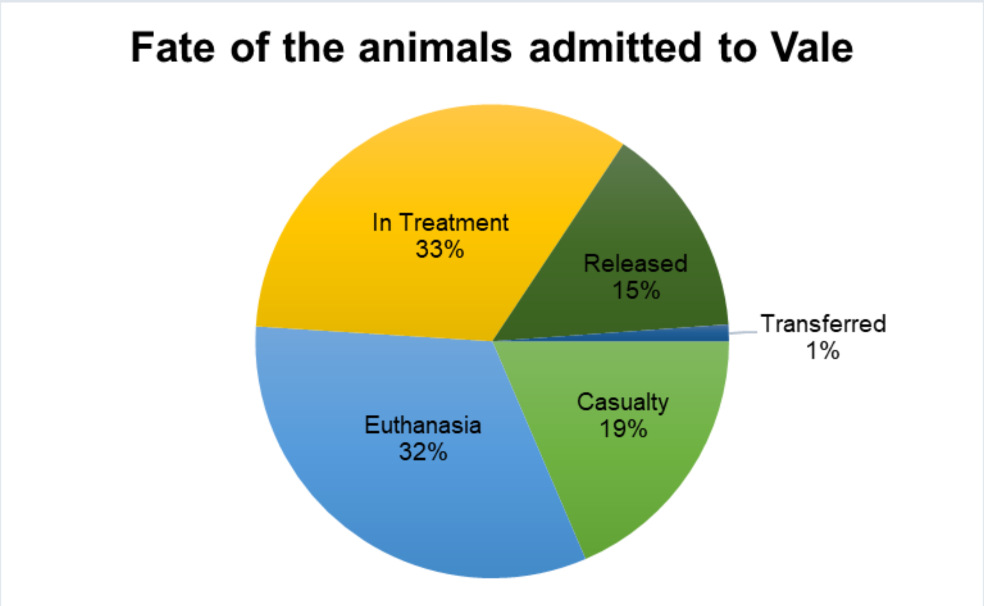

Figure 8 – Fate of the animals admitted to GREFA and respective relative frequencies.

4. Training and knowledge acquired during the externship

During her externship the author received training and gained knowledge in the main areas of Clinical Examination, Diagnostic Imaging, Surgery and Anaesthesia, Laboratory Techniques, Post-mortem Examination and Rehabilitation of wildlife animals.

Euthanasia will be explained in more depth because it is a procedure that unfortunately, must be dealt with on a daily basis at wildlife rescue centres. It is also the fate of a relatively high percentage of the treated animals at both places of externship, accounting for 37% and 32% of cases at GREFA and Vale, respectively. Because of this the author feels that is a theme that should deserve special attention.

While treating wild animals, careful attention should be taken to the species being treated and to the injuries with which the animal presents. The techniques used must be adapted in order to achieve better results when treating these animals.

The skills developed and techniques applied are similar at both places of externship, therefore the areas mentioned above will be explained in a general and common way for both centres. Whenever there are major differences between the rescue centres this will be highlighted for a clearer understanding.

4.1. Clinical Examination

The Clinical Examination of the animals includes restraining the animal, physical examination, obtaining the previous history (when possible) and determining the cause of admission. Applying first aid and emergency care is also an important part, as well as determining adequate therapeutic protocols, collecting samples and, if needed, performing X-rays.

Since these centres deal with wild animals, special precautions must be taken when handling them, specifically when it comes to restraining techniques. Bad restraint puts at risk both the patient and the person examining it.

While restraining a bird it is important to have one hand holding the chest or the head (in large birds) and the other holding the legs (with one finger between the legs so that the animal doesn't hurt itself by rubbing them together or accidently hurts itself with its nails or claws and also to avoid fractures). If possible, a towel or a hood should be placed over their head so that they are calmer and less stressed. In case of bigger birds, the handler should also use their arms to make sure that the bird doesn't open its wings. Sometimes, especially with birds of prey, leather gloves may be needed. For small birds this restraining technique can't be used due to their size, but they can be easily restrained by hand or with a small cloth or towel.

When it comes to mammals, hedgehogs and rabbits were the most common species in these centres. Hedgehogs are usually restrained by using leather gloves. Rabbits usually arrive in a state of *freezing* and are relatively easy to restrain. For larger mammalian species, like foxes or badgers, if they are young they can be restrained just by grabbing their scruff, if they are larger, sedation or volatile anaesthesia might be necessary.

Reptiles can be restrained by only using one's bare hands, but careful restraint of the head might be necessary, depending on the species being handled, to prevent bites.

The physical examination starts by collecting the animal from the carrier or box used to bring them in. It is important to notice whether the animal seems to be in a normal position for the species and if not, annotate the findings. For example, if it is not standing or if one of its limbs is in an abnormal position it is important to note this. Then the animals should be weighed, to assess if the animal is within the normal range for the species and to later establish adequate dosage of treatment, if needed. Distance observation might also be included to assess the animal's breathing, demeanour and gait, for example. Feather or fur condition can also be assessed at this stage.

To begin the physical examination itself, for birds it is easier to have them ventrodorsally on a table and start by assessing their hydration status. This is done by tenting the skin on the keel. The body score can be determined at the same time. After this, the whole keel should be felt/palpated, followed by the clavicles, both wings and legs. While doing the physical examination the joints should also be mobilised so that any possible joint fractures or luxations can be detected. If no fractures are found, the wings should also be fully extended to make sure that there is full range of movement. The head is left until last so that the animal remains calmer during the whole examination. When examining the head, the eyes should be checked to make sure they are symmetrical, and no obvious injuries are found. Both eyelids should also be examined, as well as the nictitating membrane. Both ears must also be examined, as well as the beak, nares and lastly the inside of the oral cavity.

When it comes to mammals the physical examination is very similar to the way it is done in companion animals. The biggest exception are hedgehogs, but they usually uncurl after a while of not being touched or by stroking their rump vigorously in a craniocaudal direction. After uncurling it is relatively easy to assess their gait and determine if it is normal or not. Another option is having them on the examination table and after they uncurl, they can be brought to the edge of the table, it is then possible to examine their underside and limbs.

When dealing with reptiles the hydration status is determined by the humidity of the skin and as described above, every limb as well as the head should be examined.

When animals are moving too much and a safe and thorough examination is not possible, the use of sedation or volatile anaesthesia might be necessary. For example, when it comes to hedgehogs most of the time they don't uncurl and don't allow a clinical examination and it is necessary to use these methods. For other mammals mentioned above, like foxes, badgers, etc. these methods need to be used to do a thorough and safe examination (unless they are young).

In terms of reptiles, these usually can be examined while they are awake, but again, if needed they can also be sedated or anaesthetised.

The animals' previous history is often very difficult to obtain but if the person who spotted the animal saw obvious injuries this might help with diagnosis and treatment. Determining the cause of admission can also be extremely difficult because most of the time the animals are found injured but without knowledge of what caused the injuries. However, there are cases where this is known which can be very helpful when choosing a treatment protocol.

First aid and emergency care are extremely important in animals that arrive with serious wounds and/or fractures, in shock, in respiratory distress or extremely dehydrated.

When an animal arrives with wounds that are at risk of infection it is crucial to immediately treat the wound, and sometimes suturing might also be necessary. In some cases, particularly when suturing is needed, the animal should be under anaesthesia because the treatment is too painful.

If fractures are found during the initial clinical examination their stabilization is of paramount importance and should be done immediately to prevent misalignment and further damage to the surrounding tissues. There are a wide range of bandages and splints that can be used on wild animals, but it is important to consider the species that is being dealt with when choosing the right type and dressing materials to use. For example, in birds, whose skeleton is light and adapted to flight, bandages and splints used shouldn't be too heavy. Otherwise the animal will be too uncomfortable and unbalanced, which increases the chances of the animal sustaining further injury, trying to remove its bandages and/or stop eating, possibly delaying its recovery.

Both when dealing with shock and dehydration it is very important to rehydrate the animal. Fluids can be given orally (PO), subcutaneously (SC), intravenously (IV) or intraosseously (IO) Each of these techniques has different advantages and disadvantages, and the decision to use one or the other depends on the case at hand. The author was able to perform all these types of rehydration, with exception of the intraosseous route, since this one is painful and requires a lot of experience, being used only as a last resort. Even though the author couldn't do it on a live animal, practice was possible on cadavers. Also, if the animal is in shock and/or respiratory distress, it should be decided if the animal can handle rehydration or not. The animal should be moved first to an oxygenation chamber, that should be dark and warm so that the animal can be stable before continuing any sort of examination.

Therapeutic protocols should be chosen carefully depending on the injuries with which the animal presents. Drugs can be administered through different routes such as oral, subcutaneous, intramuscular, intravenous, topical, pericoelomic, or corneal routes. If the animal arrives dehydrated it is very important to establish a rehydration schedule and continuously assess if it is still needed.

Establishing a treatment protocol also includes defining adequate nutrition. When the animal isn't eating or has a low body score it might be necessary to force feed or tube feed it.

Collecting samples from the admitted animals is very important either for diagnostic purposes or for projects being developed at the centres, for partnerships, research projects, etc. Samples collected were for haematology, biochemistry, proteinograms, parasitology, microbiology and toxicology. The author was also able to collect these different types of samples.

Radiographic Imaging is also a very important part of the Clinical Examination, but this theme will be explored in more depth in the Diagnostic Imaging section.

Besides all the procedures mentioned above, a full ophthalmologic exploration might be needed when examining a wild animal, especially when it comes to birds of prey. Also, the use of thermal imaging was available at GREFA and this was particularly useful when birds suspected of electrocution came in because it allows clear identification of which body parts are affected. The wings and feet are the most commonly affected body parts. In electrocuted birds the affected body parts present a lower temperature than the rest of the body. A thermal camera has a colour map that corresponds to the different body temperatures, for example, a normal body temperature can be defined in red tones and colder temperatures in blue tones. This technology allows early detection of electrocuted animals and treatment of this condition with higher effectiveness.

4.2. Diagnostic Imaging

At these centres Diagnostic Imaging is mainly composed by X-rays and these are an extremely important part, especially when it comes to fractures and their resolution. It also allows to detect radiographic changes of the organs that might be associated with certain diseases.

The Ventrodorsal (VD) projection is the standard projection for X-raying a bird, it usually enables a good diagnosis. In some cases, it is possible to radiograph and obtain a good VD image of a bird whilst it is conscious, but if it is necessary, sedation or light anaesthesia should be used. For this projection the bird must be put in a dorsal recumbency, then the head must be secured at the neck level with paper tape, making sure that the neck is stretched. If it is a larger animal weights should be used instead of tape, making sure that these do not cover important structures. Ideally, the head should be covered with a towel or a hood to have a higher chance of the animal staying still during the procedure and reduce stress. This is especially important if the animal is awake. Then the hind limbs should be fully extended to prevent superimposition of the coelomic cavity and taped to maintain that position. Lastly the wings should be positioned to form a 45-degree angle between each joint and secured with tape. The keel and the spine should be aligned. The VD projection provides a good radiographic image to evaluate the whole skeleton and organs.

If any doubt arises, for example, if a fracture is present and it is necessary to further assess any misplaced bone, a Laterolateral (LL) image might be needed.

