

Luisa Christina Patricio Stöfer

First evidence of sex-specific epidemiological differences in
tattoo-like skin lesions among free-ranging bottlenose
dolphins (*Tursiops truncatus*)



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dolphins (*Tursiops truncatus*)

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First evidence of sex-specific epidemiological differences in tattoo-like skin lesions among free-ranging bottlenose dolphins (*Tursiops truncatus*)

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Assinatura

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Assinatura



Abstract

This thesis aims to shed light on the fascinating world of cetacean epidemiology with focus on Cetacean poxviruses and Tattoo skin disease, by giving an in-depth state of the art, and delivering a baseline data study.

The skin of cetaceans is a multifaceted feature that offers a wealth of visible information. Epidermal lesions from trauma or disease are common, but the emergence, severity, and changes in demographic pattern of certain skin diseases may serve as a bioindicator for environmental change and wellbeing of a population. This study investigates tattoo-like skin lesions (TSL) in a bisexually philopatric coastal bottlenose dolphin (*Tursiops truncatus*) population, reporting sex-specific differences and their potential environmental implications. Data was collected in 2017 and 2018 during 145 daily boat-based surveys in Ría de Arousa-bay (NW Spain). The subsequent analysis of 10,409 photographs of 48 females and 51 males revealed an overall prevalence of 26.2%. Notably, males had a significantly higher prevalence (37.5%) compared to females (15.7%), along with increased lesion quantity in 2018. Minimum persistence time (MPT) of TSL ranged from 2.4 to 19.6 months (n=15), with males at a median of 407 days and females at 347 days. Sex-specific differences could be attributed to increased aggressive behavior and social stress but also to the very high polychlorinated biphenyl (PCB) concentrations in the area. While aggressive behavior may lead to increased cutaneous abrasions and viral entry, immunotoxic PCBs were found to be released through lactation and increased accumulation in males. Future studies should prioritize comparing populations exhibiting an equal distribution of TSL between sexes with those where males are disproportionately affected, aiming to discern differences, particularly in PCB concentration and behavioral aggression.

Keywords: Tattoo skin disease; Poxviruses; Bottlenose dolphins; Epidemiology; Health status; Skin lesions

Resumo (Portuguese)

Difícilmente algum outro grupo de animais capturou tanta atenção pública e política quanto os cetáceos. Eles não são apenas um dos taxa "carismáticos" mais populares, mas também desempenham papéis cruciais nos ecossistemas marinhos, contribuindo significativamente para a saúde e integridade do ecossistema. Sendo predadores de topo com longa vida útil e expostos a diversas ameaças antropogênicas, os cetáceos podem servir como espécies sentinelas e indicadoras, oferecendo valiosas informações sobre o estado dos ecossistemas marinhos. Com recursos limitados na ciência e um crescente foco em questões ambientais, há uma demanda para identificar as questões cruciais essenciais para avançar nos esforços de conservação de cetáceos. Em 2013, a conferência da "Society of Marine Mammalogy" resumiu e discutiu 15 tópicos de importância global para a conservação dos cetáceos. Foi destacada a importância da integração de dados de pesquisa multidisciplinar e a exploração do impacto de um ambiente em mudança, como as mudanças climáticas e a poluição antropogénica, nas áreas, estrutura populacional, uso do habitat e saúde dos cetáceos. Por fim, a importância da quantificação da dor e do sofrimento em cetáceos, examinando seu papel na saúde, respostas ao estresse e a sua relevância para os esforços de conservação.

A epidemiologia da vida selvagem ocupa, portanto, um lugar importante na pesquisa sobre conservação de cetáceos. Ela combina várias disciplinas para obter "insights" sobre as influências da ocorrência de doenças em nível populacional, abrangendo assim todos os três aspectos e destacando-se como um tópico importante nos esforços de conservação de cetáceos. Ela integra estudos sobre determinantes de doenças, como condições ambientais e fatores antropogénicos, características do hospedeiro e interações entre hospedeiros e patógenos. Esses dados são usados para desenvolver estratégias para prevenir e controlar doenças, melhorar o bem-estar animal e podem finalmente ser integrados na formulação de políticas.

Abordagens não invasivas no estudo de cetáceos em liberdade são essenciais para pesquisas éticas, minimizando perturbações nos comportamentos naturais e garantindo o bem-estar de uma população. Nesse sentido, a pele dos cetáceos, a interface entre o organismo e seu ambiente, oferece uma visão visível de vários aspectos epidemiológicos de uma população e pode ser investigada por meio de análises fotográficas. Lesões epidérmicas devido a traumas ou doenças são comuns, mas o surgimento, a gravidade e as mudanças no padrão demográfico de certas doenças de pele podem servir como um bioindicador de mudanças ambientais e do bem-estar da população.

A doença de pele de tatuagem, uma dermatopatia causada por poxvírus de cetáceos (CePVs), destaca-se como uma das doenças de pele mais frequentemente observadas em cetáceos. Foi observada em 17 espécies de odontocetos e quatro espécies de mysticetos em todos os oceanos, desde as águas árticas até as tropicais e ambientes estuarinos. A proliferação na pele e membranas mucosas resulta nas lesões escuras características, macroscopicamente visíveis, não sublimadas, com um padrão pontilhado e bordas escuras, tornando-a um alvo identificável em populações em liberdade.

A dermatopatia tipicamente expressa um padrão holoendêmico, atingindo o pico em juvenis em comparação com filhotes, provavelmente devido à perda gradual da imunidade materna e ao aumento do contacto com membros infectados do grupo. A prevalência geralmente diminui em animais maduros, presumivelmente seguindo o desenvolvimento de imunidade adquirida. Curiosamente, o padrão usual foi invertido, com mais adultos sendo afetados em cetáceos (*Delphinus delphis* e *Phocoena phocoena*) das Ilhas Britânicas em má saúde, atribuído a fatores como inanição, doenças infecciosas e parasitárias. Isso pode estar potencialmente relacionado à função imunológica comprometida influenciada pela alta exposição a bifenilos policlorados (PCBs) na região. Um padrão holoendêmico invertido pode, portanto, ser um indício de má saúde de uma população e degradação do ambiente. Da mesma forma, os sexos geralmente são afetados igualmente pela doença, mas há casos com um padrão reverso, com os machos sendo significativamente mais afetados do que as fêmeas. Em toninhas encalhadas no norte da Califórnia, a prevalência da doença de pele de tatuagem foi de 43,6% e o dobro em machos (58,1%, N = 31) em comparação com fêmeas (25%, N = 24). Este padrão epidemiológico e prevalência inesperadamente alta assemelha-se ao observado em toninhas de Burmeister (*Phocoena spinipinnis*) enredadas no Peru, mas difere do padrão observado em toninhas saudáveis das águas do Reino Unido. As descobertas no Peru foram observadas durante um período de intensas atividades pesqueiras, resultando na mortalidade direta e indireta de milhares de indivíduos, indicando assim um ambiente altamente estressante. As descobertas no norte da Califórnia podem estar associadas à residência em torno de San Francisco, o segundo porto mais movimentado da costa oeste dos EUA. Esses habitats são caracterizados por altos níveis de contaminantes e outras interações humanas, como pescarias, além de agressões entre espécies de golfinhos-nariz-de-garrafa. Diferenças entre machos e fêmeas podem ser atribuídas a fatores hormonais e comportamentais, mas também podem estar associadas às descobertas de concentrações aumentadas de bifenilos policlorados (PCB) em machos, já que as fêmeas eliminam organoclorados durante a gestação e lactação, sendo assim expostos a um maior risco imunotóxico.

Em resumo, uma mudança nos padrões epidemiológicos típicos dentro de uma população pode ser atribuída à má saúde, imunidade comprometida e estresse, favorecendo a infecção viral, persistência e recorrência. A investigação desses padrões pode contribuir para a compreensão do bem-estar das populações de cetáceos e também pode servir como bioindicador: um aumento na prevalência, gravidade e mudanças no padrão demográfico podem sinalizar mudanças ambientais mais amplas que precisam ser identificadas para estabelecer estratégias de gestão protetora.

No estudo, investigamos as diferenças específicas de gênero na prevalência, gravidade e tempo mínimo de persistência em uma população de golfinhos nariz-de-garrafa (*Tursiops truncatus*) costeira e filopátrica bissexual, residente no norte da Espanha. O objetivo foi obter dados importantes de referência, em uma área de estudo (Ría de Arousa) conhecida por sua alta produtividade oceânica e enfrentando um impacto antropogénico significativo, principalmente por cultivo de mariscos, pesca e atividades turísticas.

A metodologia é baseada em análises fotográficas, referindo-se à avaliação de lesões cutâneas semelhantes a tatuagens, sem confirmação real de poxvírus de cetáceos (CePVs). Os dados foram coletados a partir de 145 pesquisas diárias de barco em 2017 e 2018. Fotografias (10.409 imagens) de 48 fêmeas e 51 machos foram analisadas. Um catálogo foi estabelecido para identificar e quantificar adequadamente as lesões semelhantes a tatuagens. Os resultados revelaram que, no período bienal de 2017-2018, a prevalência de lesões cutâneas semelhantes a tatuagens em uma população costeira de golfinhos nariz-de-garrafa foi de 26,3%, situando-se no extremo superior entre populações em liberdade, variando de 4,5% (Estreito de Gibraltar, Espanha, N = 334) a 42,6% (Baía de Sarasota, EUA, N = 101). A metodologia entre os autores varia de avaliação fotográfica a análises laboratoriais, portanto, a comparação direta dos resultados deve ser obtida com cautela. A persistência variou de 2,4 a 19,6 meses em 15 golfinhos, com extensão potencial devido a um tamanho de amostra pequeno e período de observação restrito. Ao contrário de outros *Delphinidae* em liberdade, a prevalência foi significativamente maior em machos (37,5%) ($N_{\text{♂}} = 48$, $N_{\text{♂}}^{\text{doença}} = 18$) do que em fêmeas (15,7%) ($N_{\text{♀}} = 51$; $N_{\text{♀}}^{\text{doença}} = 8$). Além disso, a quantidade de lesões foi significativamente maior em machos do que em fêmeas, em 2018. Embora nenhuma lesão da classe de tamanho "muito grande" tenha sido observada em fêmeas, elas foram encontradas em dois machos que estavam relacionados a deficiências de saúde em estudos anteriores. Por fim, o tempo de persistência mediano foi prolongado em machos (407 dias) em comparação com fêmeas (347 dias), embora as diferenças não tenham atingido significância estatística. Diferenças específicas

de gênero na prevalência e gravidade de lesões cutâneas semelhantes a tatuagens podem ser explicadas pelo aumento do comportamento agressivo e estresse social, mas também podem estar relacionadas às concentrações muito altas de bifenilos policlorados (PCBs), ultrapassando todos os limites de toxicidade conhecidos para mamíferos marinhos, na área de estudo. Enquanto o comportamento agressivo pode levar a aumento de abrasões cutâneas e entrada viral, os PCBs imunotóxicos foram encontrados sendo libertados através da lactação e acumulação aumentada em machos.

Estudos futuros devem priorizar a comparação de populações que exibem uma distribuição igual de lesões cutâneas semelhantes a tatuagens entre os sexos com aquelas em que os machos são desproporcionalmente afetados, visando discernir diferenças, especialmente nas concentrações de PCB e padrões comportamentais. Padrões epidemiológicos de doenças de pele podem servir como indicadores importantes para o bem-estar das populações de cetáceos e saúde do ecossistema e devem ser estudados mais a fundo no futuro. Um tamanho de amostra maior e uma metodologia analítica de laboratório, bem como o monitoramento de uma população ao longo do tempo para detectar mudanças epidemiológicas, podem fornecer informações importantes adicionais sobre a doença. Este estudo lançou, portanto, as bases para pesquisas futuras nesta área na população de golfinhos nariz-de-garrafa, na Ría de Arousa.

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Table of contents

Abstract.....	I
Resumo (Portuguese).....	II
Acknowledgements.....	VI
PART 1: State of the art	12
1. Introduction.....	12
2. Cetacean poxviruses and Tattoo skin disease	14
2.1. Discovery of Cetacean poxviruses and Tattoo skin disease.....	14
2.2. Current taxonomy and host range.....	17
2.3. Affected species and distribution	19
2.4. Morphogenesis of Cetacean poxviruses.....	21
2.5. Genome of Cetacean poxviruses.....	22
3. Pathogenesis: molecular and individual aspects of the course of disease.....	22
3.1. Transmission pathways.....	22
3.2. Replication processes of poxviruses	23
3.3. Macroscopical development and persistence of Tattoo skin disease	24
3.4. Distribution of Tattoo skin disease on the body.....	25
3.5. Lesion size and number of Tattoo skin disease on the body	26
3.6. Histopathology of Tattoo skin disease	26
3.6.1. <i>Cetacean skin anatomy</i>	26
3.6.2. <i>Histopathology</i>	28
3.7. Latent infections	28
4. Epidemiology: observations at population level.....	30
4.1. Prevalence and severity of Tattoo skin disease.....	30
4.2. Demographic factors.....	30
4.2.1. <i>Age</i>	30
4.2.2. <i>Sex</i>	31
4.3. Environmental factors.....	32
4.3.1. <i>Salinity</i>	32
4.3.2. <i>Water temperature</i>	33
4.4. Anthropogenic factors.....	34
4.4.1. <i>PCBs and other immunotoxic contaminants</i>	34
4.4.2. <i>Others</i>	35
4.5. Social and behavioral factors.....	35
4.6. Methodology aspects	37
5. References of the literature review	38

PART 2: Manuscript	46
1. Introduction.....	46
2. Materials & Methods	49
2.1. Study site	49
2.2. Data collection	50
2.2.1. <i>General field approach</i>	50
2.2.2. <i>Dolphin encounter approach</i>	51
2.3. Data processing.....	52
2.4. Tattoo-like skin lesions (TSLs).....	53
2.4.1. <i>Definition of TSLs</i>	53
2.4.2. <i>Identification</i>	54
2.4.3. <i>Classification</i>	55
2.4.4. <i>Prevalence</i>	56
2.4.5. <i>Minimum persistence time (MPT)</i>	57
2.4.6. <i>Classification: Lesion size</i>	57
2.4.7. <i>Classification: Lesion distribution</i>	57
2.4.8. <i>Classification: Lesion number</i>	58
2.5. Statistical analyses	59
2.5.1. <i>Trend</i>	59
3. Results	59
3.1. Prevalence.....	59
3.2. Minimum persistence time (MPT).....	60
3.3. Lesion number	61
3.4. Lesion size	62
4. Discussion.....	63
5. Conclusion	66
6. Literature cited in the manuscript	67

Table of images

Figure 1. Global map of reported cases of tattoo skin disease in cetaceans	20
Figure 2. Macroscopic image of characteristic tattoo skin disease lesions1	25
Figure I. Map of the study area “Ría de Arousa”-bay.....	50
Figure II. T ⁺⁺ and T ⁺ tattoo-like skin lesions on <i>Tursiops truncatus</i>	54
Figure III. Illustration of the bottlenose dolphins body divided into 12 distinct sections	59
Figure IV. Prevalence of tattoo-like skin lesion during 2017 and 2018 in 99 Ría de Arousa bottlenose dolphins (<i>T. truncatus</i>).....	60
Figure V. Minimum persistence time (MPT) of tattoo-like skin lesions in a total of 15 Ría de Arousa bottlenose dolphins (<i>T. truncatus</i>).....	61
Figure VI. Tattoo-like skin lesion counts in male and female Ría de Arousa bottlenose dolphins (<i>T. truncatus</i>) in years 2017 and 2018	62
Figure VII. Distribution of tattoo-like skin lesion counts per size category among 8 female and 18 male Ría de Arousa bottlenose dolphins from 2017 to 2018	63