In the LL projection the bird must be put on one of its sides and the head must be secured as in the VD projection. The wings must be pulled over the back of the bird and secured with paper tape or weights. Then the hind limbs must be fully extended too, in this case they should be placed one in front of the other to avoid their superimposition.

Radiographic images are shown below portraying both positions described above (Figure 9A and 9B).

Figure 9A and 9B – Ventrodorsal radiographic image of a Common Kestrel (*Falco tinnunculus*) (9A) and Laterolateral radiographic image of a Lesser Kestrel (*Falco naumanni*) (9B) (images kindly provided by GREFA).

When it comes to mammals, the standard radiographic images are also the VD and LL projections and the animals are placed in the same fashion as in companion animal practice, as shown in the figures below (Figure 10A and 10B).

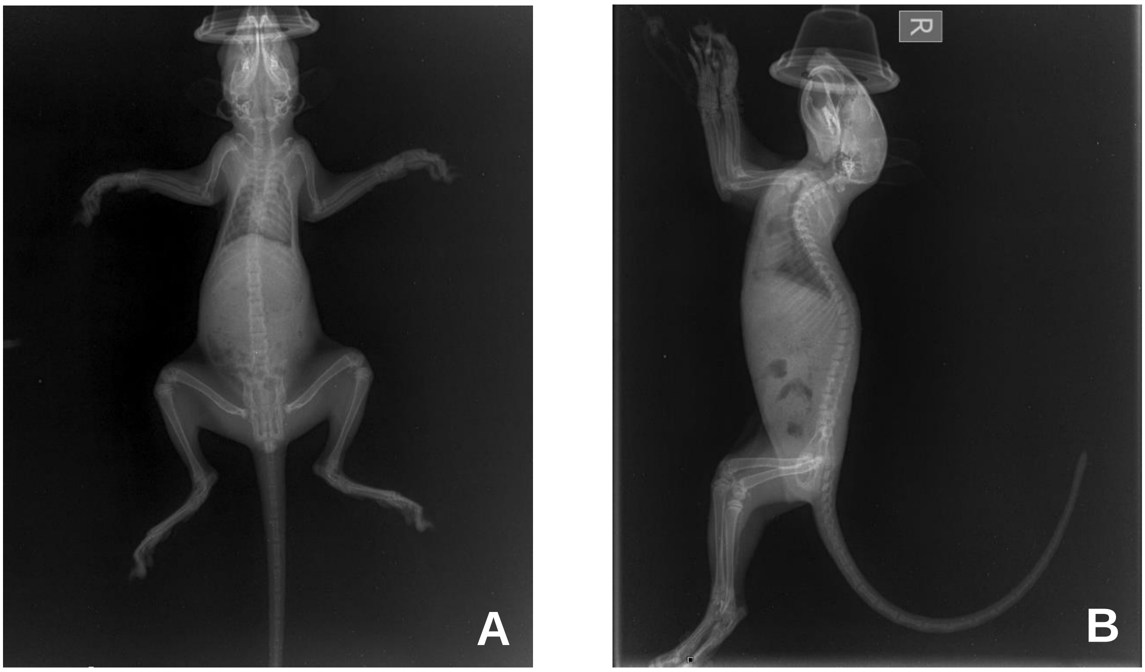

Figure 10A and 10B – Ventrodorsal (10A) and Laterolateral (10B) radiographic image of a Grey Squirrel (*Sciurus carolinensis*) (images kindly provided by Vale Wildlife Hospital & Rehabilitation Centre).

Since the most common reptiles to be admitted at these centres were turtles, the projections for this species will be explained in more depth.

When X-raying turtles, the standard projections used to obtain a diagnosis are the Dorsoventral (DV), LL and Craniocaudal (CrCa) projections. These animals usually retract completely into their shell or do not stop moving, so to obtain good radiographic images it is better to sedate or anesthetise these animals. For a DV projection it is important to extend the turtle's limbs so that a clear radiographic image is obtained. By doing so the coelomic cavity can clearly be radiographed permitting a correct diagnosis to be made. For both the LL and CrCa projections, the use of foam pads is necessary to position the animal's body correctly. For the LL projection the limbs should also be out of the shell to obtain a good radiographic image of the coelomic cavity.

Below are radiographic images obtained by these projections (Figure 11A, 11B and 11C).

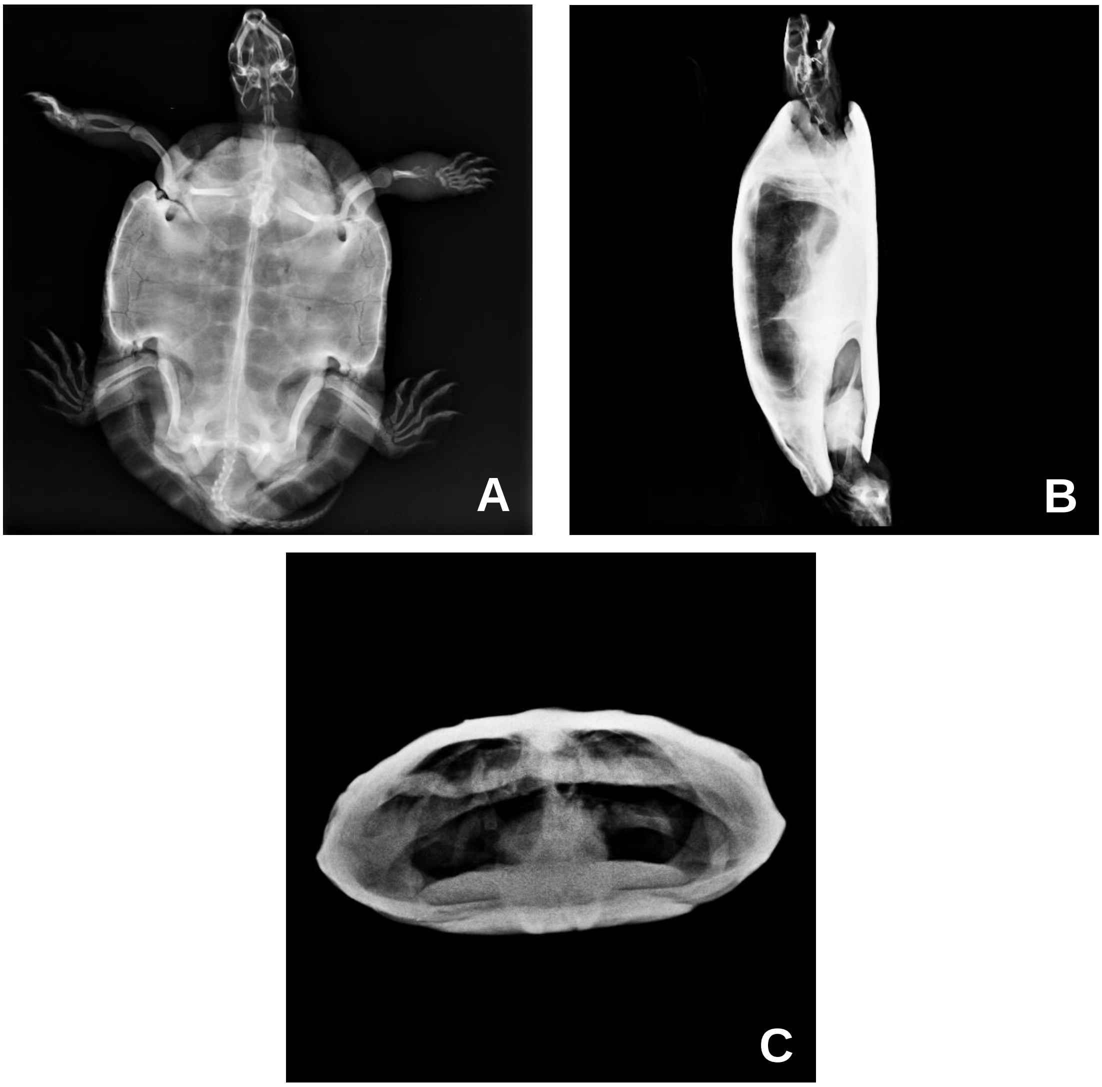

Figure 11 A, 11B and 11C – Dorsoventral (11A), Laterolateral (11B) and Cranialcaudal (11C) radiographic images of different Mediterranean Pond Turtles (*Mauremys leprosa*). In Figure 11A a fracture of the right radius and ulna can be seen. In Figure 11B a fishing hook is present in the mouth (images kindly provided by GREFA).

Radiographic diagnosis was available at both centres where the author did her externship. Since this was a service used daily for diagnosis, the author obtained a lot of experience in this area.

An ultrasound machine was also available at GREFA, this can be used when the need arises, for example, when an animal is suspected to have a cardiac defect. An ocular ultrasound was also available at this centre which is especially helpful if an animal has an eye injury and it is necessary to know which structures are affected. This is particularly helpful in nocturnal birds of prey.

4.3. Surgery and Anaesthesia

The author was able to observe and assist in some surgeries and help with anaesthetic monitoring during her training.

Anaesthesia was used not only for surgery, but also for stressful animals, suturing wounds, full clinical examinations, etc. It is extremely important to carefully control the animal's vital signs, because these can change very quickly, and a quick adjustment to the amount of anaesthetic supplied must be made. The author was able to assist and be part of the anaesthesia team in surgeries during her externship.

During surgeries, the author was also able to observe and help the veterinary surgeon. The surgeries observed included orthopaedic surgeries, for example fracture repair (like the mandibular fracture shown in Figure 12) and limb amputation, ophthalmologic surgeries and pododermatitis surgeries. Practicing surgical techniques, particularly orthopaedic ones, was possible on cadavers.

Figure 12 – Stabilisation of a mandibular fracture in a juvenile fox (*Vulpes vulpes*) (photo kindly provided by Vale Wildlife Hospital & Rehabilitation Centre).

4.4. Laboratory Techniques

Earlier on this report it was mentioned that diverse samples were taken from the animals, either for diagnostic purposes or for research projects within the centres or other institutions. The samples taken were for haematology, biochemistry, proteinograms, parasitology, microbiology and toxicology analysis. There was a big difference in the laboratory equipment available at each centre. At GREFA most of these samples can be processed on site, while at Vale only the parasitology and some haematology analysis can be processed there. Other samples must be sent to specialised laboratories.

In terms of haematology the author was able to collect the blood samples herself and prepare, fix and examine blood smears. Microhematocrit and total protein count was also performed.

When it comes to biochemistry and proteinograms, the centrifugation of the blood samples is an important step before the results can be obtained. Then, the use of a specialised biochemistry analyser is needed.

Parasitology analysis consisted mainly of the microscopic observation of faecal samples. This was particularly important because gastrointestinal parasites are one of the most common parasites in wild animals and can cause a range of clinical signs.

Microbiology samples were processed at GREFA and allow a wide range of studies to be undertaken. This type of analysis is done over consecutive days and usually has many steps. The author was able to observe and participate in some of these steps, which are shown in the figure below (Figure 13).

Figure 13 – Processing of samples for microbiologic studies (photo kindly provided by GREFA).

At both centres, one of the most important toxicology analysis that had to be made was for lead poisoning. This analysis could also be made on site at GREFA, however it had to be sent to an external laboratory at Vale.

4.5. Post-mortem examination

Post-mortem examinations are essential in veterinary medicine and they can help reach a definite diagnosis when one was not found whilst the animal was alive. They might also help find the animal's cause of death. Another advantage of performing post-mortem examinations is the knowledge gained about the animals' anatomy. The anatomy of wild animals isn't as well documented as that of domestic and farm animals, so post-mortem examinations can give us valuable information on this subject.

At GREFA there is a team of volunteers that does the post-mortem examinations of many of the admitted animals. This is usually because the cause of death or its diagnosis is unknown, samples are needed (for example, samples for pathological anatomy), and/or there is special anatomical interest in some species.

On the other hand, at Vale, post-mortem examinations are usually only performed when human intervention is suspected, for example poisoning.

4.6. Rehabilitation

Rehabilitation is particularly important when dealing with wildlife because this step allows the release of an animal into the wild with some degree of certainty that it will survive and hopefully prosper.

This function of a wildlife rescue centre starts almost as soon as the animal is admitted at the centre, by placing it in an appropriate enclosure, as well as giving it proper nutrition.

When the animal is no longer under medication and it no longer needs close attention and observation it is usually moved to a larger outside enclosure. Here it can rebuild strength and reach its ideal body score. In some cases, this part of rebuilding strength might need extra help with physiotherapy, especially with animals that suffered fractures and have muscle atrophy. This can be properly evaluated by Goniometry of affected articulations, Thermotherapy, Massage Therapy and Kinesiotherapy. More modern physiotherapy techniques can also be used for example Laser Therapy, Magnetotherapy or Ultrasound.

4.7. Euthanasia of Wildlife

Euthanasia is derived from the Greek terms *eu* meaning good and *thanatos* meaning death. This term is used to describe the killing of an animal in a way that pain and distress are minimized or eliminated and humanely terminates the animal's life [2].

A lot of the animals that arrive at a wildlife hospital have injuries that make it impossible for them to be released back into the wild with a good quality of life, which means that they would be unable to feed themselves properly and reproduce. Since the whole point of a wildlife hospital and rehabilitation centre is to release animals back into the wild and not to keep them in captivity, when the release of an animal is not viable it is more humane to perform euthanasia. The only exceptions to this rule are endangered species. In those cases, they are kept in captivity, usually taking part in captive breeding programs or environmental education programs.

Euthanising a wild animal should be a very thoughtful and responsible decision and should not be seen as the easiest option when dealing with these animals, because caring for these animals is giving them a chance to keep their life [3]. Conditions like emaciation, high presence of ectoparasites or fractures (particularly open fractures or situated close to a joint) are poor prognostic indicators and euthanasia is likely the most humane decision to make in these cases [4]. Besides these poor prognostic indicators, there are specific situations where euthanasia might be the only option for the animal:

- When death is imminent;
- Severe, deep and/or extensive injuries;

- Severe emaciation associated with debilitating injury or disease;
- Vertebral column severed and displaced;
- Open fractures (if the injury is recent and uncontaminated, an exception might be made);
- Permanent disability, for example, limb amputation, joint disorders or bone malformation;
- A bird that is blind;
- Disabled wood pigeons (this species does not generally tolerate captivity well);
- Rupture or prolapse of tissues in which deeper structures or organs are exposed, especially if contaminated;
- Untreatable systemic disease, for example, rabbits with myxomatosis;
- Most deer that aren't suitable for release;
- Animals that will suffer unacceptable levels of pain even if treated;
- Animals that will not have adequate quality of life even if they recover and are kept in captivity;
- Animals for which veterinary techniques developed for domestic animals are unsuitable;
- A long period in close confinement is needed but suitable facilities are not available [3,4].

These are the most clear-cut reasons for euthanasia, but there might be exceptions to these since each animal should be evaluated individually. Also, there might be other reasons for euthanasia besides the ones described.

The objective of euthanising an animal is to stop unnecessary suffering, so the action of killing it should not include physical and mental suffering [3,4]. For this to be achieved the technique used should result in rapid loss of consciousness followed by cardiac or respiratory arrest, and consequently loss of brain function [2]. To achieve this the American Veterinary Medical Association (AVMA) developed the *AVMA Guidelines for the Euthanasia of Animals*[2] where a description of acceptable methods of euthanasia are described. There are too many methods to include them all in this report, but the ones seen and used by the author will be described, since these correspond to the most common methods used at the places of externship. For most wild animals the preferred method is via lethal injection with barbiturates because these are highly effective as euthanasia agents, have a rapid onset of action and are applicable across a wide range of species and sizes of animals [2].

For birds, the quickest and most reliable way of performing euthanasia is by using an injectable euthanasia agent by intravenous injection, when this can be performed without causing fear or distress [2]. In birds, the preferred sites to administer the lethal injection are the medial metatarsal vein, ulnar vein or right jugular vein. General anaesthesia may be needed before injection. When peripheral circulation is compromised and IV injection is impossible intrahepatic, intracoelomic, intracardiac and intraosseous injection is also indicated, but only if the bird is unconscious or anesthetised [2,4].

Wild mammals include many species and sizes of animals and this should be taken into account when performing euthanasia. Most mammal species should be under anaesthesia prior do this procedure because this allows a good restraint of the animal and decreases the chances of distress and pain. The preferred sights to administer IV lethal injection are the cephalic or the jugular vein [4]. When IV administration is not possible, or this method will cause further distress, an intraperitoneal injection of barbiturates is also an acceptable method of euthanasia. Intracardiac injection is also a valid option but this should be limited to animals that are unconscious or anesthetised [2].

For larger mammals, like deer or boar, euthanasia using a penetrating captive bolt followed by pithing is also an acceptable method. This a relatively rapid technique and can be used for a wide range of species and sizes, however well-trained personnel and well-maintained equipment is needed [2].

In reptiles, an IV injection of barbiturates is also the preferred method of euthanasia. The most accessible veins in turtles, which was the most common reptile at these centres, are the jugular veins and it is the preferred site to administer the lethal injection. If peripheral circulation is compromised, it is also described that intracoelomic, subcutaneous lymph spaces and lymph sacs are acceptable routes of administering barbiturates [2].

III. Monograph – Botulism in Gulls

5. Introduction

Avian botulism is one of the most important diseases of migratory birds worldwide, especially aquatic birds. Botulism is caused by neurotoxins produced by a group of bacteria known as *Clostridium botulinum*, a strict anaerobe that forms dormant spores in adverse conditions [5]. This disease usually results from ingestion of food items with the toxin or when bacteria that produces the toxin colonizes the intestinal tract or infects a wound. The epizootiology of this disease in birds is quite complex and diverse, since it depends on the type of bacteria present, local environmental factors and the species of birds affected [6]. The different strains of *C. botulinum* produce seven different neurotoxins. Type C toxin is the one that usually causes botulism outbreaks in birds, but type E toxin has also caused sporadic mortalities, particularly in fish-eating birds. Type C botulism has been reported in every continent except Antarctica [5], meanwhile type E botulism outbreaks are much less frequent [6].

Gulls belong to the order Charadriiformes and to the family Laridae, which includes a wide range of species. The smaller species of this family usually feed by picking from the surface of the water, while the larger ones are scavengers or feed on fish, often preying on eggs and young of seabirds. Gulls are also attracted to fishing ports, fishing boats, landfills and sewage outfalls [7]. Gulls are considered opportunistic birds because they feed on human waste. This behaviour can represent a risk for the appearance of outbreaks of diseases like avian botulism [8].

Birds affected by avian botulism show progressive signs of weakness, paresis and flaccid paralysis of skeletal muscles [6]. Eventually, the neck muscles are affected and by this stage birds usually drown due to their inability to hold their neck and head erect. If they are not in water, the disease progresses and respiratory failure follows, ultimately leading to death [9].

This monograph includes a literature review regarding Botulism in Gulls. Also included is a clinical case of a Lesser Black-backed Gull (*Larus fuscus*) with botulism that the author followed during her externship at GREFA.

6. Aetiology

C. botulinum are Gram-positive bacteria with seven serotypes (A, B, C, D, E, F and G), that are not morphologically distinguishable, but the neurotoxins produced by them are serologically different [6,10]. Recently, two other serotypes have been discovered but further research about these might be needed [11–13]. Diverse factors influence the vegetative growth and toxin production of this bacteria, including temperature, pH, oxygen tension/redox potential, water vapour pressure and presence of suitable growth medium, as well as the strain [6,10].

In character with other clostridia, *C. botulinum* strains are strict anaerobes that produce dormant spores, resistant to physical and chemical stresses, in order to survive extreme environmental conditions and that can remain viable for long periods of time until favourable conditions allow germination. Spores germinate into vegetative cells when environmental conditions, like humidity, nutrients and anaerobiosis, are appropriate; while sporulation is triggered when in the presence of oxygen, and water and nutrients are scarce [14]. These spores are distributed widely throughout the world. Fresh water habitats, and sporadically marine environments, are where type C spores can be found, while type E is usually present in mud or sediments in or near freshwater. Both these types of spores can also be found in invertebrates, vertebrate tissues and some animal's faeces [6].

In the dormant spore state, *C. botulinum* is harmless because toxin production only happens after the spores germinate and cell division is initiated. Furthermore, only when the cells suffer autolysis does the toxin get released. Even though these neurotoxins represent a risk to animals and humans their role in the growth and physiology of the bacteria is not known, in fact there are naturally occurring isolates of this bacteria that do not produce toxins [6].

Another interesting phenomenon that occurs in type C and D strains is that neurotoxin production depends on the presence of specific bacteriophages that infect *C. botulinum*. In bacteria from these strains that are not infected with these bacteriophages, neurotoxin production is not present. Besides this, bacteriophages from type C strains can infect nontoxigenic type D strains and induce them to produce type C toxin, and vice-versa. This is due to the structural gene for the neurotoxin being located on the genome of these bacteriophages and not in the bacteria itself. In types A, B, E and F a similar mechanism has not been discovered. Bacteriophages have been found in these strains but these don't seem to influence their toxin production. This might explain why toxin production is more stable in these types than in type C and D, and why these two have a higher probability of losing their toxin production ability after several passages [6,15].

In all seven types of *C. botulinum* the location of the toxin gene is different, but the different neurotoxins have a similar structure and pharmacologic action. The toxins are produced as an inactive single-chain protein, that after lysis of the cell or its secretion from the cell, is cut by proteases or trypsin, that can be endogenous or exogenous to the bacteria. Type E *C. botulinum* is the only strain that doesn't produce endogenous proteases, so exogenous ones are necessary for activation of the toxins to happen. Since these bacterium usually develop in decomposing tissue, which is rich in proteases, this doesn't pose a problem for toxin activation, but it is something to take into account in a laboratory environment, in which addition of trypsin will probably be needed [6].

The cut single-chain protein originates a dichain molecule that is composed by a heavy (H) and a light (L) chain joined by a disulfide bond. An important role in cell penetration is played by this disulfide bond. This molecule is folded into three functional domains and each one plays a different role in the intoxication of the nerve cells that leads to paralysis. The H chain has a carboxy-terminal and an amino-terminal that are responsible for binding specifically to receptors on nerve cells and for cell penetration, respectively. And the L chain has enzymatic activity and disrupts the release of the neurotransmitter acetylcholine by acting intracellularly [6].