List of abbreviations, acronyms and symbols

Abbreviation	Explanation
BDRI	Bottlenose Dolphin Research Institute
CPV	Cetacean poxvirus (alternative abbreviation)
CePV	Cetacean poxvirus
CePV-1	Cetacean poxvirus 1
CePV-2	Cetacean poxvirus 2
DDT	Dichloro-Diphenyl-Trichloroethane
DNA	Deoxyribonucleic Acid
EEV	Extracellular Enveloped Virus
EV	Extracellular Virus
HOCs	Halogenated Organic Compounds
ICTV	International Committee on Taxonomy of Viruses

IMV	Intracellular Mature Virus
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Σ 25CBs	Sum of 25 Chlorinated Biphenyl Congeners
kb	Kilo Base Pairs
mm	Millimeters
μ m	Micrometers
N	Sample Size
PCBs	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
TSD	Tattoo skin disease
USA	United States of America
$^{\circ}$ C	Degrees Celsius
FL	Full Leap (dolphin behavior)

GPS	Global Positioning System
La Toja	A site for land-based fieldwork
PAS	Primary Activity State (referring to the main activity behavior of dolphins)
Rons	A site for land-based fieldwork
Ría de Arousa	A tidal estuary in Galicia, Spain
SST	Sea Surface Temperature

PART 1: State of the art

1. Introduction

Hardly any other group of animals has captured public and political attention as much as cetaceans (Hoyt, 2012; Parsons et al., 2015). They are not only one of the most popular, “charismatic” flagship taxa (Hoyt, 2012), but also play pivotal roles in marine ecosystems, contributing significantly to ecosystem health and integrity (Bowen, 1997; Katona & Whitehead, 1988; Parsons et al., 2015; Sergio et al., 2008). Cetacea inhabit a wide range of aquatic habitats, from all the oceans of all climate zones, shallow coasts and abyssal canyons, to river arms (Parsons et al., 2015). Their adaptability exposes them to an array of anthropogenic threats, including accidental entanglement in fishing gear, exposure to pathogens and pollutants, collisions with shipping vessels, and the pervasive impact of underwater noise (Parsons et al., 2015; Parsons et al., 2013). As top predators and exposed to the variety of anthropogenic threats, cetaceans can serve as sentinel as well as indicator species, offering valuable insights into the state of marine ecosystems (Godard-Codding et al., 2011; Moore, 2008; Parsons et al., 2015). In addition, cetaceans contribute substantially to the economy of numerous coastal communities, as the whale-watching industry alone exceeds a billion dollars annually (Hoyt & Welfare, 2002; O’Connor et al., 2009; Parsons et al., 2015; Parsons et al., 2003).

With limited resources in science and a growing focus on environmental issues, there is a demand to pinpoint the crucial questions essential for advancing conservation efforts (Parsons et al., 2015; Sutherland et al., 2011). Parsons et al. (2015) summarized 15 themes of global importance for cetacean conservation from the conference *Society of Marine Mammalogy* in 2013. It was pointed out the importance of the integration of multidisciplinary research data and the exploration of the impact of a changing environment such as climate change and anthropogenic pollution on cetacean ranges, population structure, habitat use, and health. Lastly, the importance of quantification of pain and suffering in cetaceans, examining its role in their health, stress responses, and its relevance to conservation efforts.

Wildlife epidemiology therefore takes an important place in the research of cetacean conservation: It combines several disciplines to gain insight into the influences of disease occurrence on a population level, therefore spanning all three aspects and standing as important topic within cetacean conservation efforts (Pfeiffer, 2010). It integrates studies on determinants of disease, like environmental conditions and anthropogenic factors, host characteristics and

interactions between hosts and pathogens (Pfeiffer, 2010). This information is used to develop strategies for preventing and controlling diseases and enhancing animal welfare and can finally be integrated into policymaking (Pfeiffer, 2010). Wildlife epidemiology in cetaceans encompasses several methodological approaches: Field surveys, necropsies, and health assessments in free-ranging and captive animals provide insights into disease prevalence, causes of mortality, and overall health (Pfeiffer, 2010; Van Bresseem et al., 2009; Van Bresseem & Van Waerebeek, 1996). Serological studies and molecular techniques detect antibodies against a pathogen (Blacklaws et al., 2013; Segura-Göthlin et al., 2023), while electron microscopy permits to visualize the virus (Blacklaws et al., 2013). Satellite telemetry and tagging track cetacean movements (Mate et al., 2011; van Weelden et al., 2021), while surveillance of environmental factors correlates changes with disease prevalence (Van Bresseem et al., 2009; Van Bresseem et al., 2009; Van Bresseem, Van Waerebeek, et al., 2015).

Non-invasive approaches in studying free-ranging cetaceans are essential for ethical research, minimizing disturbance to natural behaviors, and ensuring the well-being of individuals (Papastavrou & Ryan, 2023; Toms et al., 2020; Van Bresseem et al. 2022). Hereby, the skin of cetaceans, the interface between the organism and its environment, offers a visible insight into several epidemiological aspects of a population without the need of invasive methods. Epidermal lesions from trauma or disease are common, but the emergence, severity and changes in demographic pattern of certain skin diseases may serve as a bioindicator for environmental change and wellbeing of the population (Chan & Karczmarski, 2019; Mouton & Botha, 2012; Van Bresseem et al., 2009).

This thesis aims to shed a light on epidemiology in cetaceans concentrating on the skin, by diving deeper into the topic by choosing Cetacean poxviruses and Tattoo skin disease as an example, giving an in-depth state of the art and delivering a baseline data study in a coastal, free-ranging common bottlenose population (*Tursiops truncatus*) in northern Spain.

2. Cetacean poxviruses and Tattoo skin disease

Cetacean poxviruses (CePVs) stand as the pathogen behind one of the most frequently observed skin diseases in cetaceans around the globe (Van Bresseem et al., 2023). Upon infection, it can result in the characteristic, macroscopically visible, dark lesions with a dotted pattern and dark borders, giving it the name Tattoo skin disease (TSD) by Geraci and colleagues (Geraci et al., 1979; Van Bresseem et al., 2003; Van Bresseem & Van Waerebeek, 1996). The following chapters will review current knowledge of Cetacean poxviruses and Tattoo skin disease with main focus on epidemiological aspects.

2.1. Discovery of Cetacean poxviruses and Tattoo skin disease

Poxviruses have been known to affect cetacean species since the 1970s and since then, numerous studies have reported skin lesions associated with poxvirus infections in cetaceans. In 1975, Sweeney and Ridgway published a paper on "Common Diseases of Small Cetaceans", to inform on diagnosis and treatment of diseases that occur in Cetaceans held in captivity, mainly common bottlenose dolphins (*Tursiops truncatus*) (Sweeney & Ridgway, 1975). They described lesions of unknown origin, including tattoo-like skin lesions with dark stippled pattern, which were considered to be strictly epidermal lesions occurring mainly on the head and persisting for months or years (Sweeney & Ridgway, 1975).

In 1979, Flom and Houk discovered first supportable evidence of virus disease in cetaceans. Through transmission electron microscopy intracytoplasmic particles were observed in epithelial fragments from lesions in two mature Atlantic bottlenose dolphins (*T. truncatus*) held at the *Naval Oceans Systems Center*, San Diego, California (Flom & Houk, 1979). Some particles had the characteristic poxvirus dumbbell-shaped core (nucleoid) compressed between two elliptical bodies and enclosed by an outer envelope (Flom & Houk, 1979). Standard histological examination for inclusion bodies though was hindered due to inadequate biopsy tissue (Flom & Houk, 1979). Inclusion bodies are small particles inside cells visible after staining under the light microscope and often serve as diagnostic criterion in viral infections (Mark et al., 1991). The authors indicated similarity to the sheep pox subgroup and differentiated their findings of "tattoos" from lesions and virus particles of sealpox virus (Flom & Houk, 1979).

In 1979, Geraci and colleagues finally confirmed poxviruses in captive and free-ranging bottlenose dolphins (*T. truncatus*) and one stranded Atlantic white-sided dolphin

(*Lagenorhynchus acutus*). After completed histological evidence, the disease was given the name “dolphin pox” (Geraci et al., 1979). The intracytoplasmic inclusions in cells of the transition zone contained aggregations of virions in varying stages of development (Geraci et al., 1979). The virions, which measured approximately 200 by 300 nm, contained a dumbbell-shaped core with an outer membrane, an intermediate coat and an outer envelope (Geraci et al., 1979). First statements of “dolphin pox” being stress-related, induced through certain environmental conditions such as poor water quality and general health, were made and indicated that “dolphin pox” may be more than a skin disease (Geraci et al., 1979). The disease could be a visual clue to general health and stress in captive and free-ranging dolphins, delivering a possible non-invasive monitoring technique (Geraci et al., 1979).

Further research through visual assessment of “tattoos” and electron microscopy revealed unclassified poxviruses not only in common bottlenose dolphins (*T. truncatus*) and Atlantic white-sided dolphin (*L. acutus*) (Geraci et al., 1979), but in several other Odontocete species (Baker & Martin, 1992; J. R. Baker, 1992; Flom & Houk, 1979; Van Bresseem et al., 1999; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 1993) (see table 1).

Table 1. Cetacean species observed with “tattoo” skin lesions through visual assessment (= VA) and assessment of specimen of cetacean skin affected with “tattoo” skin lesions under the electron microscopy (= EM), from 1979 to 2003. Subsequent molecular analyses with confirmation of Cetacean poxviruses were first carried out in 2006 (Bracht et al., 2006).

Method	Species	Where	When (Reference)
VA & EM	Common bottlenose dolphin (<i>Tursiops truncatus</i>)	Captivity (USA) Free ranging (Florida; central Portugal)	(Flom & Houk, 1979; Geraci et al., 1979; Van Bresseem et al., 2003)
EM	Atlantic white-sided dolphin (<i>Lagenorhynchus acutus</i>)	Free ranging (stranded, Massachusetts) Captivity (Massachusetts)	(Geraci et al., 1979)
EM	Killer whale (<i>Orcinus orca</i>)	unknown	(Van Bresseem et al., 1999)

VA & EM	Dusky dolphins (<i>Lagenorhynchus obscurus</i>)	Central Peru	(Van Bressemer et al., 1993)
EM	Long-beaked common dolphins (<i>Delphinus delphis</i>)	Central Peru	(Van Bressemer & Van Waerebeek, 1996)
VA & EM	Burmeister's porpoises (<i>Phocoena spinipinnis</i>)	Central Peru	(Van Bressemer et al., 1993)
VA	Striped dolphin (<i>Stenella coeruleoalba</i>)	Mediterranean Sea	(Baker, 1992; Van Bressemer et al., 2003)
VA	White beaked dolphin (<i>Lagenorhynchus albirostris</i>)	UK	(Baker, 1992)
VA	Long-finned pilot whales (<i>Globocephala melaena</i>)	UK	(Baker, 1992)
VA & EM	Hectors dolphin (<i>Cephalorhynchus hectori</i>)	New Zealand	(Duignan et al., 2003)
VA & EM	Harbor porpoises (<i>Phocoena Phocoena</i>)	UK	(Baker & Martin, 1992; Baker, 1992)

Though “tattoo” skin lesion tissues have been repeatedly demonstrated using thin-section electron microscopy, the poxvirus-agent had not been isolated yet (Flom & Houk, 1979; Geraci et al., 1979; Smith et al., 1983).

First molecular analyses of Cetacean poxviruses were carried out by Bracht et al. in 2006. Polymerase chain reaction was used, targeting DNA polymerase and DNA topoisomerase I genes of members of the subfamily *Chordopoxvirinae*. Two different Cetacean poxvirus groups were identified in lesions of 10 Odontocetes: Cetacean poxvirus 1 (CPV-1) and Cetacean poxvirus 2 (CPV-2). Groups were assigned based on nucleotide and amino acid identities of gene fragments. CPV-1 was amplified from skin lesions of four Asian bottlenose (*Tursiops aduncus*), one Atlantic bottlenose (*T. truncatus*), two rough-toothed (*Steno bredanensis*), and two striped (*Stenella coeruleoalba*) dolphins. Nucleotide and amino acid shared 93% and 97% identities. CPV-2 was amplified from skin lesions of a bowhead whale (*Balaena mysticetus*)

and shared only 84% nucleotide and 89% amino acid identities compared to homologous sequences from the other nine Cetacean poxviruses, therefore provisionally assigned to this second group of Cetacean poxviruses. This was also the first observation of Cetacean poxviruses in a Mysticetes species. Comparison to DNA-polymerase-gene-fragments with homologous sequences of terrestrial poxviruses and deduced amino-acid-sequences revealed highest identity of CPV-1 with genus *Orthopoxvirus* and of CPV-2 of the genera *Orthopoxvirus* and *Suipoxvirus*.

Ongoing effort contributes to the understanding of the epidemiology of Tattoo skin disease in cetaceans and is described in the following chapter.

2.2. Current taxonomy and host range

Viruses are classified according to the type, structure, and replication mode of the nucleic acid, structural components and morphology of virions, strategy of gene expression, and immunological properties and behavior toward inactivating agents (*International Committee on Taxonomy of Viruses*, 2022; McInnes et al., 2023). Nomenclature and classification of viruses are regulated by the *International Committee on Taxonomy of Viruses* (abbr. ICTV) (*International Committee on Taxonomy of Viruses*, 2022). The *Poxviridae* family is divided into two subfamilies - *Chordopoxvirinae*, which encompasses poxviruses in vertebrates with eighteen genera, and *Entomopoxvirinae*, which encompasses poxviruses of invertebrates, particularly insects, with four genera (Bracht et al., 2006; International Committee on Taxonomy of Viruses, 2022).

Within each genus, there are various species associated with specific hosts and diseases. For instance, the subfamily *Chordopoxvirinae* include the genus *Orthopoxvirus*, exemplified by smallpox virus (*Variola major*), which plagued humanity for centuries until its eradication in 1977 through an extensive vaccination campaign by the *World Health Organization* (Barquet, 1997; Strassburg, 1982; World Health Organization, 1980). The genus *Capripoxvirus* encompasses lumpy skin disease virus causing nodules or lumps on the skin as well as several systemic symptoms in cattle, still afflicting parts of sub-Saharan Africa, the Middle East, and the Indian subcontinent (Bracht et al., 2006; Ireland & Binopal, 1998). *Entomopoxvirinae* include the genus *Betaentomopoxvirus* infecting Lepidoptera (butterflies and moths), exemplified by *Amsacta moorei entomopoxvirus*. *A. moorei entomopoxvirus*, obtained from *A. moorei* moth larvae, has demonstrated the ability to infect significant agricultural pests like *Estigmene acrea* and *Lymantria dispar*, suggesting its potential as a

biological control agent for pest management (Efridi & Lappin, 2023; Gencer et al., 2023; Perera et al., 2013). In summary, poxviruses exhibit a wide host range and infect various vertebrate and invertebrate animal species worldwide, including humans (G. D. Bossart & Duignan, 2018; Bracht et al., 2006; Silva et al., 2020).