Another important aspect of type C strains is that they can produce C1 or C2 toxins or only one of them. This also affects strains of type D because, as explained before, these can be induced into producing type C toxins. C1 is a neurotoxin but C2, unlike C1, does not act on the nervous system and the gene for C2 toxin is located on the bacterial genome and not a bacteriophage. C2 is a binary toxin with ADP-ribosylating activity, and it is highly lethal when injected into laboratory animals because it causes an increase in vascular permeability that induces pulmonary haemorrhage and oedema. The role of C2 toxin in avian botulism is currently unknown, but this toxin has been implicated in the pathogenesis of botulism in some animals (for example, in horses) [6,16].

7. Epidemiology

The epidemiology or epizootiology of avian botulism is very complex and diverse, depending on various factors such as environmental conditions and climatic events, but also the foraging behaviour of the bird species affected [6]. Botulism outbreaks happen in environments in which *C. botulinum* spores are present. Outbreaks can spread rapidly and lead to increasing numbers of animals affected in just a few days. In general, spore germination, cell replication and, consequently, toxin production can happen when decomposing organic material, a high protein substrate, and anaerobiosis are present. Other environmental conditions that contribute to this are warm temperatures and shallow alkaline waters with abundant invertebrate populations [6,14].

7.1. Type C Botulism

When it comes to type C avian botulism outbreaks these usually happen during the Summer and Fall, probably because for this strain optimal growth temperature is between 25 and 40ºC. But in some cases, it seems that the toxin can remain in the environment through Winter and cause outbreaks in Winter and Spring. For type C botulism, several patterns have been found and will now be described [6,14].

7.1.1. Botulism associated with Wetlands:

Outbreaks can be unpredictable and happen throughout the year, but most commonly these occur in Summer and Fall. Bird deaths can also vary a lot from year to year and from species to species, meaning that in the same location one year only a few hundred may die and in the next year thousands [5].

Botulinum spores are highly resistant to adverse environmental conditions and can remain viable for many years. These are also widely distributed in wetland habitats, and locations that have previously had botulism outbreaks are contaminated with spores and have a higher probability of outbreaks reoccurring when comparing them to locations with no previous history of botulism. Spores can also be found in the tissues of wetland inhabitants, for example, aquatic insects, molluscs, crustaceans, and vertebrates like birds and fish. In fact, birds can distribute these spores to new environments through their faeces and this explains the appearance of botulism outbreaks in wetlands that never had the disease before [5].

Wetland conditions represent a higher risk of botulism outbreaks. Water pH between 7.5 and 9, low redox potential, water temperature higher than 20ºC, low turbidity and low salinity seem to play an important role in the occurrence of botulism. Even when these conditions present themselves and the risk for botulism is high, other factors play an important role in outbreaks. For example, invertebrate density, bird density and characteristics of the sediment and water [5,8].

Human activity also increases the risk of botulism. Flooding and draining of wetlands, sewage, pesticides and other chemicals resulting from agriculture can kill aquatic life, consequently providing more substrate for toxin production [5]. A study found that the spill of treated water in wetlands represents a regular integration of nutrients and leads to eutrophication. This favours an anaerobic environment that can lead to the growth of *C. botulinum*. Eutrophication also increases phytoplankton and zooplankton productivity from Spring to Autumn, which attracts a great deal of aquatic birds and consequently represents a higher abundance of birds and risk of botulism outbreaks. Besides this, these wetlands also showed a higher prevalence of other avian pathogenic bacteria (for example, *Escherichia coli* and *Clostridium perfringens* type A) that can lead to a small number of bird deaths and whose carcasses are an ideal substrate for the growth of *C. botulinum* [8].

The association between environmental conditions and risk of avian botulism outbreaks involves complex mechanisms that remain unclear. It is thought that wetland conditions allow bacterial growth and toxin production, which represents a high-risk situation, but other factors must be present for an outbreak to occur. Toxic food items, such as decaying organic matter that contains the toxin, must be found and ingested by birds for an outbreak to actually happen. Empirical evidence seems to support this mechanism, but it has not been clearly demonstrated in natural outbreaks [6].

Depending on the species, aquatic birds can be poisoned while sifting through the mud and filtering the water to feed by ingesting decaying organic matter or dead invertebrates; or while feeding on toxin-laden zooplankton or wetland invertebrates, such as molluscs and crustaceans [6,17].

Bird carcasses resulting from birds that died by other pathogens or that were first affected by botulism play an important role in the carcass-maggot cycle, that will be explained below. This cycle is a classic example of secondary poisoning by consumption of toxin-laden invertebrates that has been researched in more depth [5].

7.1.2. Carcass-maggot Cycle:

Decomposing tissues containing *C. botulinum* spores can support high levels of toxin production. As was previously stated, vertebrates such as birds and fish can be carriers of *C. botulinum* spores in their tissues. When these carriers die, the carcasses result in an anaerobic environment and a protein rich substrate that is optimal for the germination of spores, vegetative cell growth and toxin production. Decomposition also generates high internal temperatures, independent from ambient temperatures, that allow toxin production even in cool weather [6].

Fly larvae and other invertebrates, such as worms and mussels, that feed on decomposing tissues seem to be unaffected by the botulinum toxin, and consequently concentrate this toxin in their tissues while they feed. Most aquatic birds do not directly consume a vertebrate carcass, but many would feed on maggots that have fallen off [5,14,17–19]. This mechanism has become known as the Carcass-Maggot Cycle of botulism (Figure 14), and it is thought that toxic-laden maggots pose a high risk for botulism outbreaks [5]. Fish and other terrestrial and aquatic vertebrate animals can also feed on these larvae and invertebrates, and die from ingestion of the toxin, helping to perpetuate this cycle [14].

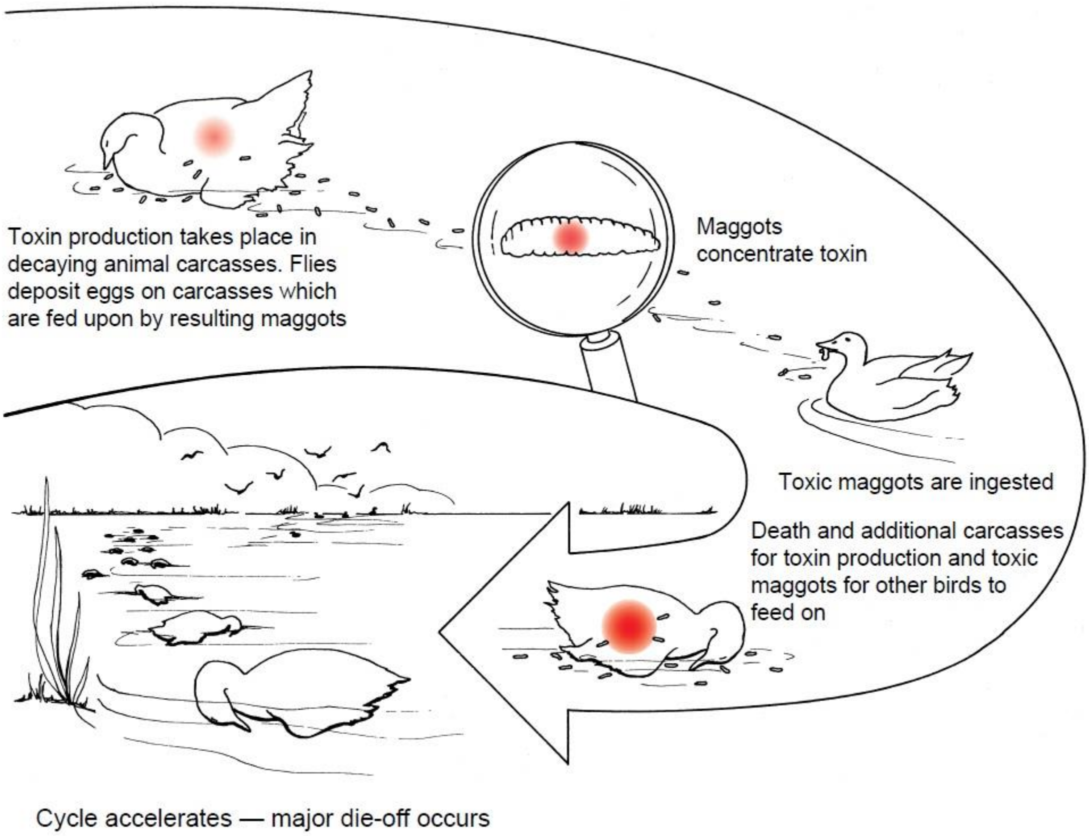

Figure 14 – Carcass-maggot cycle of avian botulism (adapted from Friend & Franson, 2001).

Botulinum spores carriers, such as the vertebrates mentioned above, that die from causes unrelated to botulism represent as high a risk as birds that have died from the disease in causing a botulism outbreak due to the carcass-maggot cycle [5].

Fly density, ambient temperature, wind speed, maggot development and maggot dispersal from carcasses are some of the factors that influence the carcass-maggot cycle. But the most critical factor in this cycle is the amount of carcasses containing botulinum spores, because this is what ultimately allows the deposition of fly eggs and the propagation of the carcass-maggot cycle [5,14,20].

7.1.3. Winter and Spring Outbreaks:

As mentioned before, most botulism outbreaks occur in the Summer and Fall due to higher temperatures being more favourable to bacterial growth and toxin production. Nevertheless, Winter and Spring type C botulism outbreaks have been reported before. Evidence shows that these outbreaks happen in locations where botulism outbreaks were reported the previous Summer or Fall. It is believed that these outbreaks result from toxin-laden maggots falling into the bottom of these wetlands and being available in the Winter/Spring for diving ducks, since these seem to be the most affected species in these outbreaks [5].

7.1.4. Type C botulism in fish-eating aquatic birds:

Type C botulism in fish-eating birds is considered infrequent and incidental when comparing it to other botulism outbreaks. Usually, botulism outbreaks in fish-eating birds involve the type E botulinum toxin. However, in 1996 a type C botulism outbreak involving fish-eating birds, like pelicans and herons, was reported in the Salton Sea (California, USA). The epizootiology of this outbreak is considered abnormal and it is believed that an invader species of fish became infected with the botulinum toxin and due to high environmental temperatures and other stressors lead to spore germination and toxin production, and consequently to the botulism outbreak [5].

7.1.5. Botulism in Gulls associated with Landfills:

Landfills and refuse disposal sites have been associated with several botulism outbreaks [6]. In the UK, type B, C, D and E of *C. botulinum* have been found in these locations (types A, F and G were not detected). It is established that botulism in wild birds, such as gulls, is usually caused by type C botulinum toxins [21].

Refuse disposal sites represent the ideal environment for growth and toxin production of *C. botulinum* since these generate high temperatures and have an abundance of rotting organic matter. Gulls are known for their scavenging habits and tendency to feed on rubbish, which corroborates the suspicion that landfills are a source of botulinum toxin for these animals [21].

However, samples taken from refuse before it was distributed to the disposal sites revealed negative results for *C. botulinum*. A probable explanation to this is that the contamination source is not the refuse itself. Since birds can carry botulinum spores in their gastrointestinal (GI) tract or on their external body surface these are probably propagated by these animals. In fact, genetic studies conducted with *C. botulinum* strains in Europe have shown that these are genetically related, which might be due to some migratory birds being spore carriers [21–23].

Gulls and other birds are foraging animals and are attracted to landfills, so it is possible that they are responsible for the contamination of these sites. Due to their behaviour gulls are especially vulnerable to suffer from botulism and their carcasses represent a high risk of environmental contamination with this toxin [21,24].

7.1.6. Botulism as a result of Toxico-infection:

A toxico-infection is the formation of toxin due to proliferative growth of *C. botulinum* in the GI tract. This type of infection happens when, for some reason, an anaerobic environment is established in the GI tract due to the animal being debilitated or when GI function or normal GI flora is disrupted [6].

It is plausible that botulism toxico-infections can also occur in wild birds, but it is difficult to distinguish this hypothesis from ingestion of the preformed toxin. Type C botulism has been found in dying aquatic birds that were simultaneously affected by lead poisoning and vitamin A deficiency that were extremely emaciated and not feeding. The habitat of these animals was contaminated with botulinum spores and it is thought that due to their debilitation their GI function and flora was disrupted, which led to the development of botulism by toxico-infection. It is unlikely that this route of infection is significant in wild birds, but it is something to take into consideration when treating wild birds affected by diseases that can have effects on the health of the GI tract [6].

7.2. Type E Botulism

Type E avian botulism is a lot less frequent than type C but there have been some cases in wild birds. This strain of *C. botulinum* seems to prefer temperate, fresh and brackish water habitats. Its optimal growth temperature is between 18 and 25ºC and its spores have a lower heat resistant temperature than type C spores [6,25].

Healthy fish routinely ingest *C. botulinum* type E and its spores, with no harmful effect since the spores seem to pass through the GI tract of the fish and germination and growth of this bacteria does not happen in or on living fish. Nevertheless, when these fish die their carcasses allow an anaerobic environment and protein rich substrate leading to spore germination and growth, and toxin production [6].

The toxin source for this type of avian botulism is still not known, but fish seem to be the most likely origin. In some situations, another possible source is blow fly larvae, but the mechanism involving these larvae does not appear to be similar to the carcass-maggot cycle for type C botulism. Shore birds have been affected by type E botulism which suggests that other sources of toxin besides fish, for example invertebrates, might be involved in the spread of this disease [6].

8. Pathogenesis

The botulinum neurotoxins are considered the most toxic substances currently known. The lethal dose for this toxin varies according to the toxin type and the species affected [6,26].

This neurotoxin usually forms a complex with one or more nontoxin proteins. In the absence of the nontoxin proteins, the neurotoxin is significantly less toxic than when associated with these proteins. This suggests that the neurotoxin and nontoxin proteins complex acts to protect the botulinum neurotoxin from the acid and proteolytic enzymes present in the GI tract. These proteins may also have a role in transporting the neurotoxin across the gut membrane to the circulatory system. The way this transport happens is still not clear [6].

Once in the circulatory system, botulinum neurotoxins target the peripheral nervous system and they mainly act in the neuromuscular junction. At this site, they inhibit the release of the neurotransmitter acetylcholine (ACh), which results in paralytic effects. Furthermore, the neurotoxins also act on the autonomic ganglia, postganglionic parasympathetic sites, postganglionic sympathetic nerves that release ACh and the adrenal glands. However, the blood-brain barrier is not crossed by this toxin due to its large size. The high toxicity of botulinum neurotoxins is explained by their high affinity to presynaptic membranes and their persistent and specific inhibition of ACh release. Typically, these neurotoxins do not kill nerve cells but interfere with their synaptic transmission [6,26,27].

When the botulinum neurotoxin reaches the neuromuscular junction, a process of multiple stages happens in order for the toxin to penetrate the membrane of the nerve cells.

Firstly, the neurotoxin binds itself irreversibly to the presynaptic nerve cell membrane [6]. This ability is due to the carboxy-terminal of the H chain of the neurotoxin molecule, which has folding and binding properties [14]. Then, the neurotoxins are internalized into the cell via an endosome through receptor-mediated endocytosis. After the internalization of the neurotoxin, the amino-terminal of the H chain allows the translocation of the L chain across the membrane of the endosome and into the cytosol of the nerve cell. This translocation happens because the disulfide bond between the L and H chain is reduced, and consequently a drop in the endosomal pH occurs. This pH drop stimulates a conformational change in the neurotoxin molecule which enables the escape of the L chain across the membrane of the endosome into the cytosol [6,14].

The last stage of intoxication happens at this point and it involves enzymatic cleavage of proteins connected to neurotransmitter release. The L chain acts as a zinc-dependent endoprotease that cleaves and inactivates essential proteins involved in the docking and fusion of synaptic vesicles to the cell membrane. This blocks the release of the ACh by the cell and results in the flaccid paralysis and autonomic dysfunction characteristic for botulism. The three proteins targeted by botulinum neurotoxins are known as SNARE (soluble n-ethylmaleimide sensitive factor attachment protein receptor) proteins. These proteins are the SNAP-25 (synaptosomal-associated protein of 25kDa), VAMP (vesicle-associated membrane protein; also known as synaptobrevin) and syntaxin. Each type of botulinum neurotoxin specifically targets one of these proteins and catalytic active sites. Types B, D, F and G cleave VAMP proteins; Types A, C1 and E cleave SNAP-25 proteins. Besides cleaving SNAP-25, botulinum neurotoxin Type C1, the one primarily implicated in avian botulism, also cleaves syntaxin and is the only type able to do so. The inactivation of any of these proteins destabilizes or prevents the formation of functional SNARE complexes, which consequently inhibits vesicular fusion and neurotransmitter release [6,14,26,27].

Different species and tissues have distinct isoforms of the SNARE proteins. Of these, only some are vulnerable to the proteolytic activity of botulinum neurotoxins. This might explain why some species are affected by some types of botulinum neurotoxins but not by others. For example, botulism in humans is usually caused by serotypes A, B, E, and, rarely, F; while in domestic animals, livestock, poultry and wildlife, botulism is usually associated with serotypes A, B, C1 and D. The absence of certain membrane receptors may also account for the resistance of some species to this toxin. This may be the reason why scavenging birds, such as crows, ravens and vultures seem to be resistant to botulism [6,26].

9. Clinical Signs

Clinical signs can occur within hours or days of exposure to the botulinum neurotoxins. Several factors affect the severity and duration of clinical signs, including the amount of ingested toxin, the type of toxin consumed, the species affected and individual sensitivity. The rapidity of onset of the clinical signs is directly proportional to the toxin dose. Death or recovery from this disease is also dependent on the neurotoxin dose ingested [6,26].

The most common clinical sign in botulism is a progressive, symmetrical and flaccid paralysis of skeletal muscles. Paresis starts in the hind limbs and progresses cranially. The first sign of avian botulism is weak flight, with birds being reluctant to move and developing a stumbling gait. Birds progressively get weaker, eventually being unable to fly and stand. Occasionally, they can be seen standing and walking on their tarsometatarsi. Dropped wings and recumbency reflect the weakened state of these animals. Due to their inability to move properly, affected birds drag themselves forward with their wings and beak. Usually these animals remain mentally alert and aggressive even though they are paralysed [6,26,28,29]. However, in later stages of the disease, animals severely affected become moribund and unresponsive. Their neck muscles become affected and they are unable to hold their neck and head erect, this sign is known as “limberneck”.

Respiratory muscle paralysis follows, resulting in death [6,26,29]. Moribund birds may present dyspnoeic, slow and open-mouthed breathing, as well as ventroflexion of the head [29]. Paralysis of the nictitating membrane may also be present. This may lead to partially or completely closed eyelids and affected birds can look comatose [6,9,26]. Even though it is rare, affected birds can also present nystagmus [29]. Occasionally, animals can also have anorexia, hypersalivation, nasal and ocular discharge, and green diarrhoea [6,30,31]. Environmental factors (for example wind, excessive heat and extreme weather) can exacerbate the clinical signs and incapacitated animals exposed to the sun and heat can quickly dehydrate [6].

Hunter et al. (1970) defined three categories for the affected birds which can be used to evaluate treatment options and the probability of survival: Class I - birds were bright and alert, walking but flightless; Class II - birds had difficulty walking and problems in holding their heads erect; Class III - birds were prostate and almost totally paralyzed [32]. Affected birds that present clinical signs of the last category usually do not survive [9,33,34].

It is likely that the full range of physiological effects and clinical signs of botulism in wild birds remains unknown. These may have implications in survival after treatment and release. In humans, some clinical signs remain up to two or three months after treatment. However, the different serotypes act differently, and susceptibility is different according to the species affected, so extrapolations between species and serotypes should be made carefully [6].

10. Pathology

No obvious lesions are usually found in animals that died from botulism [6,35]. Congestion and/or hyperaemia of various organs may be present but this a nonspecific lesion [6]. If affected birds die due to drowning, lesions related to this cause of death can be found [9].

Subcutaneous tissues can be dry and stringy because of dehydration [29]. The crop, proventriculus and ventriculus may be empty and bile-stained, and the gall bladder full. The colon may be distended with urates and faeces. Since affected birds are incapable of moving and feeding themselves, these lesions are consistent with their inability to feed and drink or anorexia [6,29].

The limbs and pectoral muscles may show acute muscle damage due to repeated attempts to ambulate and prolonged sternal recumbency [29,36].

Even though it is rare, the GI tract can have content that can pinpoint the disease epizootiology. For example, if maggots are found in the GI tract, this is highly suggestive of type

C botulism; on the other hand, if fish is found, type E botulism may be the cause of death of the animal [6].

11. Diagnosis

Botulism diagnosis is mostly presumptive and based on the clinical signs showed by affected birds. The absence of pathologic findings during post-mortem examination of the cadavers of affected birds is another common finding for this disease. Definitive diagnosis for botulism would be the isolation of botulinum neurotoxins from serum/tissues of afflicted birds [6,26]. However, these birds usually have low quantities of neurotoxin in their blood that are not detectable by current methods [6]. Detection of botulinum neurotoxins or *C. botulinum* from bird intestines, cecum or other tissues may be helpful to reach a diagnosis. But *C. botulinum* can be found in the GI tract of healthy birds so testing these tissues has a lower value. Moreover, a definitive diagnosis cannot be achieved by isolating botulinum neurotoxins or *C. botulinum* from carcass tissues because this bacterium can proliferate and spread from the GI tract to the surrounding tissue [26].

The mouse bioassay (MBA) is the gold standard test for diagnosing avian botulism. To perform this test, blood samples must be collected from afflicted birds or from the heart of recently dead birds and centrifuged. Afterwards, the serum fraction is inoculated into two groups of laboratory mice, in which one has been administered botulism antitoxin. If the mouse administered with botulism neurotoxin survives and the other mouse dies or shows signs of this disease (for example, hind limb paralysis, pilo-erection, contracted abdomen or dyspnoea) the sample was positive for botulinum neurotoxin. After this test, the serotype can also be identified using the mouse neutralization test, where type-specific antitoxin is used. Another alternative would be to use the type-specific antitoxin in the first test, since type C botulinum neurotoxin is the toxin usually involved in avian botulism. MBA is still the most common test used to diagnose botulism because it can detect biologically active neurotoxin. It is also highly sensitive for small sample volumes, but false positives can occur. Nevertheless, alternative diagnostic tests are being explored due to MBA being time consuming, its ethical issues with using live animals and its cost [6,9,26,37–40].