Cetacean poxviruses (CePVs) belong to the family *Poxviridae* (Delhon, 2022; Luciani et al., 2022). It is hereby noted that the abbreviation for Cetacean poxvirus varies between authors, either as CePV or CPV. Within the *Poxviridae* family, CePVs are classified in the subfamily *Chordopoxvirinae* (Rodrigues et al., 2020; Upton et al., 2003). Notably, there is an ongoing effort to establish a more specific taxonomic designation for these viruses. As of the latest information, CePVs are tentatively assigned to the genus *Cetaceanpoxvirus*, a classification that is awaiting final acceptance by the *International Committee on Taxonomy of Viruses* (ICTV) (Blacklaws et al., 2013; Bracht et al., 2006; Rodrigues et al., 2020; Van Bresseem et al., 2022). The taxonomy of CePVs is characterized by their genetic diversity, with different clades identified based on genetic analyses, particularly involving partial sequences of DNA polymerase and DNA topoisomerase I genes, as well as SYBR green real-time PCR assay (Barnett et al., 2015; Blacklaws et al., 2013; Bracht et al., 2006; Fiorito et al., 2015; Luciani et al., 2022; Sacristán et al., 2018; Van Bresseem et al., 2022). Notably, two main clades have been identified: CePV-1 and CePV-2 (Blacklaws et al., 2013; Bracht et al., 2006; Fiorito et al., 2015). These clades distinguish between Cetacean poxviruses found in Odontocetes (toothed whales; CePV-1) and Mysticetes (baleen whales; CePV-2), respectively (Blacklaws et al., 2013; Bracht et al., 2006; Fiorito et al., 2015). CePV-1 encompasses strains recovered from *Delphinidae* (dolphins) and *Phocoenidae* (porpoises) across various oceanic regions (Blacklaws et al., 2013; Bracht et al., 2006; Luciani et al., 2022; Rodrigues et al., 2020; Sacristán et al., 2018; Van Bresseem et al., 2022). These strains exhibit a high degree of genetic homogeneity within the *Delphinidae* family, irrespective of the host species or the ocean province, although *Phocoenidae* strains seem to form a separate lineage (Barnett et al., 2015; Blacklaws et al., 2013). CePV-2, on the other hand, includes strains isolated from Mysticetes. One strain was identified in skin collected from a bowhead whale (*Balaena mysticetus*) without apparent lesions (Bracht et al., 2006; Van Bresseem et al., 2022). The second was from an adult female southern right whale (*Eubalaena australis*) with raised, circular skin lesions upon stranding at Peninsula Valdes, Argentina (Fiorito et al., 2015; Van Bresseem et al., 2022). However, the study of CePVs in baleen whales is challenging due to difficulties in recognizing lesions and obtaining diagnostic samples (Van Bresseem et al., 2022). A recent study by Rodrigues et al., 2020 unveiled the complete sequence of a Cetacean

poxvirus, CePV-TA, recovered from tattoo skin lesions in a captive Indo-Pacific bottlenose dolphin (*Tursiops aduncus*). Through phylogenetic analyses, the study establishes CePV-TA as the sister group to a clade including *Centapoxvirus*, *Orthopoxvirus*, and clade II poxviruses, which include viruses of artiodactyls (*Cervidpoxvirus*, *Suipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, and *Yatapoxvirus*).

2.3. Affected species and distribution

Cetacean poxviruses have been studied in various Odontocetes and four Mysticetes species inhabiting the coastlines of Chile, Peru, Argentina, Brazil, the United States, Canada, the United Kingdom, Portugal, Italy, New Zealand, Australia, Oman and other areas of the globe (see Figure 1) (Bossart et al., 2015; Cocumelli et al., 2018; Fiorito et al., 2015; Hamilton & Marx, 2005; Hart et al., 2012; Powell et al., 2018; Sacristán et al., 2018; Segura-Göthlin et al., 2021; Tomo & Kemper, 2022; Van Bresseem et al., 2003, 2009; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022; Van Bresseem et al., 2015).

Reported cases of tattoo skin disease or tattoo-like skin lesions in cetaceans

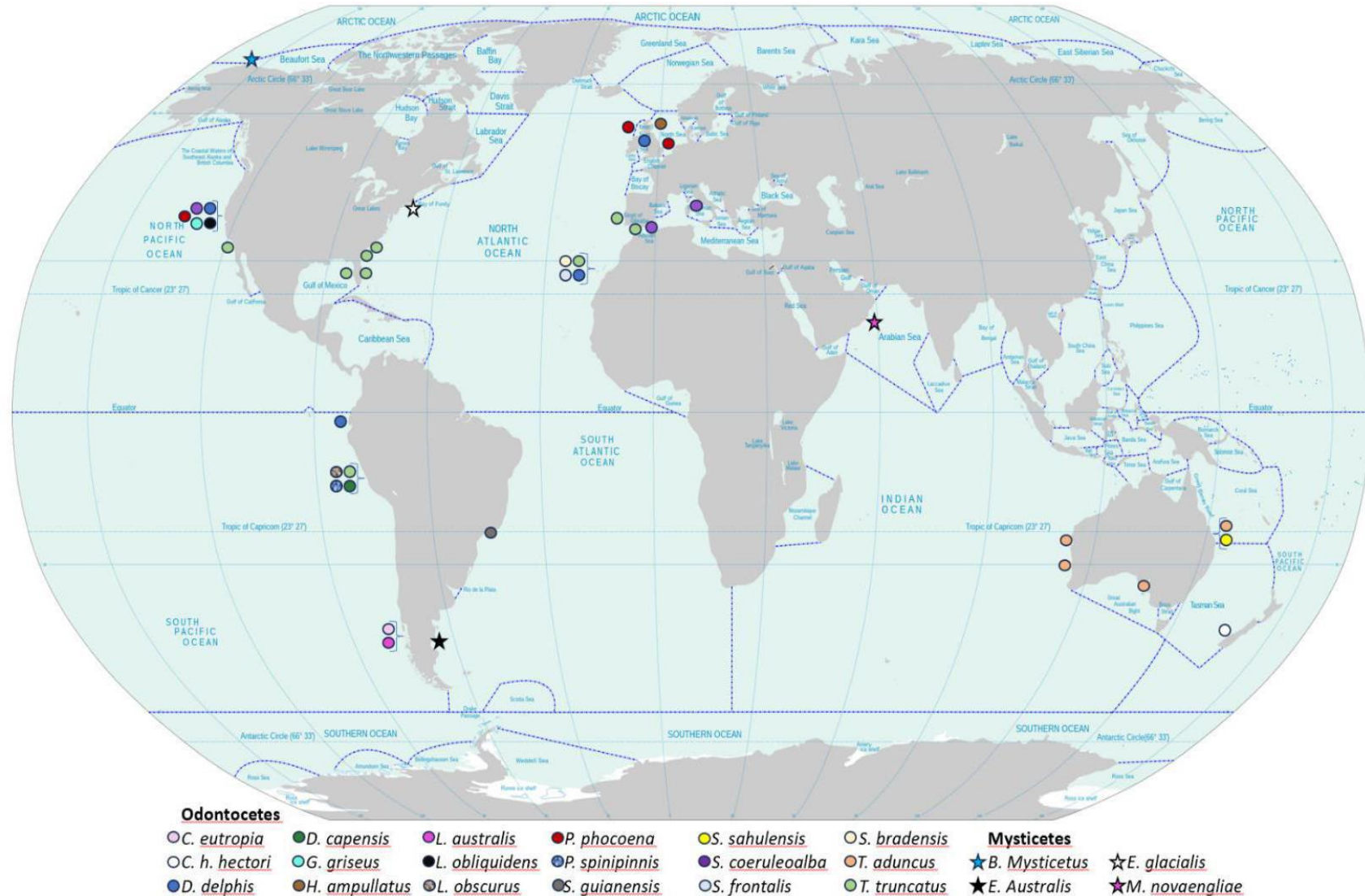


Figure 1. Reported cases of tattoo skin disease or tattoo-like skin lesions in cetaceans. The legend features dots representing Odontocetes species and stars representing Mysticetes species. Distinct colors differentiate between species within each suborder.

Derived from IHO 23-3rd: Limits of Oceans and Seas, Special Publication 23, 3rd Edition 1953, published by the International Hydrographic Organization. Wikimedia Commons, 5 April 2021, commons.wikimedia.org/wiki/File:Oceans_and_seas_boundaries_map-fr.svg. Modifications made by Pinpin, Jugger90 and Dziban303. Legend added by Luisa Christina Patricio Stöfer, 20.11.2023.

2.4. Morphogenesis of Cetacean poxviruses

Viruses are infectious organic structures that differ fundamentally from living microorganisms due to their lack of cellular organization and mode of reproduction (Correa et al., 2021; Dimmock et al., 2015). They consist either of deoxyribonucleic acid (DNA; DNA viruses) or ribonucleic acid (RNA; RNA viruses) (Schulz, 2020). Within the host cell, these nucleic acids govern the replication of the virus, while outside the host cell - in the released virus particle or virion - they are consistently enclosed by a protein envelope known as the capsid (Weinbauer, 2004). Some viruses also have an additional lipid envelope derived from the host cell membrane, aiding in entry of a host cell (Schulz, 2020; Weinbauer, 2004).

Poxviruses are among the largest known pathogenic double-stranded DNA-viruses (Delhon, 2022; Efridi & Lappin, 2023). They are characterized by their unique spheroidal to brick-shaped morphology and 220-450 nm length (140-260 nm width, 140-260 nm thick) and can therefore be seen under a light microscope at higher magnifications (McInnes et al., 2023). Different stages of virions of CePV-1 were measured under the transmission microscope with lengths of 200 to 300 nm (Geraci et al., 1979) and in CePV-2 in southern right whales (*Eubalaena australis*) with lengths of 200 to 320 nm (150 nm in diameter) (Fiorito et al., 2015). Three main viral structures can be identified within a virion: core, membrane and lateral bodies (Cocumelli et al., 2018; Geraci et al., 1979). The virion is enveloped by an irregular surface membrane enclosing a biconcave to cylindrical core which contains the large linear double-stranded DNA genome, along with various proteins and enzymes which form a nucleoprotein complex, necessary for viral replication (Cocumelli et al., 2018; Greseth & Traktman, 2022; McInnes et al., 2023). On the concave side between core and envelope, one or two proteinaceous elongated structures termed lateral bodies are found (Bidgood et al., 2022; Condit et al., 2006; McInnes et al., 2023). The intracellular mature virus (IMV) may form a cylindrical nucleoprotein complex which forms an Z-structure along the long virion axis (McInnes et al., 2023). Intracellular enveloped viruses (IEV) are formed when the virion is additionally wrapped in a double layer of intracellular membranes (McInnes et al., 2023). This stage mainly occurs within mammalian poxviruses and is therefore likely to occur within *Cetaceanpoxvirus*, while avian viruses skip this intermediate formation (McInnes et al., 2023). When externalized, extracellular virions (EV) are formed, where the outer membrane layer is fused and lost with the cell membrane (McInnes et al., 2023). EVs contain specific envelope-proteins, antigenically different from IMVs (McInnes et al., 2023). Two main pathways have been observed: formation of cell-associated enveloped virus (CEV) or formation of extracellular enveloped virus (EEV)

(McInnes et al., 2023). While CEV are formed by binding to the cell surface, EEV is formed by release into the extracellular matrix (McInnes et al., 2023). The exact pathway of *Cetaceanpoxvirus* is yet unknown.

2.5. Genome of Cetacean poxviruses

The genomically linked *Poxviridae* family is known to exhibit a genome with lengths of 128-375 kilo base pairs (kb), the termini of which are covalently closed and consist of inverted complementary sequences of different lengths (Efridi & Lappin, 2023; McInnes et al., 2023). The complete genome sequence of a Cetacean poxvirus was isolated from a captive Indo-Pacific bottlenose dolphin (*Tursiops aduncus*), named CePV-TA, and found to be only 121,769 base pairs (bp), therefore being one of the smallest poxvirus genomes known (Rodrigues et al., 2020). It has a high A + T content of 71.5% and a low G + C content of 28.5% (Rodrigues et al., 2020). The genome features inverted terminal repeats and terminal hairpin palindromic sequences (Rodrigues et al., 2020). Among its 120 open reading frames, 80 encode proteins shared by all members of the *Chordopoxvirinae* subfamily (Rodrigues et al., 2020). Notably, CePV-TA has five open reading frames coding for immune evasion proteins (CePV-TA-4, CePV-TA-117 encodes apoptosis regulator M11 L-like proteins, CePV-TA-20, CePV-TA-21 encodes anti-apoptotic proteins, CePV-TA-113 encodes an anti-inflammatory protein), inhibiting the host's innate immune response (Rodrigues et al., 2020). Unique to CePV-TA is the presence of two copies of the E3L gene, which, in vitro, hinders interferon antiviral enzyme activation (Rodrigues et al., 2020).

3. Pathogenesis: molecular and individual aspects of the course of disease

3.1. Transmission pathways

Poxviruses have the ability to initiate infection through a variety of direct and indirect pathways and involves a complex interplay between the virus and the hosts immune response (Mark et al., 1991; McInnes et al., 2023). Generally, pathogens of the subfamily *Chordopoxvirinae* cause infections that commonly lead to the development of lesions, skin nodules, or a widespread rash, which contain high concentrations of infectious particles (Mark et al., 1991; McInnes et al., 2023). The spread of the virus to new hosts often occurs through the release of infectious material from these lesions (Mark et al., 1991). This direct skin contact, known as smear infection, is exemplified by cases of localized molluscum contagiosum (genus

Molluscipoxvirus) infections in humans (Hanson & Diven, 2003; Mark et al., 1991). Other direct pathways are droplet transmission via the respiratory tract, such as in variola virus infections in humans, and oral transmission, as observed in cases of ectromelia virus (genus *Orthopoxvirus*) infections in mice (Mark et al., 1991). Contaminated environments and mechanical transmission via insect bites (insect as vector) serve as indirect routes for poxvirus transmission (Mark et al., 1991). Poxviruses exhibit resilience in the environment, remaining viable for extended periods in dried scabs or other virus-laden materials (Mark et al., 1991). This efficiency in transmission, ability to undergo antigenic variation, and manipulation of host cell functions contribute to their pathogenic properties and their quest to establish infections in various hosts (Koonin et al., 2021; Schulz, 2020; Weinbauer, 2004).

Transmission mechanisms specifically of Cetacean poxviruses need to be further explored, but as described, poxviruses from the subfamily *Chordopoxvirinae* can enter their hosts through skin abrasions or the respiratory and oral pathways (Barnett et al., 2015; Mark et al., 1991; McInnes et al., 2023; Powell et al., 2020). Tattoo skin lesions are regularly observed superposed on tooth rakes and other superficial epidermal injuries, suggesting that these may favor viral entry (Segura-Göthlin et al., 2023; Van Bresseem et al., 2018, 2022). The potential correlation of Tattoo skin disease and tooth rakes provides a suspected inoculation route that needs to be further explored (Van Bresseem et al., 2018). Additionally, prior contact with Tattoo skin disease-positive individuals shows to be a significant predictor of Tattoo skin disease, likely through direct transmission within the social network (Powell et al., 2020).

3.2. Replication processes of poxviruses

This section provides an overview of replication processes observed in the *Poxviridae* family, given that replication of Cetacean poxviruses remains not specifically studied.