One of these alternatives is the enzyme-linked immunosorbent assay (ELISA) technique. Avoiding the use of live animals and lower costs are some of the advantages of this technique over the MBA; but specialized equipment is needed to process the samples. Furthermore, blood and tissue samples from dead animals are not always appropriate for ELISA testing due to contamination of the samples. However, ELISA is helpful when large volumes of samples need processing and with adequate amount of serum, this test’s sensitivity is similar to the sensitivity of MBA [6,26,41]. This technique still faces difficulties due to neurotoxin-associated proteins, which hinder the detection of botulinum neurotoxins. Research is still being conducted in order to overcome this issue [37].

Polymerase chain reaction (PCR) has been used to detect type C toxico-infections, but this type of infection is not the most relevant one in wild birds. Furthermore, this method wouldn't reveal if *C. botulinum* was actively replicating in the live tissues or had recently been ingested with the neurotoxin by the animal [6]. Recent studies showed good results for diagnosing botulism in birds using livers, which can be helpful in identifying botulism outbreaks [42,43].

Other techniques have been tested to diagnose botulism but not all of them are suitable for diagnosis in wild animals. In order for them to be used in this context, they would have to be available at a low cost and be easy to use. Furthermore, they would have to be able to detect botulinum neurotoxins in complex sample matrices, like serum and faeces [37].

12. Differential Diagnoses

The differential diagnoses for botulism include diseases that affect a large number of aquatic birds and that present with similar neurological signs, particularly progressive paresis or paralysis. The most common differentials for botulism are:

- Newcastle Disease: paralysis of the legs and wings can be unilateral or bilateral; pathologically, this virus causes lesions in the central nervous system, kidneys, alimentary tract, and/or respiratory system; definitive diagnosis is achieved through viral isolation from faeces or swabs of trachea or cloacae and/or tissue samples.

- Avian Cholera (*Pasteurella multocida*): most wild birds are found dead with no previous sign of disease; petechial haemorrhages may be found on the epicardium and serosal membranes, and small foci of necrosis in the liver.

- Salmonellosis: nonspecific clinical signs of septicaemia like ruffled feathers, dyspnoea, shivering, weakness and lethargy; diarrhoea if enteritis is developed; faeces, cloacal swabs or tissues can be collected for bacteria isolation; gross lesions compatible with septicaemia (for example, congested lungs and kidneys) and enteritis (congestion, ulceration and/or necrosis of the intestinal tract).

- Aspergillosis: in birds with chronic aspergillosis clinical signs include emaciation, reduced activity and stamina, inability to fly, dyspnoea or tachypnoea, vomiting and diarrhoea, and, if infections have invaded the nervous system, ataxia, torticollis, etc.; endoscopy and radiography may help in diagnosing sick animals; upon post-mortem examination, caseonecrotic plaques in the air sacs are usually present.

- Sarcocystosis: birds show neurological deficits like tremors, dropped wings, incapability to fly and ventroflexion of the neck; during the post-mortem examination, parasitic cysts, the size of rice grains, are visible upon removal of the skin.

- Algal Toxicosis: neurological signs, such as incapability to fly, paralysis and tremors may be present; usually associated with the natural phenomena known as Harmful Algal Blooms; detection of the toxin producing organism in the GI tract or of the toxin in tissue samples confirms the diagnosis.

- Chemical Toxicity: the most common being lead poisoning and clinical signs include weakness, weight loss, lethargy, anorexia, ataxia, head tremors, circling, head tilt, dropped wings, paresis, hyperesthesia, paralysis, blindness, convulsions; blood analysis for lead to confirm the diagnosis.

- Pesticides: varied clinical signs but the most common are anorexia, regurgitation, vomiting and diarrhoea, central nervous system signs, convulsions, dyspnoea and cyanosis; no specific gross lesions found upon post-mortem examination; diagnosis through detection of pesticides in GI contents or other tissues.

- Nutritional Imbalances: usually related to thiamine (Vitamin B1) deficiency; clinical signs included generalized weakness, loss of voice, diarrhoea, paralysis of the wings and legs and tremors; diagnosis through measurement of thiamine and enzymes in several tissues [6,30,44].

13. Treatment

Treatment of gulls intoxicated with the botulinum neurotoxin is usually very successful and has a high survival rate, even in animals with advanced clinical signs [3,6].

Ideally, primary therapy should include botulinum antitoxin administration at a dosage of 0.05 to 1 mL/day. Progression of the disease may be prevented if the antitoxin is given in early stages of the disease, since it is only protective against toxin in general circulation. However, the use of the antitoxin in wild birds is very rarely used due to the costs involved and also the logistics, especially if dealing with a large outbreak. Moreover, the effects of the antitoxin are transient, and birds treated with it may become intoxicated again. Nevertheless, for endangered species the benefits of this therapy may outweigh its costs [6,26,30,45].

Supportive therapy is the most common treatment for botulism in wild birds. The use of drenches, purgatives and laxatives may help in expelling any bacterium and toxin present in the GI tract. Since affected birds usually are anorexic, tube feeding is also indicated. Intravenous or subcutaneous support with glucose-saline solution and electrolyte is also indicated [4,6,26,30]. Administering vitamins B12 and B1 is advised, since these have neuroprotective effects and are appetite stimulants [4,30,46]. Antibiotics can also be used, even though botulism is usually an intoxication and not a toxico-infection. These are used in case *C. botulinum* was ingested but also to prevent secondary infections. Aminoglycosides, tetracycline, and procaine penicillin should be avoided because these drugs potentiate neuromuscular weakness. Metronidazole should also not be used because *C. botulinum* is not sensitive to this antimicrobial, and it seems to be associated to a higher risk of botulism [26,30]. A study shows that Clostridia seem to be susceptible to amoxicillin, bacitracin, and enrofloxacin [47].

Sick birds should be kept in a quiet and stress-free environment. Fresh water and food should also be available, taking care that the dishes these are placed in are not too deep since sick animals can drown in them. Adequate bedding must be provided in order to avoid pressure sores and lesions to the keel [3,4,26].

14. Control and Prevention

Birds that recover from botulism do not gain immunity against this disease and can be affected by this disease again. Vaccines have been developed for birds and morbidity and mortality are lower in vaccinated birds than in unvaccinated birds. However, the level of protection of these vaccines is limited and reduces over time [6,33,48–50]. Unfortunately, vaccination of wild birds is impractical but might be an option for small populations of endangered species that have a high risk of exposure to the botulinum neurotoxin and that can be safely captured [6,26,36].

Considering the epidemiology of botulism and ubiquity and resistance of botulism spores, reducing or eliminating the agent of this disease is not feasible. The potential for environmental contamination is associated with the proliferation of *C. botulinum* in the carcasses of dead vertebrate and invertebrate animals. Therefore, prevention of outbreaks should be aimed at reducing this potential. In order to achieve this, carcasses should be removed, and sick birds should be collected for treatment in a wildlife rehabilitation centre or for humane veterinary euthanasia. Furthermore, adequate management of locations with high risk for this disease, with special focus on reducing deaths of invertebrates and vertebrates, is also advisable in preventing outbreaks [9,26,51]. To achieve this goal, some of the preventive measures that can be applied, particularly in lakes or ponds, are:

- Maintaining good circulation of water.
- Maintaining healthy communities of oxygenating plants (these prevent the formation of an anaerobic environment).
- Prevention of the water level falling in the bodies of water, preventing deoxygenation and the exposure of putrefying material.
- Removal of decaying plant material (including leaves) from the water.
- If appropriate, removal of silts by pump action (in the face of an incident this may temporarily exacerbate the disease due to agitation of material).
- Document environmental conditions, specific areas of outbreaks, and dates of occurrence and cessation.
- Searching for and removal of dead animals in high risk periods (for example, warm summer months).
- Avoid routine flooding (may exacerbate invertebrate deaths).
- Removal of overhead power lines (higher risk of electrocutions and bird deaths).
- Avoid sewage and wastewater discharges.
- Avoid or remove any factors that may increase deaths in susceptible locations [9,26,51,52].

The removal of carcasses is one of the most important and effective preventive measures that can be applied, particularly because it can be used in wetlands and landfills and not only in lakes or ponds. Quick removal of carcasses slows down the rate of avian outbreaks. Unfortunately, this measure is labour intensive and costly, and might be inefficient when the outbreak covers a very large geographical area [40].

15. Public Health Considerations

Botulism in humans has never been associated with botulism in wild birds. Humans do not appear to be susceptible to type C botulism [53] and only one case has been reported in humans [26,54]. However, type C botulism has been reported in non-human primates, after ingesting contaminated meat [55,56] and a study found that *in vitro* samples of human muscle can become paralysed by this serotype [53]. Type E botulism affects humans, but it is usually connected to the consumption of fish and other marine products [6,57].

At present time, it is rare for botulism type C to affect humans, but this may change in the future because bacteria can evolve and adapt to infect new hosts. Gulls are important vectors in the distribution of botulism spores. These animals are also opportunistic and their presence in urban areas is becoming more common. This behaviour can become a risk factor of botulism in humans, so sharing information between public health entities and veterinarians and/or wildlife rescue centres is very important to prevent outbreaks and monitor this disease. Furthermore, bodies of water with environmental conditions favourable for botulism spore's development can also have a higher prevalence of other pathogenic bacteria, such as *E. coli* and *C. perfringens* type A, which can also affect humans [8]. So, water quality monitorization of bodies of water is equally important for preventing and controlling this disease.

When it comes to domestic animals, there have been some cases where these have contracted type C botulism related to wild birds. There are some reports of dogs developing botulism after coming into contact with carcasses of wild birds [58,59]. A case involving cattle was also reported, where these contracted the disease while grazing near a lake with a botulism outbreak in wild birds [60].

Botulism transmission from bird to predators may also be a possibility. There has been a case where a fox and a weasel had botulism in association with an outbreak in wild birds [26].

16. Impacts on Wildlife Population

Avian botulism is probably one of the most significant diseases of aquatic birds, but its effects on wildlife populations remain poorly understood. There are no studies that adequately evaluate the impact of botulism in wild birds [6]. Retrieval of carcasses during outbreaks, a measure commonly applied during these, could be used to try and determine the impact of botulism on populations but this technique seems to underestimate the real number of birds affected [61].

Several species can be affected by botulism outbreaks. Species that are numerous, geographically widespread and have a high reproductive potential, like mallards, may be able to cope with high losses due to this disease; but other species, specially endangered ones, may be impacted more significantly [5,6]. In shorebirds, like gulls, the impact of botulism is practically unknown [6].

17. Case Report

Species: Lesser Black-backed Gull (*Larus fuscus*)

Age: Subadult

Sex: Unknown

Weight: 916g

17.1. Previous history

On November third, 2018, this gull was collected from the Santillana reservoir (Madrid, Spain), and taken to GREFA to be treated. It was collected because it was incapable of flying and had blood on its beak.

17.2. Clinical examination

Upon clinical examination it was determined that this gull was thin, unable to move its hind limbs and hypothermic (37.4ºC). However, it was alert and tried to bite. Due to the time of year (Autumn) and the clinical signs presented, a presumptive diagnosis of botulism was proposed.

17.3. Treatment

After establishing the presumptive diagnosis of botulism for this gull, the standard protocol used at GREFA for treatment of this disease was applied:

- IV Rehydration at 2% of body weight, with 1:1 Duphalyte® and Ringer's lactate solution (for at least three days)

- Enrofloxacin 15 mg/Kg PO (for at least five days)
- Vitamin B 30 mg/Kg PO (until it starts to eat by itself and/or stands)
- Buprenorphine 0.06 mg/Kg IM or Tramadol 15 mg/Kg PO (for at least three days)
- Vitamin E + Selenium 0.1 mg/Kg IM (Single dose)
- Itraconazole 5 mg/Kg PO (during its stay at the Hospital department)

This treatment protocol follows what is described in the literature: intravenous rehydration, antibiotics and Vitamin B. But some drugs were also added as a consequence of previous experience with this disease at this centre. Buprenorphine or Tramadol are given as analgesics because these animals usually have spent long periods of time in recumbency and may result in discomfort or damage to their keel and pectoral muscles. Upon post-mortem examination of animals previously admitted due to botulism, these had exhibited small necrotic foci on their liver, and it was decided to administer Vitamin E to all the gulls admitted with botulism because this vitamin has hepatoprotective properties. The combination of Vitamin E + Selenium is also administered to help to prevent capture myopathy, that these birds can develop due to the stress of being handled [62]. Itraconazole is administered at a 5 mg/kg dose as a preventive measure for Aspergillosis, since it is thought that some aquatic birds developed this disease at this centre due to the stress of being kept in captivity.

Tube feeding was also necessary, since these birds usually have anorexia. To achieve this, Hill's® Prescription Diet® a/d® Canine/Feline was used mixed with a small amount Glucose 5% in Water making up a consistency that can be easily administered by a feeding tube. This is done until the animal starts to feed by itself. Nevertheless, small quantities of diverse food (small fish, cow heart slices and chopped chick) in a shallow dish were left in its enclosure to see if the animal started to eat by itself. Regular weighing was also performed to make sure that the animal did not lose too much weight.

This gull was also hypothermic, so it was put in a heated enclosure and its temperature was taken every day until it stabilised. The bedding in its enclosure was also adapted to suit the state of the animal and to prevent pressure sores by using towels (Figure 15).

Figure 15 – Lesser Black-backed Gull (*Larus fuscus*) with botulism in an adapted enclosure.

On the fourth day of treatment, the hydration status was re-evaluated to assess if a new rehydration schedule should be started or not. The animal was still dehydrated and not eating by itself, so a new three-day rehydration schedule was started, but at 1% of body weight.

On the seventh day, when the animal was about to be collected to be medicated and tube fed, it was noticed that it had eaten some of the food and was standing. To avoid stressing the animal, it was not picked up nor tube fed, and the medication (Enrofloxacin, Itraconazole and Vitamin B) was left on its food. Even though the gull was standing it was still stumbling.

On the next day, the animal was still able to stand, was very active and alert, and ate by itself. It was then decided to move it to an enclosure without heating and the only medication that remained was the Itraconazole and the Vitamin B.

By the tenth day the gull had kept improving so it was transferred to an outside enclosure. Since it kept eating by itself, Vitamin B was no longer given. The only remaining medication was the Itraconazole.

The animal continued being treated at the Hospital department until it was feeding well and gaining weight. On day 14 of treatment the animal was transferred to the Rehabilitation department. There, it was put in a large outside enclosure with a pond, in which other gulls were also being rehabilitated. The animals stay in this department until they are able to fly and feed well. After three months of being at the centre (on February third, 2019), this Lesser Black-Backed Gull was released into the wild.

17.4. Discussion

During the author's externship at GREFA, 13 gulls were admitted with a suspicion of being affected by botulism (ten Lesser Black-backed Gulls [*L. fuscus*] and three Black-headed Gulls [*Larus ridibundus*]). Of these, ten were released back into the wild; two were euthanised because they were moribund; and one died while being treated. With this knowledge, it is possible to conclude that the treatment of gulls suspected of being affected by botulism is highly successful at this centre.

At GREFA, diagnosis of botulism is only presumptive since a laboratory diagnosis is too costly and time consuming. However, a study conducted at this centre in previous years was able to confirm a botulism outbreak from fly larvae collected at the location where affected gulls were collected [31]. Even though laboratorial diagnosis is not always possible at this centre, previous experience and the effectiveness of the treatment protocol established at this centre supports the presumptive diagnosis of botulism.

The source of botulism is hard to pinpoint, but the study mentioned before also tried to determine possible sources. This study found that most gulls feed in refuse disposal sites during the day, and during the night rest in reservoirs and lagoons near the city. The Santillana reservoir, where this gull was collected, was found to be one of the most important sites where botulinic gulls are usually discovered [31]. These findings support the epidemiology of this disease, but it is hard to say for certain if the botulism outbreaks in the Madrid area are associated with wetlands, with landfills, the carcass-maggot cycle or to fish-eating. It might be a combination of one or more of these hypotheses, especially when taking into consideration that gulls are important reservoirs and carriers of botulinum spores. Furthermore, this study also concluded that most cases of botulism happen in Autumn, which further supports the epidemiology of this disease [31].

18. Conclusion

The author's externship at both centres, GREFA and Vale, proved to be extremely important for obtaining knowledge and practice with wild animals. A wide range of techniques were successfully achieved and, when dealing with wildlife, creativity to adapt these techniques to the different species can be surprisingly helpful. The author was also able to work with species she was previously unfamiliar with. Further understanding of the threats that human activity poses to wildlife was also eye-opening, particularly in cases where animals were shot or electrocuted. Appreciation of the effects that climate change can cause on wildlife was also a surprise during the author's externship, particularly in the hatching of birds. There were some cases where birds hatched later than usual, probably due to high temperatures later in the year.

Botulism was chosen as the theme for this monograph because it is one of the main suspected causes of admission of birds that are not directly related to human activity and is a naturally occurring disease.

Botulism is one of the most important diseases of wild birds in the world. Its effects on wildlife populations remain unclear and no reliable techniques have been discovered in order to understand its impact. From previous studies it is possible to say that endangered species are particularly vulnerable to this disease. Botulism can cause high mortality rates, which are dangerous to the survival of these species. With a better knowledge about botulism, the easier it will be to prevent and control outbreaks and losses of important species. Most gull species are not endangered, but they are one of the species that can be affected by botulism and are frequently treated in wildlife hospital centres because of this disease. This was the case when the author was training at GREFA. Gulls are more commonly treated due to botulism than other more endangered birds, but the knowledge and practice obtained while treating these animals can prove to be helpful if another endangered species is admitted with botulism.

Nowadays, global warming affects fauna and flora around the world. When taking into account the epidemiology of botulism, where high temperatures are essential for the growth of botulinum spores, it is a real possibility that outbreaks can become more common and far reaching. The fact that, in recent years, botulism outbreaks have been reported in locations where there were no previous records of this disease may be due to climate change. Efforts to avoid further climate change may be just as important as other measures to prevent botulism outbreaks.

IV. References

1. Tomlinson A (2016) Wildlife casualties and the veterinary surgeon. In *BSAVA Manual of Wildlife Casualties,* 2nd edition, ed Mullineaux, E. & Keeble, E., British Small Animal Veterinary Association, Gloucester, UK. 978 1 905319 80 0, pp. 1-2.

2. Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin M, Meyer R, Miller D, Shearer J & Yanong R (2013) AVMA Guidelines for the Euthanasia of Animals: 2013 Edition, American Veterinary Medical Association, Schaumburg, IL, USA. 978-1-882691-21-0, pp. 6, 65-66, 76-79.

3. Meredith A (2016) Wildlife triage and decision-making. In *BSAVA Manual of Wildlife Casualties*, 2nd edition, ed. Mullineaux, E. & Keeble, E., British Small Animal Veterinary Association, Gloucester, UK. 978 1 905319 80 0, pp. 33-34.

4. Stocker L (2005) Practical Wildlife Care, 2nd edition, Blackwell Publishing, Oxford, UK, 13 978-1-4051-2749-3, pp. 10-11.

5. Rocke TE (2007) The global importance of avian botulism. In *Waterbirds around the world* ed. Boere, G.C., Galbraith, C.A. & Stroud, D.A., TSO Scotland Ltd., Edinburgh, UK, 0 11 497333 4, pp. 422-426.

6. Rocke T & Bollinger T (2007) Avian Botulism. In *Infectious Diseases of Wild Birds* ed. Thomas, N.J., Hunter, D.B. & Atkinson, C.T., Blackwell Publishing, Ames, IA, USA, 978-0-8138-2812-1, pp. 377, 380-383, 391-408.

7. Svensson L (2009) Collins Bird Guide, 2nd edition, HarperCollinsPublishers, London, UK, 978 0 00 726814 6, pp. 178.

8. Anza I, Vidal D, Laguna C, Díaz-Sánchez S, Sergio Sánchez, Chicote A, Florín M & Mateo R (2014) Eutrophication and bacterial pathogens as risk factors for avian botulism outbreaks in wetlands receiving effluents from urban wastewater treatment plants. *Applied and Environmental Microbiology*, **80**(14): 4251-4259.

9. Friend M & Franson JC (2001) Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds, U.S. Geological Survey, Washington, D.C., USA, 0-607-88096-1, pp. 271-280.

10. Stringer SC, Webb MD & Peck MW (2011) Lag time variability in individual spores of *Clostridium botulinum*. *Food Microbiology*, **28**(2): 228-235.

11. Maslanka SE, Lúquez C, Dykes JK, Tepp WH, Pier CL, Pellett S, Raphael BH, Kalb SR, Barr JR, Rao A & Johnson EA (2016) A novel botulinum neurotoxin, previously reported as Serotype H, has a hybrid-like structure with regions of similarity to the structures of serotypes A and F and is neutralized with Serotype A antitoxin. *Journal of Infectious Diseases*, **213**(3): 379-385.

12. Zhang S, Masuyer G, Zhang J, Shen Y, Henriksson L, Miyashita S, Martínez-Carranza M, Dong M & Stenmark P (2017) Identification and characterization of a novel botulinum neurotoxin. *Nature Communications*, **8**: 1-10.

13. Pellett S, Tepp WH, Bradshaw M, Kalb SR, Dykes JK, Lin G, Nawrocki EM, Pier CL, Barr JR, Maslanka SE & Johnson EA (2016) Purification and Characterization of Botulinum Neurotoxin FA from a genetically modified *Clostridium botulinum* strain. *mSphere*, **1**(1): 1-18.

14. Rossetto O, Pirazzini M & Montecucco C (2014) Botulinum neurotoxins: Genetic, structural and mechanistic insights. *Nature Reviews Microbiology*, **12**(8): 535-549.

15. Woudstra C, Skarin H, Anniballi F, Fenicia L, Bano L, Drigo I, Koene M, Bäyon-Auboyer MH, Buffereau JP, De Medici D & Fach P (2012) Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe. *Applied and Environmental Microbiology*, **78**(9): 3120-3127.

16. Woudstra C, Le Maréchal C, Souillard R, Anniballi F, Auricchio B, Bano L, Bayon-Auboyer MH, Koene M, Mermoud I, Brito RB, Lobato FCF, Silva ROS, Dorner MB & Fach P (2018) Investigation of *Clostridium botulinum* group III's mobilome content. *Anaerobe*, **49**: 71-77.