Generally, virions spread extracellularly by transmission, but rely on a suitable host cell for replication as they lack an own metabolism (Koonin et al., 2021; Weinbauer, 2004). They therefore are strictly intracellular parasites that reproduce exclusively within organism cells whose metabolic apparatus they use for replication (Schulz, 2020). In contrast to other DNA-viruses, the replication cycle and assemblage of *Poxviridae* occurs exclusively in the cytoplasm of the host cell (Efridi & Lappin, 2023). Therefore, poxviruses cannot use all the cellular factors found in the cell nucleus (such as DNA- and RNA-polymerases) but must code for the corresponding virus-specific enzymes (Greseth & Traktman, 2022). The virion attaches through electrostatic interactions to specific entities - mainly glycosaminoglycans and laminin - of the

host cell surface (McInnes et al., 2023). After adsorption and penetration – achieved through either fusion of the virus envelope with the cell plasma membrane or phagocytosis and subsequent membrane fusion - the virus core and DNA are released into the cellular cytoplasm in a two-stage process (Moss, 2006). Fusion is induced by a highly conserved viral protein complex (McInnes et al., 2023). The transcription and processing (capping, polyadenylation at 3' termini, no splicing) of the primary mRNAs takes place in the intact virus cores, which contain the necessary transcription factors (McInnes et al., 2023). After breaking up the cores, early genes are transcribed which encode several proteins such as enzymes involved in replicating the genome (Moss, 2013). DNA replication takes place with expression of intermediate genes, encoding late transcription factors (McInnes et al., 2023; Moss, 2013). Lastly transcription of the late genes can be expressed during the post-replicative phase (Moss, 2013). DNA-replication occurs at the so-called viral factories, recognizable as type B basophilic inclusion bodies (Damon, 2011; McInnes et al., 2023). No RNA-primer is required for initiation, but a free 3'-OH end is produced in one of the DNA strands proximal to hairpin formation by strand breakage (self-priming) (Baroudy et al., 1982, 1983; Moss, 2013; Moyer & Graves, 1981). The elongation proceeds unidirectionally with strand displacement, DNA refolding in the area of the inverted terminal repeats and the formation of concatemeric molecules (concatemers), which are resolved into genomes of unit length when the virus particles are assembled (McInnes et al., 2023; Moss, 2013).

3.3. Macroscopical development and persistence of Tattoo skin disease

Pathogens of the subfamily *Chordopoxvirinae* cause infections that commonly lead to the development of lesions, skin nodules, or a widespread rash, with potentially fatal outcomes in certain cases (McInnes et al., 2023). Cetacean poxviruses replicate in the skin, generating localized or generalized lesions of variable duration (Van Bresseem et al., 2022).

Lesions typically present as flat or slightly raised, varying in shape from round to elliptical, and ranging in size from small to larger irregular blemishes that may merge (see Figure 2) (Geraci et al., 1979; Van Bresseem et al., 2003, 2009). Presence of CePV-1 was found in white-fringed, black-fringed, and ring lesions (Geraci et al., 1979; Segura-Göthlin et al., 2023). The lesions can have depressed black centers, forming tattoo-like stippled patterns, giving it the name “Tattoo skin disease” due to its characteristic appearance (see Figure 2 and 3 in Segura-Göthlin et al., 2023) (Geraci et al., 1979; Van Bresseem et al., 2003; Van Bresseem & Van Waerebeek, 1996). Healing stages appear light gray in color.

Observed persistence time of the tattoo skin lesions varies from 3 to 65 months (Powell et al., 2018; Van Bressem et al., 2003; Van Bressem et al., 2018). Several factors may contribute to the prolonged persistence of Cetacean poxviruses in the skin (Van Bressem et al., 2022). Cetacean epidermal cell kinetics exhibit a slow turnover rate, with bottlenose dolphins showing a calculated transit time of 73 days (Hicks et al., 1985; Van Bressem et al., 2022). This extended retention of infected cells could facilitate ongoing transmission within the epidermis over months (Van Bressem et al., 2022). This trend aligns with the observed immune evasion strategies in Cetacean poxviruses, similar to what has been documented in other poxviruses (Shisler, 2015; Van Bressem et al., 2018; Yousif & Al-Naeem, 2012). Additionally, factors such as limited immune surveillance in the epidermis and the presence of at least five genes predicted to encode immune evasion proteins (see section “genome of Cetacean poxviruses”), inhibiting the host's innate immune response in the viral genome may contribute to the prolonged presence of Cetacean poxviruses (Rodrigues et al., 2020). The variations in immune fitness could also account for differences in severity and persistence observed among cetacean populations and communities (Van Bressem et al., 2022).



Figure 2. Macroscopic image of characteristic tattoo skin disease lesions (Van Bressem, unpublished work).

3.4. Distribution of Tattoo skin disease on the body

Tattoo skin lesions have been observed to occur across the entire body of cetaceans, while

photographic assessment often is biased towards dorsal body parts that break the waters surface more often (Powell et al., 2018). In Burmeister's (*Phocoena spinipinnis*) and harbor porpoises (*Phocoena phocoena*), tattoo skin lesions are more commonly found on the head, though they can also affect other areas like the trunk, flippers, and peduncle (Van Bresseem et al., 2009; Van Bresseem et al., 2022). In a specific case of a juvenile male porpoise with poor body condition, severe nephrolithiasis, and parasitic pneumonia, generalized skin lesions were observed throughout the body, suggesting severe immunodeficiency (Van Bresseem et al., 2009).

3.5. Lesion size and number of Tattoo skin disease on the body

Lesion size varies from very small (e.g. 5 mm or 12 x 8 mm) to very large (e.g. 225 x 225 mm or > 750 mm) (Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2018). Adult small cetaceans in the southeastern Pacific, northeastern Atlantic, and Portugal's Sado Estuary have been observed to display very large tattoo skin lesions, reminiscent of “progressive vaccinia”, a serious complication of smallpox vaccination in humans with immunological deficiencies (Van Bresseem et al., 2003; Van Bresseem et al., 2009). This suggests an impaired immune response, with an instance of pneumonia diagnosed in affected adult Burmeister's porpoise (*Phocoena spinipinnis*) from central Chile (Van Bresseem et al., 2009). The persistent and extensive nature of very large tattoo skin lesions over three years in adult bottlenose dolphins (*Tursiops truncatus*) from the Sado Estuary in Portugal further implies underlying immune deficiencies (Van Bresseem et al., 2003).

Tattoo skin lesion number per individual was observed to range from 1 to more than 50 in various odontocete species (Van Bresseem et al., 2009).

3.6. Histopathology of Tattoo skin disease

3.6.1. Cetacean skin anatomy. This section aims to provide an overview over the skin anatomy of cetaceans (see Figure 1 in Mouton & Botha, 2012), to better understand the subsequent section about histopathological differences the skin faces with poxviral infection.

The skin of cetaceans can be distinguished into epidermis, dermis and hypodermis (Geraci et al., 1979; Parry, 1949). The epidermis, the outermost layer of the skin, is remarkably thicker compared to that of other mammals (Mouton & Botha, 2012). To put it in perspective, it surpasses human epidermal thickness by 15 to 20 times (Mouton & Botha, 2012). It is further divided into the more superficial stratum externum and the deeper stratum germinativum

(Geraci et al., 1979; Parry, 1949). The stratum externum is often termed the parakeratotic layer (Geraci et al., 1979; Mouton & Botha, 2012). It consists of stratified squamous epithelium of moderately flattened cells with retained elongated nuclei and prominent organelles, including mitochondria, indicative of a form of parakeratosis (Geraci et al., 1979; Mouton & Botha, 2012; Spearman, 1972). This cornified layer likely plays a role in waterproofing the skin of these mammals (Mouton & Botha, 2012; Spearman, 1972): approximately 95% of the epidermis constitutes of Lipokeratinocytes - scleroproteins responsible for mechanical support in epithelial cells, producing both keratin and lipid droplets (Menon et al., 1986; Mouton & Botha, 2012; Schweizer et al., 2006; Zabka & Romano, 2003). These macromolecules are mechanically hard, chemically unreactive, insoluble, fibrous, and exceptionally tough due to numerous disulfide cross linkages (Mouton & Botha, 2012; Sharma & Rajak, 2003). Lipids enhance the ability of Lipokeratinocytes to act as a physical barrier in an hypertonic environment and contribute to the unique buoyancy, streamlining, insulation, and caloric characteristics of cetacean skin (Menon et al., 1986; Mouton & Botha, 2012). This physical barrier serves as the first line of defense against adverse environmental conditions such as heat, radiation, water loss, and pathogen penetration (Mouton & Botha, 2012). Using bottlenose dolphins (*Tursiops truncatus*) as model, it was found that the epidermal layer of cetaceans has a large capacity for cell population and a long turn over time, accompanied by rapid sloughing (Hicks et al., 1985; Mouton & Botha, 2012). These characteristics account for the unusual thickness, as well as the smooth surface of the skin, thereby enhancing the barrier properties and ability to limit attachment by microbes (Geraci et al., 1979; Hicks et al., 1985; Mouton & Botha, 2012). The stratum germinativum of the epidermis consists of deeper cylindrical cells (Mouton & Botha, 2012; Parry, 1949).

The dermis lies beneath the epidermis and is a connective tissue layer that provides structural support and facilitating sensory perception. The dermis consists of white fibers and features rete pegs (“dermal papillary ridges”) interdigitating with epidermal papillae (Mouton & Botha, 2012; Parry, 1949). This layer is highly innervated particularly in areas like the head, blowhole, perianal, and genital slit (Cozzi et al., 2016). Hair follicles, except for a few neonatal rostral vibrissae, and adnexal glands, besides the mammary glands, are absent (Cozzi et al., 2016).

One of the most distinctive features of dolphin skin is the presence of a thick layer of subcutaneous fat, the lipid-rich hypodermis, also known as blubber (Mouton & Botha, 2012; Struntz et al., 2004). The thick and fatty hypodermis merges into the dermis (Mouton & Botha, 2012), serving multiple purposes including thermoregulation, buoyancy control, and

metabolic energy storage (Struntz et al., 2004). The blubber also contributes to the smooth surface of the skin, improving streamlining and locomotion through reducing drag in the water (Struntz et al., 2004).

Histopathology. The histopathological aspects of tattoo skin lesions show variation but typically demonstrate some distinctive characteristics (Geraci et al., 1979; Segura-Göthlin et al., 2023): the stratum externum displays hyperkeratosis, along with ballooning degeneration characterized by the formation of intracellular edema with large inflated cells (see Figure 2 in Cocumelli et al., 2018) (Geraci et al., 1979; Segura-Göthlin et al., 2023; Van Bresseem et al., 2022). Hyperkeratosis manifests as increased keratinization, characterized by a multiplication in cell layers with marked compression, which results in downward thickening (Segura-Göthlin et al., 2023). It is typically associated with mild focal hyperpigmentation (Segura-Göthlin et al., 2023). In more chronic lesions, there is focal pitting and disruption of the surface layer, allowing entry of bacteria, fungi, protozoa and other opportunists. In these, there can be focally extensive necrosis in the stratum spinosum and externum with more marked leukocytic infiltration (Duignan et al., 2018; Geraci et al., 1979; Van Bresseem et al., 2022). Coinfection with herpesvirus can display a combination of typical histological changes occurring for each of the infections, including diffuse acanthosis and multifocal ballooning degeneration with associated hyperkeratosis (Segura-Göthlin et al., 2023). Lesions with herpesvirus coinfections commonly lack the typical umbrella-like arrangement in intracytoplasmic inclusion bodies (Segura-Göthlin et al., 2023).

The stratum spinosum shows thickening (acanthosis) as well as small eosinophilic intracytoplasmic inclusion bodies which house the poxvirus particles within vacuolized keratinocytes (see figure 3 in Van Bresseem et al., 2022) (Geraci et al., 1979; Van Bresseem et al., 2022). Vacuolized cells are concentrated in multiple focal points at the dermal-epidermal junction and around superficial dermal blood vessels or form linear columns (Segura-Göthlin et al., 2023; Van Bresseem et al., 2022). On rare occasions, these linear columns extend laterally, creating multifocal cones (Segura-Göthlin et al., 2023). In higher resolution under the transmission microscope, the individual virions, recognizable by their characteristic dumbbell-shaped core, can be seen in cells of the zone between the stratum spinosum and the compact periphery (Duignan et al., 2018; Flom & Houk, 1979; Geraci et al., 1979; Van Bresseem et al., 2022). Occasionally, the inflammation also includes some macrophages, eosinophils and neutrophils (Van Bresseem et al., 2022).

Uninfected epidermal tissue near the lesion can exhibit compression and a displacement of cell orientation (Geraci et al., 1979).

3.7. Latent infections

Viral infections can proceed acute, persistent or latent (Schulz, 2020). Acute viral infections have a rapid onset and short duration, often leading to recovery with immunity (Schulz, 2020). Persistent infections last longer, with continuous low-level replication, while latent infections involve periods of dormancy followed by occasional reactivation and symptoms (Schulz, 2020). Poxvirus-infections are often acute and can be either localized or systemic, with outcomes ranging from inapparent and non-lethal to highly lethal with a swift onset (Mark et al., 1991). For instance, ectromelia virus infection in laboratory mice can manifest as systemic with no apparent symptoms, low mortality, and minimal morbidity, or as highly lethal with death occurring within ten days (Esteban & Buller, 2005; Mark et al., 1991). On the other hand, molluscum contagiosum virus tends to replicate only in the stratum spinosum of the human epidermis, without significant involvement of the dermis, and typically does not spread systemically from the initial site of infection (Leung et al., 2017).

Tattoo skin disease can last weeks to years, with continuous replication in the skin. It has been observed to express in an holoendemic pattern with a decrease in prevalence in mature animals, presumably following the development of acquired immunity (Powell et al., 2018; Van Bresseem et al., 2003, 2009; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022). Therefore, infection is assumed to be acute or persistent and nonlethal (Van Bresseem et al., 2009). However, an unexpected discovery emerged recently, as molecular analyses unveiled that bottlenose dolphins (*Tursiops truncatus*) in captivity were tested positive for Cetacean poxvirus (Ce-PV 1) without any of the individuals in the facility showing macroscopically visible lesions of Tattoo skin disease (Segura-Göthlin et al., 2023). This discovery challenged previous assumptions and raised questions about the pathogenesis of the virus, suggesting that individuals without clinical signs of the disease may still harbor the virus, which is, it may manifest as latent infection. Additionally, Tattoo skin disease can recur, likely when the immune system is compromised, either because the virus was repeatedly transmitted or it was never fully removed from the individual's organism (Mouton & Botha, 2012; Van Bresseem et al., 2009). Genetic similarity of CePV-1 sequences obtained from both tattoo-like skin lesions and healthy skin samples suggests a potential persistence of the virus through generations among captive cetaceans since the introduction of wild-caught individuals in the 90s (Segura-Göthlin et al., 2023). The study explores the possibility of subclinical infections, where the virus may be present or even proliferating without causing visible symptoms. The low viral loads found in these samples could be a reason why the skin disease did not develop and a

route of entry through damaged skin may have been necessary for the development of the lesion (Segura-Göthlin et al., 2023). However, false-positive data was suggested through contamination due to circulating cell-free DNA brought from the environment or presence of viral particles in the water interfering with the sample, and therefore is still not proven (Opota et al., 2015; Segura-Göthlin et al., 2023).

4. Epidemiology: observations at population level

4.1. Prevalence and severity of Tattoo skin disease

Prevalences of Tattoo skin disease in several cetacean populations vary widely from 3.6% (*Delphinus delphis*; central coast of Ecuador) to 62.3% (*Phocoena spinipinnis*; central coast of Peru) (Van Bresseem et al., 2009; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022). Differences in prevalence and severity in studies and of the disease may occur due to various factors such as differences in study methodology, population structure and demographics, general health condition, as well as environmental conditions and anthropogenic threats - possibly causing physiological stress and in turn leading to a compromised immune system - that will be discussed in subsequent sections (Cocumelli et al., 2018; Croft et al., 2020; Fury & Reif, 2012; Hall et al., 2006; Hart et al., 2012; Powell et al., 2018; Segura-Göthlin et al., 2023; Van Bresseem et al., 2009; Van Bresseem et al., 2022). In a study exploring the occurrence of Tattoo skin disease in various cetacean species across oceanic, coastal, and estuarine settings, no distinct trends were found in relation to geography or host phylogeny (Van Bresseem et al., 2009).

4.2. Demographic factors

4.2.1. Age. An holoendemic pattern of Tattoo skin disease or tattoo-like skin lesions has been observed in several cases: although the infection is ubiquitous, prevalence of Tattoo skin disease varies with age, peaking in juveniles compared to calves, likely because of the gradual loss of maternal immunity and increased contacts with infected pod members (Powell et al., 2018; Van Bresseem et al., 2003; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022). Prevalence generally decreases in mature animals, presumably following the development of acquired immunity (Powell et al., 2018; Van Bresseem et al., 2003, 2009; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022).

In free-ranging Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) (N = 1700) from Shark Bay, the average age of an individual with tattoo skin lesions was 26.6 months and prevalence of the disease in adults was very low (0.33%, N = 303, (Powell et al., 2018). They found that yearlings (1-2 years) had a higher incidence of these lesions compared to younger and older calves (Powell et al., 2018). It was considered uncertain whether the observed age pattern during the calf period is linked to disease exposure or the development of the immune system (Powell et al., 2018). The prevalence was low in calves below one year, possibly due to maternal immunity, and in individuals over two years, suggesting acquired immunity (N = 68 calves, 4 juveniles, 3 adults) (Langer, 2009; Powell et al., 2018; Van Bresseem et al., 2003; Van Bresseem & Van Waerebeek, 1996). This pattern aligned with findings in other small cetaceans (Powell et al., 2018).

Another study investigated Tattoo skin disease in 17 odontocetes species (N = 1392; free-ranging or dead) mainly through photographs (and obduction of dead animals). Three age classes were categorized: calves (up to 6-9 months), juveniles (older calves, juveniles, and subadults), and adults (sexually mature animals). Tattoo skin disease was not found in calves (except for in one striped dolphin specimen from the Mediterranean Sea) (Van Bresseem & Van Waerebeek, 1996). Interestingly, juveniles showed a higher Tattoo skin disease probability than adults in most samples, except for cetaceans (*Delphinus delphis* and *Phocoena phocoena*) in poor health from the British Isles and Chilean dolphin (*Cephalorhynchus eutropia*) from southern Chile. Adults might have developed active immunity, except in instances of poor health, where the usual pattern was inverted, possibly indicating a weakened immune system (Van Bresseem et al., 2009). An inverted holoendemic pattern could therefore be a hint to poor health of a population (Van Bresseem et al., 2009).

The prevalence of Tattoo skin disease in 257 captive common bottlenose dolphins varied among different age groups, with the highest occurrence (23.1%) among 4- to 8-year-old females, though this difference was not statistically significant (Van Bresseem et al., 2018). Male dolphins showed high prevalence across all age classes (Van Bresseem et al., 2018). The youngest dolphins with Tattoo skin disease were 14- and 15-month-old females born in the Netherlands, suggesting potential maternal immunity in younger individuals (Van Bresseem et al., 2018). Adults, ranging from 9 to 29 years, exhibited large lesions, with the median tattoo skin lesion size increasing in adults from year 9/10 onwards (Van Bresseem et al., 2018).

4.2.2. Sex. In most study cases, Tattoo skin disease prevalence and severity was observed to affect both sexes equally. There have been three cases, that showed a prevalence of Tattoo skin

disease two to three times higher in males than females, with increased severity: in Burmeister's Porpoises (*Phocoena spinipinnis*) off Peru, common bottlenose dolphins (*Tursiops truncatus*) in captivity, and non-migratory Humpback Whales (*Megaptera novaeangliae*) from the Arabian Sea of Oman (with less significance), sexual variation in susceptibility to Tattoo skin disease has only been observed. Differences between males and females may be explained due to different hormonal and behavioral responses as well as increased toxin levels. In males, changes in testosterone levels due to sexual maturation, reproduction, and seasonal changes can lead to increased stress levels in males through competition and aggressive interactions among males for access to females (cutaneous abrasions male-male interactions) (Connor & Smolker, 1995; Van Bresseem et al., 2015; Yamamoto et al., 2015). Increased stress as well as disruption of the skin's integrity due to increased number of rake marks compared to females may lead to increased prevalence and severity of infection of Tattoo skin disease. This may also explain male-female differences in captive dolphins where stress-related behaviors may increase in males. Additionally, adult females showed significantly lower $\Sigma 25\text{CBs}$ (Sum of 25 Chlorinated Biphenyl Congeners) levels than adult males in a study which is explained by female off-loading of organochlorines during gestation and lactation (Jepson et al. 2005). Other toxins might also alter hormonal and therefore behavioral changes that increase aggression or may favor immunosuppression in males. Differences in social organization of male and female porpoises and, from there, risk of infection, could also play a role (Van Bresseem & Van Waerebeek, 1996) as well as habitat separation between males and females with habitat dominance of males (Van Bresseem et al., 2015).

Male cetaceans have been known to show higher aggression than females, resulting in more tooth rake marks than females. This may lead to increased susceptibility to the disease.

4.3. Environmental factors

4.3.1. Salinity. Tattoo skin disease does not seem to be linked to salinity levels (Hart et al., 2012; Van Bresseem et al., 2009; Van Bresseem et al., 2022). The disease occurs in cetacean species inhabiting environments with varying salinity levels, although it has to be noted that to date, freshwater dolphins have not been documented with the disease (Van Bresseem et al., 2022). Nevertheless, in two estuarine populations of bottlenose dolphins (*Tursiops aduncus*) in New South Wales (northeast Australia), ten new cases of tattoo like skin lesions were observed in a total of 46 individuals after flood events in 2004 and 2005, where no dolphin poxvirus-like lesions had been observed before (Fury & Reif, 2012). Flood events in the study

area (Clarence and Richmond River) led to high input of freshwater and sediments resulting in lower salinity, pH and dissolved oxygen levels, increased turbidity, and changes in water temperature (Fury & Reif, 2012). Dramatic decline in salinity in the study area was suggested to create physiological stress and increased incidence of poxvirus infections (Fury & Reif, 2012). However, the complexity arises from the various changing parameters, making it difficult to determine whether the salinity decline alone, or other factors contributed to the onset of Tattoo skin disease. Additionally, sample size was small, and data was not entirely consistent in that the latent period between the flood event and the appearance of lesions was variable and following floods in the Richmond River were not followed by cases of Tattoo skin disease (Fury & Reif, 2012). While salinity might indirectly play a role in overall environmental health, it is likely not a direct determinant of Tattoo skin disease. Drastic environmental changes like flood events yet may contribute to the development of Tattoo skin disease (Fury & Reif, 2012).

4.3.2. Water temperature. High prevalence of Tattoo skin disease above 20% has been reported in different temperature zones ranging from 12.1°C to 25.8°C, suggesting that its distribution is not solely dependent on water temperature (Van Bresseem et al., 2022). But changes from higher to lower temperatures had been observed to favor occurrence of Tattoo skin disease (Croft et al., 2020; Hart et al., 2012). For instance, a study using photo-identification in three bottlenose dolphin (*T. truncatus*) communities along the southeastern United States coast showed that water temperature was significantly associated with the occurrence of dark-fringed, tattoo-like and vesicular lesion types (Hart et al., 2012). The regression models for these lesions indicated a corresponding 12%, 5%, and 16% increase in the likelihood of occurrence for each one-degree Celsius decrease in water temperature (Hart et al., 2012). It was discussed that the viability of pathogens and the susceptibility of dolphins might increase when water temperatures fall within a specific range. One result was not consistent though: lowest skin lesion occurrence was observed for warmer months October and July, but April had higher skin lesion occurrence than February, although February was considered the coldest month. This indicated that skin lesion appearance may not solely be influenced by water temperature or that there's a possibility of a delay between exposure to cold water and the actual appearance of clinical signs of disease (Hart et al., 2012). In another study in 25 captive bottlenose dolphins (*Tursiops truncatus*), the effect of water temperature on the extent of tattoo skin lesions was explored (Croft et al., 2020). Increased water temperatures from 21-24°C to 25.5-26.5 °C led to significant decrease in extent and appearance of tattoo skin lesions after two to three weeks and to complete resolution after five to six weeks in 16 dolphins (Croft et al., 2020). In one facility with 3 individuals, water temperature was decreased back to previous temperatures from 26.1°C

to 21°C which led to recurrence and significant increase in extent of tattoo skin lesions in all dolphins after 4-5 weeks (Croft et al., 2020).

4.4. Anthropogenic factors

4.4.1. PCBs and other immunotoxic contaminants. Chemicals of anthropogenic origin like halogenated organic compounds (HOCs) are carried across the atmosphere and along rivers and streams, ultimately reaching the coastal and open oceans worldwide (Chen et al., 2023). Cetaceans, being at the top of the marine food chain, can accumulate high concentrations of these chemical pollutants mainly in their blubber, through the consumption of contaminated prey (Cossaboon et al., 2019; Mackintosh et al., 2016; Shaul et al., 2015; Van Bresseem et al., 2022). Concentration and distribution of these pollutants differ among communities of the same species residing in various ocean provinces, resulting in varied exposure levels: coastal populations are likely to harbor elevated levels of chemical pollutants in their blubber (Cossaboon et al., 2019; Mackintosh et al., 2016; Shaul et al., 2015; Van Bresseem et al., 2022) and especially the western Mediterranean Sea and south-west Iberian Peninsula, are global polychlorinated biphenyl (PCB)-hotspots for marine mammals (Jepson et al., 2016). Exposure to HOCs, including PCBs, dichloro-diphenyl-trichloroethane (DDT), and its derivatives, can cause immune suppression and reproductive function impairment in Cetaceans due to being potent endocrine disruptors (Cocumelli et al., 2018; Fossi et al., 2003; Hammond et al., 2005). Immune suppression caused by these contaminants have been linked to increased susceptibility of skin diseases or an exacerbation of severity of clinical signs of skin lesions (De Guise et al., 1995; Levin et al., 2007; Maldini et al., 2010). For example, in common bottlenose dolphins (*Tursiops truncatus*), elevated levels of HOCs in the blubber are linked to heightened vulnerability to lobomycosis (laccariosis), a fungal disease triggered by *Paracoccidioides ceti*, and may also be linked to increasing Tattoo skin disease-susceptibility (Reif et al., 2009; Van Bresseem et al., 2022; Vilela et al., 2021). For instance, harbor porpoises (*Phocoena phocoena*) observed in poor health condition around the British Isles displayed high PCB levels (Hall et al., 2006; Jepson et al., 2005), along with high prevalence of Tattoo skin disease and numerous, sometimes large, tattoo skin lesions (Van Bresseem et al., 2009; Van Bresseem et al., 2022). Similarly, two stranded striped dolphins (*Stenella coeruleoalba*) with Tattoo skin disease of poxviral etiology in the Mediterranean Sea, Italy, showed elevated levels of PCBs and DDTs in their blubber, liver and skeletal muscle. One dolphin exhibited substantial lesions of around 200 mm, hinting at a potential involvement of these contaminants in the progression and severity of the disease (Cocumelli et al., 2018; Van

Bressemer et al., 2022). However, more research is required to explore the connection between immunotoxic contaminants, disease occurrence, and its severity (Van Bressemer et al., 2022).

4.4.2. Others. An increase in the prevalence and severity of Tattoo skin disease or demographic shifts may indicate compromised immune function, which may be linked to environmental degradation of anthropogenic cause (Maldini et al., 2010; Powell et al., 2018; Van Bressemer & Van Waerebeek, 1996; Wilson et al., 1999). For instance, unexpected, elevated prevalence of Tattoo skin disease was documented in a study on free-ranging Dusky dolphins (*Lagenorhynchus obscurus*) and Burmeister's porpoise (*Phocoena spinipinnis*) from Peru (Van Bressemer et al., 2009; Van Bressemer et al., 2022). These findings were observed during a period of extensive fisheries activities, resulting in the direct and indirect mortality of thousands of individuals (Van Bressemer et al., 2022; Van Waerebeek et al., 1997). Additionally, a recent study by Van Bressemer et al. (2023) showed prevalence of Tattoo skin disease of 43.6% in 55 porpoises from the eastern North Pacific, similar to previous studies in net entangled Burmeister's porpoises (*P. spinipinnis*) in poor health condition off Peru (Van Bressemer & Van Waerebeek, 1996). The factors contributing to the elevated prevalence in porpoises in the eastern North Pacific remain unclear. However, potential explanations could be linked to stressors associated with their residence in and around San Francisco, the second busiest shipping and boating harbor on the US west coast. These habitats are characterized by high levels of contaminants, and other human interactions, such as with fisheries, as well as inter-species aggression from common bottlenose dolphins, may also play a role (Cope et al., 2021; Sutton et al., 2019; Van Bressemer et al., 2022; Wilkin, 2012; Wu et al., 2017).

The complex interplay of these and other anthropogenic factors, such as climate change and habitat degradation, may create conditions (contribution to injuries (e.g. net entanglement), increased stress (e.g. disruption of habitat, lack of food availability), and compromised immune systems) conducive to the emergence and spread of Tattoo skin disease among cetacean populations, that need to be further explored (Van Bressemer et al., 2015).

4.5. Social and behavioral factors

Social interactions can lead to infectious disease transmissions (Altizer et al., 2003; Powell et al., 2020). In a 34-year longitudinal study on Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) in Shark Bay, Australia, correlation between network structure and disease status for Cetacean poxvirus was investigated (Powell et al., 2020). Number of unique associates of each individual was calculated in the first year of life for individuals that had Tattoo skin disease

during their second year of life and showed no significant association with disease status (Powell et al., 2020). Time spent socializing and time spent in groups did also not differ by disease status (Powell et al., 2020). Therefore, these behavioral factors do not seem to influence disease status. However, individuals with Tattoo skin disease showed a significant higher proportion of symptomatic associates in the first year of life than individuals that did not develop Tattoo skin disease, meaning the proportion of symptomatic associates was significantly associated with Tattoo skin disease status (Powell et al., 2020). Average group size in Shark Bay bottlenose dolphins is small (2.7 adults), but perhaps there is a “tipping point” in group size such that time spent in large groups is positively associated with Tattoo skin disease infection status, whereas being in smaller groups is not (Powell et al., 2020), which should be further investigated.

Increased susceptibility to infection of Tattoo skin disease is likely caused by disruptions of the skin’s integrity, favoring viral entry (Mark et al., 1991; McInnes et al., 2023; Powell et al., 2018; Segura-Göthlin et al., 2023; Van Bresseem et al., 2018). Susceptibility to Cetacean poxvirus, leading to Tattoo skin disease, has been associated with tooth rake marks resulting from intra- or inter-species social interactions, suggesting potential transmission through bites or facilitated by damaged skin (Segura-Göthlin et al., 2023; Van Bresseem et al., 2018). Tooth rake marks serve as indirect measure of conspecific aggression encounters (Scott et al., 2005). Therefore, aggressive behavior may be one factor contributing to the occurrence of Tattoo skin disease. Aggressive social behavior in turn can be altered due to various changing social dynamics and environmental factors. These include changes in social composition and group size, seasonal mating and sexual selection, territorial disputes and competition for resources - especially in areas with limited resources -, maternal protection, or environmental changes that impact social dynamics such as human-induced disturbances. But also, other behavioral causes of skin abrasion or small injuries, such as coral rubbing or preying between mussel farms may facilitate Tattoo skin disease transmission.

In summary, Tattoo skin disease is likely to be influenced by socio-behavioral factors, such as prior contact with Tattoo skin disease-positive individuals and aggressive behavior, increasing susceptibility to disease through stress and disruption of the skin through tooth rake marks (Powell et al., 2020). Alterations in aggressive behavior may in turn be linked to several other social and environmental factors.