17. Anza I, Vidal D, Feliu J, Crespo E & Mateo R (2016) Differences in the Vulnerability of Waterbird Species to Botulism Outbreaks in Mediterranean Wetlands: an Assessment of Ecological and Physiological Factors. *Applied and Environmental Microbiology*, **82**(10): 3092-3099.

18. Soos C & Wobeser G (2006) Identification of Primary Substrate in the Initiation of Avian Botulism Outbreaks. *Journal of Wildlife Management*, **70**(1): 43-53.

19. Duncan RM & Jensen WI (1976) A Relationship Between Avian Carcasses and Living Invertebrates in the Epizootiology of Avian Botulism. *Journal of Wildlife Diseases*, **12**(1): 116-126.

20. Reed TM & Rocke TE (1992) The Role of Avian Carcasses in Botulism Epizootics. *Wildlife Society Bulletin*, **20**(2): 175-182.

21. Ortiz NE & Smith GR (1994) Landfill sites, botulism and gulls. *Epidemiology and Infection*, **112**(2): 385-391.

22. Anza I, Skarin H, Vidal D, Lindberg A, Båverud V & Mateo R (2014) The same clade of *Clostridium botulinum* strains is causing avian botulism in southern and northern Europe. *Anaerobe*, **26**: 20-23.

23. Woudstra C, Le Maréchal C, Souillard R, Bayon-Auboyer MH, Mermoud I, Desoutter D & Fach P (2016) New insights into the genetic diversity of *Clostridium botulinum* group III through extensive genome exploration. *Frontiers in Microbiology*, **7**(757): 1-10.

24. Coulson JC (2015) Re-Evaluation of the Role of Landfills and Culling in the Historic Changes in the Herring Gull (*Larus argentatus*) Population in Great Britain . *Waterbirds*, **38**(4): 339-354.

25. Hatheway CL (1995) Botulism: The present status of the disease. *Current Topics in Microbiology and Immunology*, **195**: 55-75.

26. Coffield JA & Whelchel DD (2012) Botulinum neurotoxins. In *Veterinary Toxicology*, 2nd Edition, ed. Gupta, R.C., Elsevier Inc., USA, 978-0-12-385926-6, pp. 937-949.

27. Simpson LL (2004) Identification of the Major Steps in Botulinum Toxin Action. *Annual Review of Pharmacology and Toxicology*, **44**: 167-193.

28. Desta S, Melaku M & Abdela N (2016) Botulinum Toxin and Its Biological Significance: A Review. *World Applied Sciences Journal*, **34**(7): 854-864.

29. Neimanis A, Gavier-Widén D, Leighton F, Bollinger T, Rocke T & Mörner T (2007) An Outbreak of Type C Botulism in Herring Gulls (*Larus argentatus*) in Southeastern Sweden. *Journal of Wildlife Diseases*, **43**(3): 327-336.

30. Samour J (2016) Avian Medicine, Elsevier Ltd, St Louis, MO, USA. 978-0-7234-3832-8, pp. 279-280.

31. Salis AMH (2017) Botulismo en Aves Acuáticas ingresadas en el Hospital de Fauna Silvestre de GREFA: Estudio Retrospectivo. Trabajo Fin de Grado en Veterinaria, Universidad Complutense De Madrid, Spain, pp 17-20.

32. Hunter BF, Clark WE, Perkins PJ & Coleman PR (1970) Applied Botulism Research including Management Recommendations—A Progress Report. *California Department of Fish and Game*, 87 pp.

33. Martinez R & Wobeser G (1999) Immunization of ducks for type C botulism. *Journal of Wildlife Diseases*, **35**(4): 710-715.

34. Mereb G, Rosetti F, Castelli E, Ganuza R, Ottavioni L, Cavallero D & Calderón A (1999) Description of a botulism type C outbreak among waterbirds in the province of La Pampa, Argentina. *Anaerobe*, **5**(3-4): 191-193.

35. Shin N-R, Byun S hwan, Chun JH, Shin JH, Kim YJ, Kim J-H, Rhie G, Chung HM, Mo I-P & Yoo C-K (2010) An Outbreak of Type C Botulism in Waterbirds: Incheon, Korea. *Journal of Wildlife Diseases*, **46**(3): 912-917.

36. Work TM, Klavitter JL, Reynolds MH & Blehert D (2010) Avian botulism: A case study in translocated endangered laysan ducks (*Anas laysanensis*) on midway atoll. *Journal of Wildlife Diseases*, **46**(2): 499-506.

37. Hobbs RJ, Thomas CA, Halliwell J & Gwenin CD (2019) Rapid Detection of Botulinum Neurotoxins—A Review. *Toxins*, **11**(7): 418.

38. Thirunavukkarasu N, Johnson E, Pillai S, Hodge D, Stanker L, Wentz T, Singh B, Venkateswaran K, McNutt P, Adler M, Brown E, Hammack T, Burr D & Sharma S (2018) Botulinum Neurotoxin Detection Methods for Public Health Response and Surveillance. *Frontiers in Bioengineering and Biotechnology*, **6**: 1-12.

39. Hansbauer EM, Skiba M, Endermann T, Weisemann J, Stern D, Dorner MB, Finkenwirth F, Wolf J, Luginbühl W, Messelhäußer U, Bellanger L, Woudstra C, Rummel A, Fach P & Dorner BG (2016) Detection, differentiation, and identification of botulinum neurotoxin serotypes C, CD, D, and DC by highly specific immunoassays and mass spectrometry. *Analyst*, **141**(18): 5281-5297.

40. Maréchal CL, Woudstra C & Fach P (2016) Botulism. In *Clostridial Diseases of Animals* ed. Uzal, F.A., Songer J.G., Prescott J.F. & Popoff M.R., John Wiley & Sons, Inc., Ames, IA, USA, 9781118728406, pp. 318-319.

41. Zhang Y, Lou J, Jenko KL, Marks JD & Varnum SM (2012) Simultaneous and sensitive detection of six serotypes of botulinum neurotoxin using enzyme-linked immunosorbent assay-based protein antibody microarrays. *Analytical Biochemistry*, **430**(2): 185-192.

42. Le Maréchal C, Ballan V, Rouxel S, Bayon-Auboyer MH, Baudouard MA, Morvan H, Houard E, Poëzevara T, Souillard R, Woudstra C, Le Bouquin S, Fach P & Chemaly M (2016) Livers provide a reliable matrix for real-time PCR confirmation of avian botulism. *Anaerobe*, **38**: 7-13.

43. Le Maréchal C, Rouxel S, Ballan V, Houard E, Poezevara T, Bayon-Auboyer MH, Souillard R, Morvan H, Baudouard MA, Woudstra C, Mazuet C, Le Bouquin S, Fach P, Popoff M & Chemaly M (2017) Development and validation of a new reliable method for the diagnosis of avian botulism. *PLoS One*, **12**(1): 1-17.

44. Soares SPV (2010) Portugal, Paretic syndrome in gulls (Laridae) in the south of Portugal. Dissertação de Mestrado Integrado em Medicina Veterinária, Universidade de Lisboa, Portugal, pp. 10-63.

45. Carpenter JW (2017) Exotic Animal Formulary, 5th Edit, Elsevier Inc., St Louis, MO, USA. 978-0-323-44450-7, pp. 362.

46. Song XJ (2017) Analgesic and Neuroprotective Effects of B Vitamins. In *Nutritional Modulators of Pain in the Aging Population* ed. Watson, R.R. & Zibadi, S., Elsevier, St Louis, MO, USA, 978-0-12-805186-3, pp. 255-264.

47. Fan Y-C, Wang C-L, Wang C, Chen T-C, Chou C-H & Tsai H-J (2016) Incidence and Antimicrobial Susceptibility to Clostridium perfringens in Premarket Broilers in Taiwan . *Avian Diseases*, **60**(2): 444-449.

48. Rocke TE, Samuel MD, Swift PK & Yarris GS (2000) Efficacy of a type C botulism vaccine in green-winged teal. *Journal of Wildlife Diseases*, **36**(3): 489-493.

49. Arimitsu H, Lee JC, Sakaguchi Y, Hayakawa Y, Hayashi M, Nakaura M, Takai H, Lin SN, Mukamoto M, Murphy T & Oguma K (2004) Vaccination with recombinant whole heavy chain fragments of Clostridium botulinum type C and D neurotoxins. *Clinical and Diagnostic Laboratory Immunology*, **11**(3): 496-502.

50. Silva ROS, Gómez SYM, Medeiros LB, Marques MVR, Silva ASG, Mureb EN, Oliveira Junior CA, Favoretto SM, Lobato FCF & Martins NRS (2017) Antitoxin therapy of natural avian botulism outbreaks occurred in Brazil. *Anaerobe*, **48**: 115-117.

51. APHA (2019) Avian botulism in UK wild waterbirds, accessed 23 Septembre 2019, retrieved from <http://apha.defra.gov.uk/documents/surveillance/diseases/avian-botulism.pdf>

52. Son K, Kim Yk, Woo C, Wang S-J, Kim Y, Oem J-K, Jheong W & Jeong J (2018) Minimizing an outbreak of avian botulism (Clostridium botulinum type C) in Incheon, South Korea. *Journal of Veterinary Medical Science,* **80**(3): 553-556.

53. Coffield JA, Bakry N, Zhang RD, Carlson J, Gomella LG & Simpson LL (1997) In vitro characterization of botulinum toxin types A, C and D action on human tissues: Combined

electrophysiologic, pharmacologic and molecular biologic approaches. *Journal of Pharmacology and Experimental Therapeutics,* **280**(3): 1489-1498.

54. Oguma K, Yokota K, Hayashi S, Takeshi K, Kumagai M, Itoh N, Tachi N & Chiba S (1990) Infant botulism due to Clostridium botulinum type C toxin. *Lancet*, **336**(8728): 1449-1450.

55. Smart J, Roberts T, McCullagh K, Lucke V & Pearson H (1980) An outbreak of type C botulism in captive monkeys. *Veterinary Record*, **107**(19): 445-446.

56. Smith GR, Brambell MR, Gaynor WT, Greed G & de Meurichy W (1985) Botulism in zoological collections. *Veterinary Record*, **117**(20): 534-534.

57. Horowitz BZ (2010) Type E botulism. *Clinical Toxicology*, **48**(9): 880-895.

58. Farrow BRH, Murrell WG, Revington ML, Stewart BJ & Zuber RM (1983) Type C botulism in young dogs. *Australian Veterinary Journal*, **60**(12): 374-377.

59. Barsanti J, Walser M, Hatheway C, Bowen J & Crowell W (1978) Type C botulism in American Foxhounds. *Journal of the American Veterinary Medical Association,* **172**(7): 809-13.

60. Wobeser G, Baptiste K, Clark EG & Deyo AW (1997) Type C botulism in cattle in association with a botulism die-off in waterfowl in Saskatchewan. *The Canadian Veterinary Journal = La Revue Veterinaire Canadienne,* **38**(12): 782.

61. Cliplef DJ & Wobeser G (1993) Observations on Waterfowl Carcasses during a Botulism Epizootic. *Journal of Wildlife Diseases*, **29**(1): 8-14.

62. Breed D, Meyer LCR, Steyl JCA, Goddard A, Burroughs R & Kohn TA (2019) Conserving wildlife in a changing world: Understanding capture myopathy—a malignant outcome of stress during capture and translocation. *Conservation Physiology*, **7**(1): 1-21.