4.6. Methodology aspects

Methodology varies between studies, making direct comparison of prevalence difficult. Methods to identify lesions have relied on photo-identification, stranding, by-catch and molecular data (Hart et al., 2012). While photo-identification allows an estimate of the prevalence of free-ranging populations, factors such as photo quality and percent body coverage in the photographs may play a crucial role in the detection of tattoo-like skin lesions (Toms et al., 2020). Other studies analyze carcasses of stranded or bycatch animals in the laboratory, and thus have the possibility of examination of lesions on the entire body, depending on the condition of the carcasses (Barnett et al., 2015; Cocumelli et al., 2018). Only molecular analyses and electron microscopy offer a reliable tool to confirm Cetacean poxviruses, whereas other methods target observation of tattoo-like skin lesions without final confirmation of Cetacean poxviruses (Blacklaws et al., 2013; Cocumelli et al., 2018; Toms et al., 2020). Cetacean poxvirus-positive individuals without visible clinical pathology therefore are not considered in most studies.

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PART 2: Manuscript

First evidence of sex-specific epidemiological differences in tattoo-like skin lesions among free-ranging bottlenose dolphins (*Tursiops truncatus*)

ABSTRACT: The skin of cetaceans is a multifaceted feature that offers a wealth of visible information. Epidermal lesions from trauma or disease are common, but the emergence, severity, and changes in demographic pattern of certain skin diseases may serve as a bioindicator for environmental change and wellbeing of a population. This study investigates tattoo skin disease-like lesions (TSL) in a bisexually philopatric coastal bottlenose dolphin (*Tursiops truncatus*) population, reporting sex-specific differences and their potential environmental implications. Data was collected in 2017 and 2018 during 145 daily boat-based surveys in Ría de Arousa-bay (NW Spain). The subsequent analysis of 10,409 photographs of 48 females and 51 males revealed an overall prevalence of 26.2%. Notably, males had a significantly higher prevalence (37.5%) compared to females (15.7%), along with increased lesion quantity in 2018. Minimum persistence time (MPT) of TSL ranged from 2.4 to 19.6 months (n=15), with males at a median of 407 days and females at 347 days. These sex-specific differences could be attributed to increased aggressive behavior and social stress but also to the very high polychlorinated biphenyl (PCB) concentrations in the area. The study highlights the importance of correlating sex disparities and environmental factors in future research on cetacean skin diseases.

Keywords: Tattoo skin disease; Poxviruses; Bottlenose dolphins; Epidemiology; Health status; Skin lesions

1. INTRODUCTION

The skin of cetaceans serves as both a direct interface with their environment and a barrier, making it a multifaceted source of information for research across various scientific disciplines (Segura-Göthlin et al., 2021). Any alterations of the skin, from physical injuries to pathogenic diseases, can inform about the health and wellbeing of these marine mammals but also aids as bioindicator for environmental change, providing insights into the ecological health of marine

ecosystems (Chan & Karczmarski, 2019; Maldini et al., 2010; Powell et al., 2018; Van Bresseem et al., 2009).

Tattoo skin disease (TSD), a dermatopathy caused by Cetacean poxviruses (CePVs), stands as one of the most frequently observed skin disease in cetaceans (Segura-Göthlin et al., 2021). It appears as characteristic, macroscopically visible, dark lesions with a stippled pattern and dark irregular borders, making it an identifiable study target in free-ranging populations (Geraci et al., 1979; Van Bresseem et al., 2003). Rarely lethal, TSD can still affect individuals for months or even years (Van Bresseem et al., 2009). Its emergence, severity and changes in demographic pattern may indicate compromised immune function, making it a potential bioindicator for both environmental shifts and anthropogenic influences, impacting the overall wellbeing of a population (Maldini et al., 2010; Powell et al., 2018; Van Bresseem & Van Waerebeek, 1996; Wilson et al., 1999). In essence, investigating tattoo skin disease dynamics has potential to provide valuable data for marine conservation and management efforts.

Prevalence of TSD varies widely between populations and characteristically expresses in an holoendemic pattern, with a peak of prevalence in juveniles, that is likely associated with the gradual loss of maternal immunity and increased contacts with infected pod members (Powell et al., 2018; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022). In mature animals prevalence generally decreases, indicating the development of acquired immunity (Powell et al., 2018; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2022). However, in cases of poor health such as starvation, other infectious and parasitic diseases, a reversal of the usual pattern is observed with more adults being affected, that may be attributed to compromised immune function (Hall et al., 2006; Jepson et al., 2005; Van Bresseem et al., 2009). Sexes are usually affected equally, but instances expressed a reversed

pattern, with males being significantly more affected than females (Van Bresseem et al., 2009a; Van Bresseem et al., 2022; Van Bresseem & Van Waerebeek, 1996). Differences are accompanied by relatively high prevalence and may be associated with high levels of contaminants and other human interactions, such as extensive fisheries activities resulting in the direct and indirect mortality of thousands of individuals, as well as inter-species aggression, therefore indicating a highly stressful environment (Cope et al., 2021; Sutton et al., 2019; Van Bresseem et al., 2022; Van Waerebeek et al., 1997; Wilkin, 2012; Wu et al., 2017). Contaminants such as polychlorinated biphenyls (PCBs) have immunosuppressive effects, increasing susceptibility to infectious diseases (Jepson et al., 2016). Males have been found with higher PCB-levels than females which is explained by female off-loading of organochlorines during gestation and lactation, therefore increasingly being exposed to immunotoxic risk (Jepson et al., 2005). The investigation of these patterns may contribute to the understanding of the well-being of cetacean populations and may as well serve as bioindicator: an increase in prevalence, severity and changes in demographic pattern may signal broader environmental changes that need to be identified in order to establish protective management and mitigation strategies.

Epidemiological research in cetaceans encompasses several methodological approaches: Field surveys, necropsies, and health assessments in free-ranging and captive animals provide insights into disease prevalence, causes of mortality, and overall health (Pfeiffer, 2010; Van Bresseem et al., 2009; Van Bresseem & Van Waerebeek, 1996). Serological studies and molecular techniques detect antibodies against a pathogen (Blacklaws et al., 2013; Segura-Göthlin et al., 2023), while electron microscopy permits to visualize the virus (Blacklaws et al., 2013). Satellite telemetry and tagging track cetacean movements (Mate et al., 2011; Van Weelden et al., 2021), while surveillance of environmental factors aid to study correlations with disease prevalence (Van Bresseem et al., 2009; Van Bresseem et al., 2009; Van Bresseem,

Van Waerebeek, et al., 2015). Non-invasive approaches in studying free-ranging cetaceans are essential for ethical research, minimizing disturbance to natural behaviors, and ensuring the well-being of individuals (Papastavrou & Ryan, 2023; Toms et al., 2020; Van Bressemer et al., 2022). Hereby, the skin of cetaceans offers a visible insight into several epidemiological aspects of a population without the need of invasive methods (Chan & Karczmarski, 2019; Mouton & Botha, 2012; Van Bressemer et al., 2009).

In this study the aim is to deliver important baseline data in a bisexually philopatric coastal bottlenose dolphin population (*Tursiops truncatus*), resident in the Northeast Atlantic off the coast of northern Spain. We investigate prevalence, severity and minimal persistence time of TSD based on photographic assessment. We refer to assessment of tattoo-like skin lesions (TSL), without the actual confirmation of Cetacean poxvirus (CePV).

2. MATERIALS & METHODS

2.1. Study site

The Bottlenose Dolphin Research Institute (BDRI, www.thebdri.com) is an independent marine research center located in O Grove, Galicia, northern Spain. As part of the Iberian-NW-African upwelling system the area is characterized by being one of the most productive oceanic regions in the world, providing habitat for a diverse (mega-)fauna (Álvarez-Salgado, 2007). The coastline includes four flooded tectonic valleys called the “Rías Baixas” which due to their natural characteristics, are highly suitable for mussel farming, traditionally on characteristic wooden rafts, called the “bateas” (Figueiras et al., 2002). The area therefore is anthropogenically highly affected, which is enhanced due to fisheries and tourism activities (Methion & Díaz López, 2019b). The south-western Iberian Peninsula is considered as global PCB-“hotspot” for marine mammals, markedly exceeding all known marine mammal PCB toxicity thresholds (>40 mg/kg) (Jepson et al., 2016). The study was carried out in the largest of Rías Baixas, named Ría de Arousa (see Figure I), covering an area of approximately 239 km²

(Prego et al., 1999). The Ría de Arousa hosts the Umia-O Grove intertidal complex which is included in the Ramsar Convention in the list of Wetlands of International Importance (Régos, 2011).

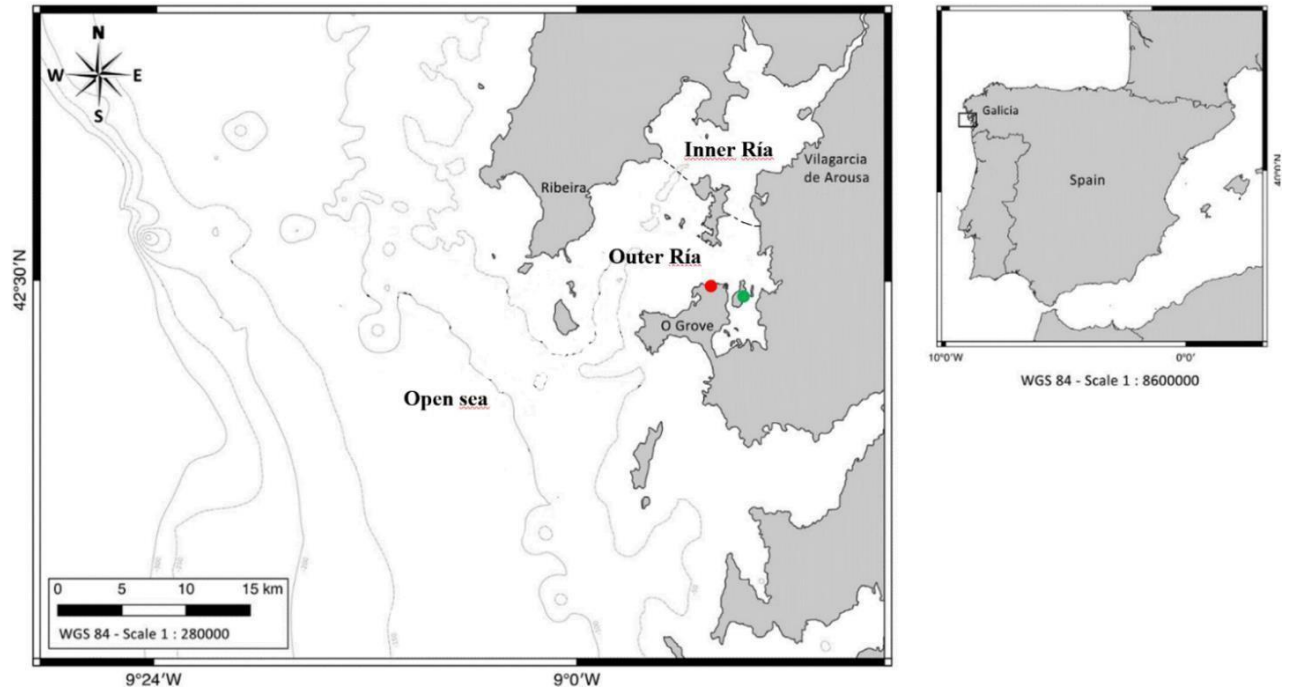


Figure I. Map of the study area Ría de Arousa inlet (divided in Inner and Outer Ría) and open waters. The dots mark the two land-based fieldwork sites in Rons (red) and La Toja (green). For this study, observations were conducted in the inner and outer Ría (by Díaz López et al., 2019; modified).

2.2. Data collection

2.2.1. General field approach. Data collection followed the detailed methodology established by the BDRI, as described in Methion and Díaz López 2018 and Methion and Díaz López 2019. It took place year-round between January 2017 and December 2018 during 145 daily boat-based surveys in Ría de Arousa-bay (NW Spain). Fieldwork was conducted consistently from a 12-meter research vessel powered by two 180-hp inboard engines and from a 5.5-meter rigid hull inflatable powered by a 50-hp outboard engine over the 24-month period and when weather conditions allowed (Methion & Díaz López, 2018). Suitable weather conditions entailed clear visibility without rain or fog and calm sea conditions rating less than 3 on the Douglas sea force scale (Methion & Díaz López, 2019a). Survey routes were prepared and planned previously,

with the aim to fully cover the study area (Methion & Díaz López, 2018). However, variations in the geographic distribution of effort were possible based on prevailing weather conditions (Methion & Díaz López, 2018). The vessels operated at a constant speed of 6 knots ($\sim 11.11 \text{ km h}^{-1}$) with a minimum number of three skilled observers (Methion & Díaz López, 2018). Observers utilized both the naked eye and 10 x 50 binoculars for a comprehensive 360° scan of the sea surface in order to detect common bottlenose dolphins (*Tursiops truncatus*) (Methion & Díaz López, 2018).

2.2.2. Dolphin encounter approach. Upon encounter of *T. truncatus*, efforts were made to minimize disturbance to their behavior (Methion & Díaz López, 2018). This involved a gradual and slow approach of the vessel towards the observed group, that could consist of only a solitary dolphin or any aggregation of *T. truncatus* that exhibited shared behavioral activities and interactions within a 100-meter radius (Methion & Díaz López, 2019a). The encounter endured until reformation of the group, losing the group, or emergence of adverse weather conditions (Methion & Díaz López, 2019a). Each encounter was recorded as each one sighting, with date and subsequent sighting number. Crucial encounter details, encompassing the initial and final times, location (Universal Transverse Mercator, UTM, coordinates), as well as estimations of group size and composition, were documented meanwhile (Methion & Díaz López, 2019a).

Estimations of group size and composition entailed photo-identification, assessment of predominant behavior and classification of sex and age of each individual observed within a group (Methion & Díaz López, 2019a). Photographic documentation for photo-identification purposes aimed to capture both sides of the dorsal fin of each dolphin present, using digital single-lens reflex (SLR) cameras equipped with a 35- to 300-mm telephoto zoom lens (Methion & Díaz López, 2019a). This recording was carried out as far as possible for each dolphin, regardless of age class, degree of markings, or behavior (Methion & Díaz López, 2019a). Photo-identification later supported the final assessment of the group size of each encounter, which

was observed in the field (Díaz López & Shirai, 2008; Methion & Díaz López, 2018). Photographic documentation for skin mark analyses aimed to capture as much body surface possible by serial image recordings of each individual within a group.

Sex and age classification was carried out for each identified dolphin within a group, either by direct observation or by photographic evidence (Methion & Díaz López, 2018). Males were identified based on the observation of an erection, the absence of mammary slits or by the distance of >2.5 cm between genital and anal slits (Methion & Díaz López, 2018). Females were identified based on the observation of mammary slits or the presence of a dependent calf in an infant position to its mother (Methion & Díaz López, 2018). Age classification followed the detailed methodology of Díaz López and Methion 2017, and was divided into “newborn”, “immature” and “adult”. The “newborn” age category was defined as individuals with fetal lines less than 1.5 m in length, swimming in infant position in relation to their mother and expressing uncoordinated surface behavior (Methion & Díaz López, 2018). The “immatures” age category was defined as individuals with two-thirds or less the length of an adult with few rake marks and skin lesions, in close association with an adult but never in infant position (Methion & Díaz López, 2018). The “adults” age category was defined as individuals with darker skin coloration and more than 2.5 m length (Methion & Díaz López, 2018).

The dominant behavior within a group was analyzed following the detailed methodology in Methion and Díaz López 2019, and divided into the four categories of foraging, resting, socializing and travelling.

2.3. Data processing

To process the serial photographs taken during fieldwork, they were transferred on internal computers and sorted by date and dolphin sighting into separate folders. Photographs that did not contain the object (dolphin), were discarded. The remained photographs were used for photo-identification of *T. truncatus*. Photo-identification based on distinct features or markings

primarily on the dorsal fin from high-quality photographs and analysis was carried out only for clearly identified individuals to prevent misidentification (Methion & Díaz López, 2019a; Würsig et al., 1990). Further details can be found in Methion and Díaz López 2018.

For skin mark analyses, each identified dolphin was assigned a separate folder, that included images of the individual divided in folders per date and sighting number. If there were several individuals in one photograph, the image was labelled according to the position of the dolphin. For this study, only sexed individuals had been chosen for TSL analyses. For further dataset refinement at least three best qualitative sightings and at best three sightings per season were chosen in terms of estimated body coverage and photographic quality. Those individuals that did not have a sufficient number of qualitative sightings were discarded. The best sightings were identified as follows: in the first step, those sightings were selected containing photographs with the largest estimated body surface coverage and photographs with good photo quality (see section *Classification: photo quality scoring*). In the second step, the three best sightings in terms of body coverage and photo quality were selected for each season. Seasons had been divided into Spring (April, May, June), Summer (July, August, September), Autumn (October, November, December) and Winter (January, February, March).

2.4. Tattoo-like skin lesions (TSLs)

In summary, 99 Individuals and 10,409 photographs of 48 females and 51 males had been selected for TSL analyses.

2.4.1. Definition of TSLs: In this paper, we refer to TSLs rather than Tattoo skin lesions or Tattoo skin disease, as no electron microscopy or histology was carried out to actually confirm the agent of Tattoo skin disease, Cetacean poxviruses (CePVs). We therefore refer to the term TSL, as the typical and characteristic features of Tattoo skin disease lesions were found, which had been associated with CePVs in several previous studies. Lesions are a "damage", "injury" or "pathological change" and can be the result of chemical and physical trauma or, as

this study case, disease.

2.4.2. Identification. TSLs were identified and counted per individual based on degree of certainty of the skin lesion. They were categorized into i) T^{++} = lesions with a high level of certainty entailing all typical characteristics: dark stippled pattern (numerous small dots or specks) and irregular dark border, dark to yellowish in color and flush with the skin or slightly raised; and ii) T^+ = lesions with characteristic T^{++} features, but not all features listed for T^{++} lesions (see Figure II) (Toms et al., 2020; Van Bressemer et al., 2003). Individuals were considered as positive, if they had at least one T^{++} or T^+ lesion on their body. Following the suggestions of Toms et al. (2020), multiple photos available for the individual and each visible body part per sighting were used for identification to improve rater certainty and reliability. This helped confirm that discolorations were indeed on the animal and not due to external factors like water or glare, particularly when assessing small or light-colored lesions.



Figure II. Example of T^{++} (left) and a T^+ (right) tattoo-like skin lesions on a *Tursiops truncatus*.

2.4.3. Classification: photographic quality scoring for lesion number, distribution and size.

If TSLs were identified, photographs were analyzed according to a modified standardized photo-ID method, to further classify the TSLs in distribution, quantity and size (for

standardized photo-ID method see Urian et al., 2015). This was because the edges of a skin lesion needed to be clearly defined and both angle and focus quality could reduce certainty (Toms et al., 2020). Photographs were therefore scored for quality based on the five criteria focus/clarity; contrast; disturbances (waves, spray, sun reflection); angle and distance of the dolphin to the frame. Each criterion was rated from 0 to 2 resulting (see Table I) in the overall scoring categories discarded (0-2), low (3-4), average (5- 7) and high (8-10). Therefore, only images rating in sum at least 3 were used for analyses. It could happen that due to one of the five categories, the photograph was not usable and discarded, for example when reflection was fully covering the body part with zero visibility.

If a dolphin was found in a sighting with one or more TSLs, the same affected body parts were searched for and examined in all other sightings. This allowed the use of sightings of relatively low photo quality to contribute to the identification of TSLs, as a lesser level of detail was necessary to recognize the TSL.

Table I. Definitions of photo quality scoring categories. The assessment based on the observed body part.

	<i>0</i>	<i>1</i>	<i>2</i>
<i>Focus</i>	Not in focus.	The majority of the observed body part is focused but unsharp.	The majority of the observed body part is in sharp focus.
<i>Contrast</i>	No distinguishable features, either from its environment or within the body. Either too bright or too dark.	Shows features and is distinguishable from the environment, but either dark or bright so that not all details are visible.	The observed body part is well distinguishable from its environment and well illuminated, showing clear details of the body.

Disturbances

Strong disturbances in form of reflection, water spray, waves or other objects covering the majority of the body part.	Some disturbances in form of reflection, water spray, waves or other objects but not covering the majority of the observed body part.	No disturbances on the observed body part.
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<i>Distance of the object to the camera</i>	Dolphins body on less than 1/8 of the photograph.	Dolphins body on more than 1/8 and up to 1/4 of the photograph.	Dolphins body on more than 1/4 of the photograph.
<i>Angle of the object to the camera</i>	45° - 90° angle to the camera.	10° - 45° angle to the camera.	0° (parallel) - 10° to the camera.

2.4.4. Prevalence. The prevalence, determined from the examination of all 99 individuals, was calculated by dividing the number of positive cases (individuals with at least one T++ or T+ lesion) by the total number of individuals examined. This calculation was conducted for both, the two-year period spanning 2017-2018 and each individual year within that period (2017 and 2018).

2.4.5. Minimum persistence time (MPT). MPT was defined as the first and the last date of TSLs observed on an individual during the study period, so the maximum time span that an individual was observed with TSLs with either same lesions or recurrence of new lesions over time (Van Bresseem et al., 2003). It was determined in individuals of which infected area(s) were observed at least in a three month time frame or with evidence of healed TSL. The first and last sighting of TSLs does not imply that an individual was not infected before or after, which is why the minimum persistence time is given.

2.4.6. Classification: Lesion size. To determine approximate size (maximum diameter) for each TSL lesion the qualitative best photo (see section 2.4.5.) per sighting was used. Lesion size was classed following the methodology of Van Bresseem et al. 2015 in small (lesions smaller than half of the eyes diameter, up to 15 mm), medium (diameter less than twice the eyes diameter, 15-50 mm), large (more than twice the eyes diameter and up to half the base length of the dorsal fin, 51-115 mm) and very large (bigger than half the base length of the dorsal fin) lesions. To estimate the size of the lesions, the tool "Dating Measure" was used to measure the proportions of the eye and the dorsal fin, respectively, and to compare them with the measured lesion. This

was always based on the largest possible diameter found for each lesion.

2.4.7. Classification: Lesion distribution. To determine approximate TSL distribution the qualitative best photo per sighting was used. To quantify lesion distribution of TSLs, the dolphins' body was divided into 12 anatomical sections (see Figure III), each defined and proportionally measured with "Dating Measure" tool. The border between upper and lower body sections run horizontally along the mouth line, to the upper base of the pectoral fins, and further to the upper base of the flukes. Vertically, the jaw is limited by a line running downwards at the end of the mouth line. Dorsally, the boundary is formed by the bowed base of the head. The throat section is bounded vertically and dorsally by the upper bases of the pectoral fins. The chest section is bounded vertically by the front flexion of the dorsal fin. The belly section begins after the chest section and is bounded vertically by the color transition from light belly section to dark ventral peduncle section. The following ventral peduncle section runs up to the flukes section, where the tailstock is raised and pointed and is laterally delimited by the flexion of the flukes. The upper sections begin with the jaw section, followed by the head section, of which the posterior border runs vertically around from the upper base of the pectoral fins. The anterior section follows the head section, and is limited posterior by the front flexion of the dorsal fin. The dorsal fin is limited by its front, back and lateral bend. The mid flank section follows the anterior section and is bounded posteriorly by the first back bend of the dorsal fin and runs vertically down up to the belly section. This is followed by the anterior peduncle section and the posterior peduncle section. The anterior peduncle section makes up about 2/3 while the posterior peduncle section makes up relatively 1/3. The posterior peduncle section is delimited posteriorly from the fluke, and is raised and pointed as a tailstock.

Lesions that were located in the transition zone between two body part sections were assigned to only one body part to avoid double counting, with preference to the body part that was already associated with other TSL lesions. In unclear cases several photographs were taken into observation to minimize angle effects.

2.4.8. Classification: Lesion number. Total lesion number per individual was estimated by the maximum number of lesions counted on the body in total (sum of maximum number of lesions per body part) during a 3-month timeframe. The counting of the lesions required clear demarcations: Very small dark and irregular lesions were often found in close proximity to TSLs. However, these were not counted as they were not considered in detail and therefore did not meet all of the criteria for a T⁺⁺ or T⁺ TSLs. Often, also fusions of TSLs were found, that was when each herd was tried to differentiate and counted each, giving approximate minimum quantification.

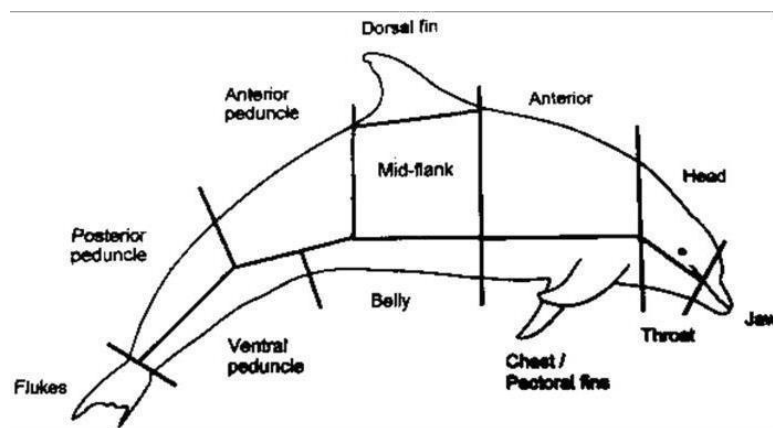


Figure III. Schematic illustration of the dolphins' (*Tursiops truncatus*) body in 12 distinct sections for assessment of tattoo-like skin lesion distribution (by Scott et al., 2005, modified).

2.5. Statistical analyses.

Statistical analysis was carried out using R program (R Core Team, 2022). Prevalence comparison between sexes and years was carried out through Pearson's Chi-squared test with Yates' continuity correction. Comparison of minimum persistence time, lesion number, and lesion size category (XL, L, M, S) between sexes and years was carried out using Wilcoxon rank sum test with continuity correction.

2.5.1. Trend. A trend is defined as observable tendency of the data results, but without statistical significance.

3. RESULTS

3.1. Prevalence

The total prevalence of TSLs from 2017 to 2018 was 26.3% ($N_{\text{total}} = 99$; $N_{\text{disease}} = 26$). Males constituted a significantly higher prevalence at 37.5% ($N_{\text{♂}} = 48$, $N_{\text{♂disease}} = 18$) compared to that of females at 15.7% ($N_{\text{♀}} = 51$; $N_{\text{♀disease}} = 8$) (χ^2 with Yates' continuity correction = 5.0017; 1, $N_{\text{total}} = 99$, $p = 0.02532$).

Overall prevalence in 2017 was 32.8% ($N_{\text{total}} = 64$, $N_{\text{disease}} = 21$) and in 2018 22.4% ($N_{\text{total}} = 76$, $N_{\text{disease}} = 17$). The decreasing trend between years did not express as statistically significant (χ^2 with Yates' continuity correction = 1.4246; 1, $N = 140$, $p = 0.2326$). Prevalence in males was 44.1% in 2017 ($N_{\text{♂}} = 34$, $N_{\text{♂disease}} = 15$) and 30.6% in 2018 ($N_{\text{♂}} = 36$, $N_{\text{♂disease}} = 11$) while in females it was 20% in 2017 ($N_{\text{♀}} = 30$; $N_{\text{♀disease}} = 6$) and 15% in 2018 ($N_{\text{♀}} = 40$; $N_{\text{♀disease}} = 6$). Within each year, no statistical difference in prevalence between sexes was found (2017: $\chi^2 = 3.1821$; 1, $N_{\text{total}} = 64$, $p = 0,07445$; 2018: $\chi^2 = 1.8204$; 1, $N_{\text{total}} = 76$, $p = 0.1773$). The two-year period likely accumulated the trend due to an increased sample size.

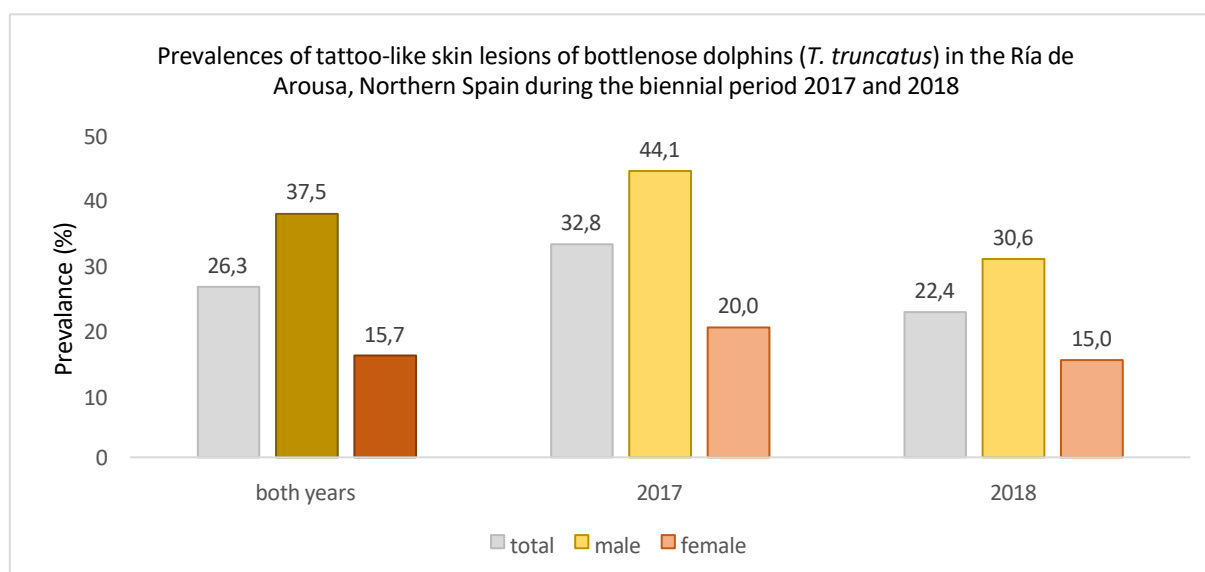


Figure IV. Total and sex-specific prevalence (%) of tattoo-like skin lesions during 2017 and 2018 and for both years 2017-2018 in a northeast Atlantic bottlenose dolphin (*T. truncatus*) population (Ría de Arousa, northern Spain). Darker bars stand for significant differences between males and females.

3.2. Minimum persistence time (MPT)

MPT varied from 2.4 to 19.6 months in 15 dolphins. Figure V illustrates the median MPT of TSLs during the biennial period 2017 to 2018. The overall median MPT is 386 days ($N_{\text{total}} = 15$), with males at a median of 407.5 days ($N_{\text{males}} = 10$) and females with 343 days ($N_{\text{females}} = 5$), with no statistical significance between sexes ($W = 28$; $N = 15$, $p = 0.5135$). Persistence time may be prolonged and need further assessment in previous or subsequent years. Longest MPT was found to be 595 days in a male individual and 514 days in a female individual while shortest MPT was found to be 88 days in a male individual and 73 days in a female individual.

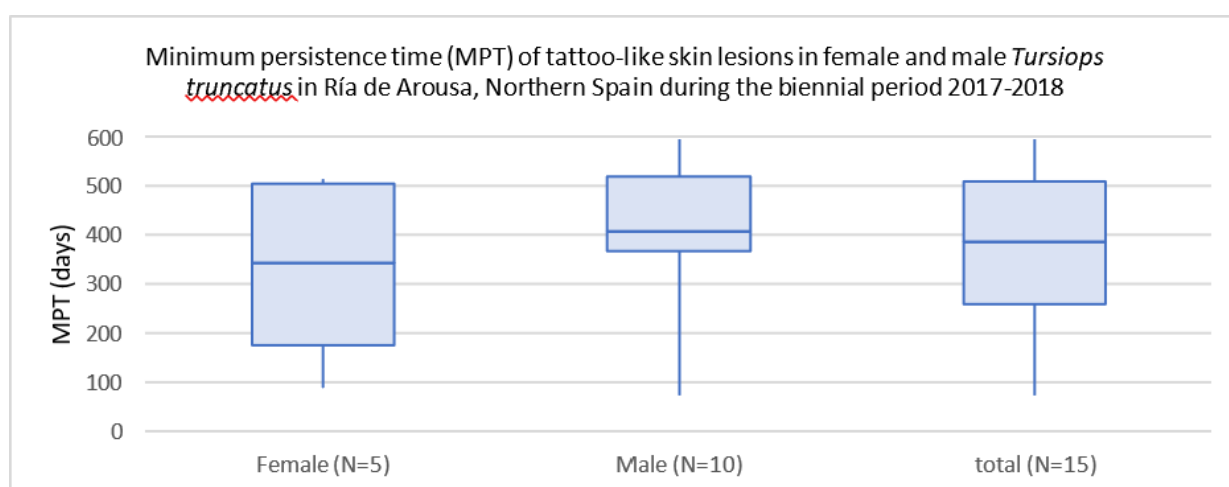


Figure V. Minimum persistence time (MPT) of tattoo-like skin lesions in a total 15 bottlenose dolphins (*T. truncatus*), 5 females and 10 males, from the northeast Atlantic (Ría de Arousa, northern Spain) displayed in days, examined during the two-year period 2017-2018. The figure displays minimum numbers, and persistence time may be prolonged.

3.3. Lesion number

Number of TSLs per individual varied between 1 to 93. In many cases, counting lesions within one sighting would only cover a few body parts. Within the three-month time span, increased body examination was possible, but new lesions may have appeared, and old lesions may have healed, resulting in a dynamic estimate. Most TSLs were observed on male individuals, with highest lesion number being 93, followed by 32, and subsequent counts of 27, 25 and 15 lesions, respectively. Comparatively, highest lesion number observed on a female was 5.

In 2018, lesion number was significantly higher in males compared to females ($W = 11.5$; $N = 17$, $p = 0.03076$). Lesion number in the two-year period 2017-2018 ($W = 38$; $N = 26$, $p = 0.06085$) as well as in 2017 ($W = 30$; $N = 21$, $p = 0.2551$) did not differ statistically significant between sexes (see Figure VI).

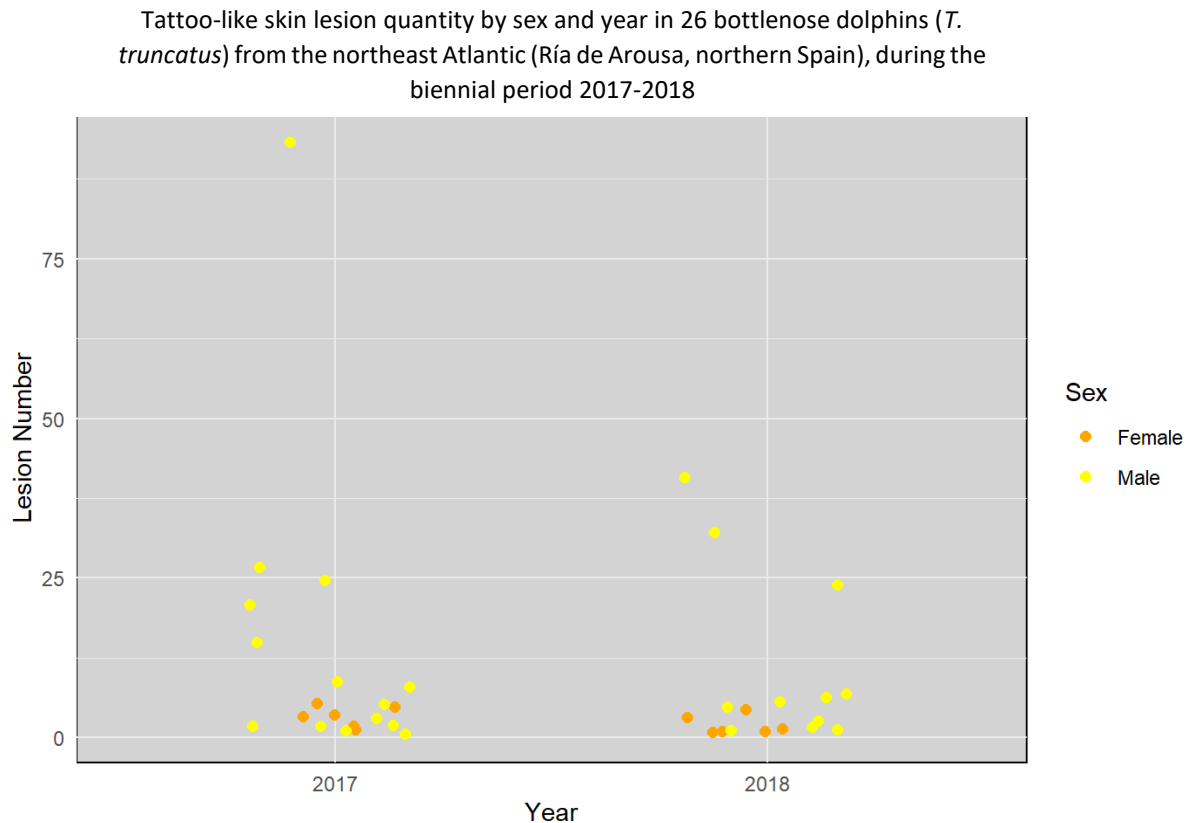


Figure VI. Tattoo-like skin lesion counts in male and female bottlenose dolphins (*T. truncatus*) in years 2017 and 2018. In 2018, tattoo-like skin lesion counts are significantly higher in males compared to females ($W = 11.5$; $N = 17$, $p = 0.03076$), but not in 2017 ($W = 30$; $N = 21$, $p = 0.2551$).

3.4. Lesion size

Medium-sized lesions were found most frequently on 25 individuals, followed by small lesions on 20 individuals and large lesions on 17 individuals. Very large lesions were found in 2 male individuals. On average the count of lesions per size category increased with decreasing size, meaning that the smaller the size category, the more lesions were counted (see Figure VII).

Although that all size categories displayed an increased trend in number of lesions for males,

these differences could not be evaluated as statistically significant (Very large: $W = 64$; $N = 26$, $p = 0.367$; Large: $W = 49.5$; $N = 26$, $p = 0.1957$; Medium : $W = 61.5$; $N = 26$, $p = 0.5571$; Small: $W = 44$; $N = 26$, $p = 0.0934$).

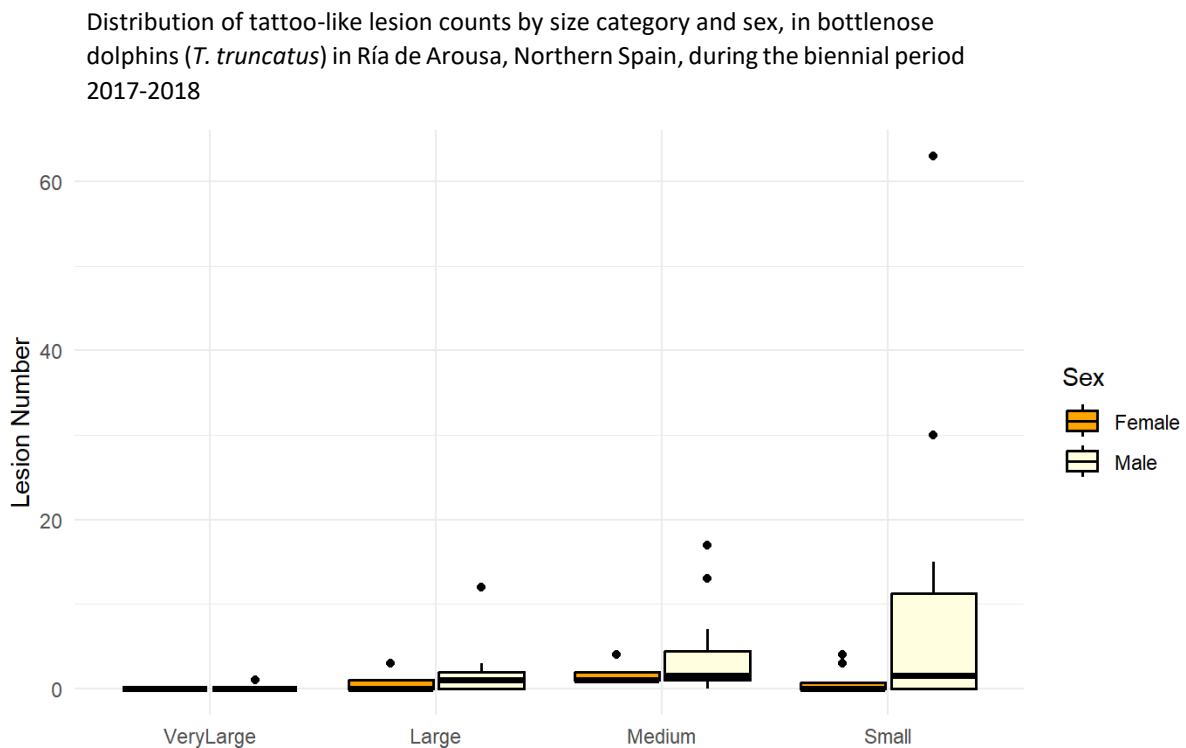


Figure VII. Boxplot visualizing the distribution of tattoo-like skin lesion counts per size category among 8 female and 18 male bottlenose dolphins (*T. truncatus*) from the Ría de Arousa, northern Spain, during the biennial period 2017 to 2018.

4. DISCUSSION

The current study generates and provides epidemiological baseline data of TSLs in Ría de Arousa common bottlenose dolphins (*T. truncatus*). It reveals that male individuals are more affected than female individuals in this population. This finding is the first of its kind in free-living *T. truncatus* and has previously only been observed in captive animals. It raises the question of what circumstances have led to this demographic shift.

Prevalences of Tattoo skin disease in free-ranging *T. truncatus*-populations around the world

vary widely from 4.5% (Strait of Gibraltar, Spain, N = 334) to 42.6% (Sarasota Bay, USA, N = 101) (Hart et al., 2012; Van Bresseem et al., 2022). Our finding lies within the upper range, with a prevalence of 26.3% from the 2017 to 2018 period. Methodology between authors varies from photographic assessment to laboratory analyses, therefore direct comparison of results needs to be obtained with caution. Minimum persistence time ranged between 2.4 and 19.6 months in 15 dolphins. Observational period was restricted to a 24-month observational period and could therefore be significantly extended. Persistence time in other *T. truncatus*-populations varied from 3 to 65 months (Powell et al., 2018; Van Bresseem et al., 2022). Epidermal cells of *T. truncatus* have a slow turnover rate of 73 days, allowing prolonged presence and potential transmission of CePV due to factors like limited immune surveillance and viral genes encoding immune evasion proteins, contributing to varied severity and persistence among cetacean populations (Hicks et al., 1985; Van Bresseem et al., 2022; Rodrigues et al., 2020). Lesion number per individual varied between 1 to 91, but due to lack of full body coverage and photo quality it is presumed that not all TSLs have been counted. Medium-sized lesions were found most frequently on 25 individuals, followed by large lesions on 16 individuals and small lesions on 13 individuals. One very large lesion was found in each two individuals, which had been linked to health deficiencies in previous studies (Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2018).

The basic assumption is that an increase in the prevalence, severity and persistence of Tattoo skin disease could be significantly influenced by stress. Stress can evidently have a negative effect on the immune system of *T. truncatus* and thus increase susceptibility to infection (Fair et al., 2014; Reif et al., 2009). Physiological stress can be induced in different ways, such as due to environmental factors, social interaction and population changes and anthropogenic influences. More specific examples that have been discussed to influence Tattoo skin disease epidemiology may be toxin exposure, climatic change (change in water temperature, sudden

flood events), intra- and interspecific aggression that additionally favors viral entry, reduced resting times due to vessel traffic and noise and lack of food availability due to fisheries (Hart et al., 2012; Maldini et al., 2010; Powell et al., 2018; Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2015; Wilson et al., 1999). Larger population size may contribute to the transmission of the disease. Changes in prevalence, severity and persistence of TSLs should therefore be monitored throughout subsequent years, to identify and correlate stressors that may favor viral infection, severity and prolonged persistence.

In addition to the relatively high prevalence, male individuals are more affected than female individuals in this population: Males constitute a significantly higher prevalence at 37.5% compared to that of females at 15.7% as well as increased severity (lesion quantity) in 2018. Each one very large lesion was found in two male individuals, indicating compromised immune function in these individuals (Van Bresseem & Van Waerebeek, 1996; Van Bresseem et al., 2018). The median persistence time for TSLs in *T. truncatus* was 407.5 days in males and 343 days in females. Due to the complexity of possible influences on the disease, it is difficult to identify factors that may contribute to the shift. Differences in hormonal and local behavioral responses could favor infection in males. Changes in testosterone levels due to sexual maturation, reproduction, and seasonal changes can lead to increased stress levels in males through competition and aggressive interactions among males for access to females, leading to cutaneous abrasions (Connor & Smolker, 1995; Van Bresseem et al., 2015; Yamamoto et al., 2015). Increased stress as well as disruption of the skin's integrity due to increased number of rake marks may favor viral entry and infection. This may also explain

male-female differences in captive dolphins where stress-related behaviors may increase in males. Dolphin populations can strongly vary in behavioral aspects and males in our study area may impose “riskier” behavior in order to develop Tattoo skin disease. Nevertheless, this was not observed as factors in any other free-ranging populations. The southwestern Iberian

Peninsula, including the study area, is considered as one of the global PCB-hotspots (Jepson et al., 2016). *T. truncatus* from the northwestern Atlantic from the area have been found with PCB-concentrations exceeding all toxicity-thresholds known for marine mammals (Jepson et al., 2016). Interestingly, males showed higher PCB-concentrations than female *T. truncatus*, similar to the increased prevalence and severity of TSLs in males. Though not significant, this increased PCB-concentrations in males could lead to increased exposure to immunotoxic risk, and therefore susceptibility to the disease. Lower PCB-levels in females could be explained by female off-loading of organochlorines during gestation and lactation (Jepson et al. 2005). This process has been observed in several dolphin species and may lead to the lower PCB-concentrations.

5. CONCLUSION

In conclusion, investigation of epidemiological patterns of TSLs may serve as bioindicator for environmental change and health status of dolphin populations. Future studies should prioritize comparing populations exhibiting an equal distribution of TSLs between sexes with those where males are disproportionately affected, aiming to discern differences, particularly in PCBs-concentration and behavioral patterns.

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