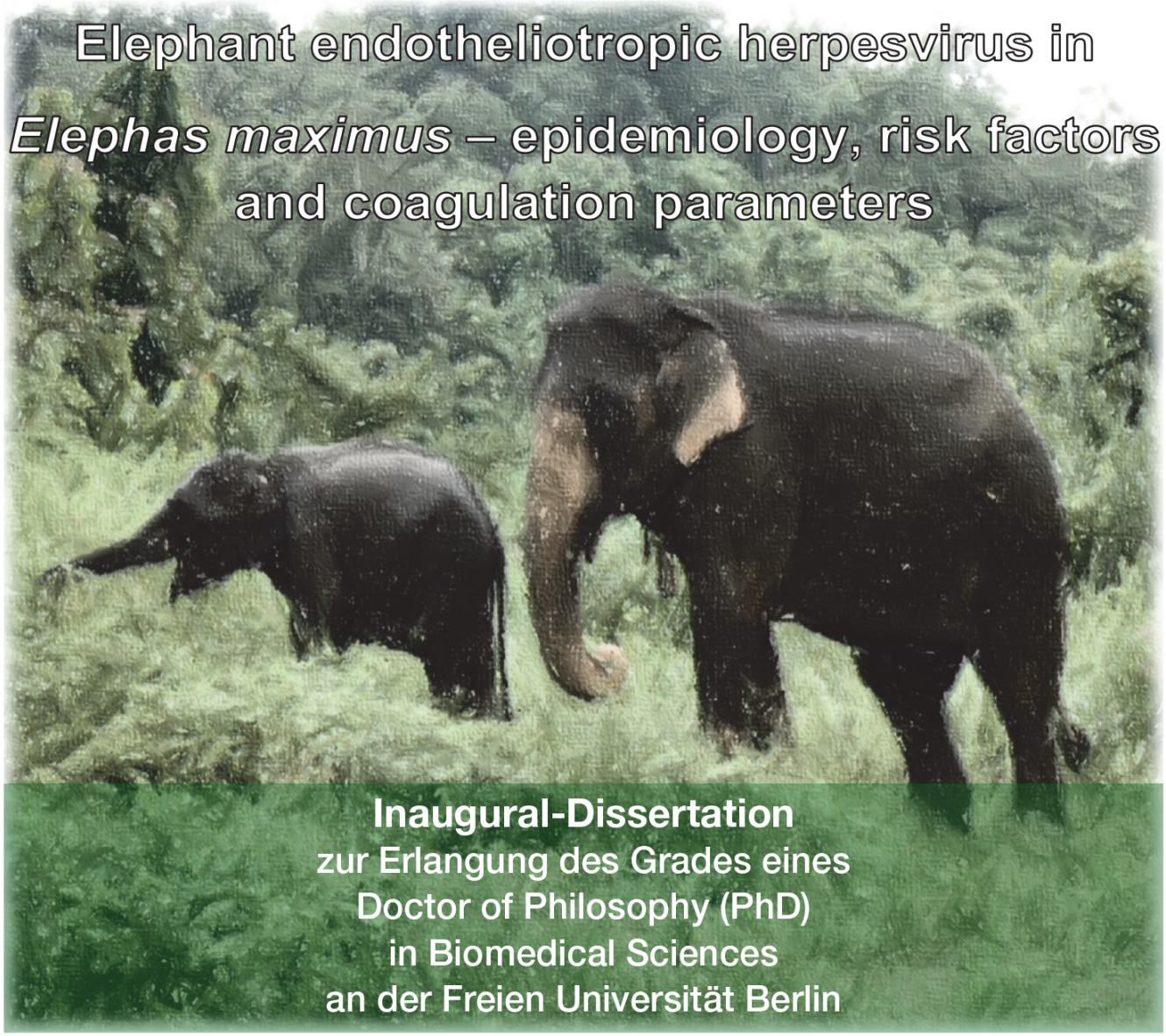
Aus dem Leibniz-Institut für Zoo- und Wildtierforschung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Elephant endotheliotropic herpesvirus in *Elephas maximus* – epidemiology, risk factors and coagulation parameters



Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der Freien Universität Berlin

vorgelegt von
Sónia Alexandra de Jesus Fontes
Tierärztin, DVM
aus Lissabon, Portugal

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List of abbreviations

Abbreviation	Full-term
A	Adenine
ANOVA	(Univariate) analysis of variance
aPTT	Activated partial thromboplastin time
Arg	Arginine
C	Cytosine
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
EAZA	European Association of Zoos and Aquaria
EDTA	Ethylenediaminetetraacetic acid
EEHV	Elephant endotheliotropic herpesvirus
EEP	European Endangered Species Programme
ELISA	Enzyme-linked immunosorbent assay
F7	Coagulation factor VII gene
G	Guanine
gB	glycoprotein B
Gln	Glutamine
HD	Haemorrhagic disease
IUCN	International Union for Conservation of Nature
IZW	Leibniz Institute for Zoo and Wildlife Research
Leu	Leucine
LIPS	Luciferase immunoprecipitation system
MVA	Modified Vaccina Ankara
OR	Odds ratio
PCR	Polymerase chain reaction
POC	Point-of-care
Pro	Proline
PROVEAN	Protein Variation Effect Analyser
PT	Prothrombin time
SD	Standard deviation
SIFT	Sorting Intolerant from Tolerant
SNP	Single nucleotide polymorphism
T	Thymine
Val	Valine
ZIMS	Zoological Information Management system

To the most magnificent being I had the lucky chance to learn from…

“They say an elephant never forgets. What they don’t tell you is, you never forget an elephant.”
– Bill Murray

CHAPTER 1

General Introduction

1.1. The Asian elephant's (problematic) status

Used by mankind for many centuries, and being the giant war soldier that determined the direction of so many imperial battles (Sukumar 2006), this magnificent animal – the Asian elephant – is nowadays critically endangered.

The Asian elephant belongs to the Animal Kingdom, Phylum Chordata, Class Mammalia, Order Proboscidea, Family Elephantidae and Genus Elephas (Linné 1758). The Asian elephant is known as *Elephas maximus*, with four extant subspecies (Fernando et al. 2003; Sukumar 2006): *E. m. indicus* (Indian), *E. m. maximus* (Sri Lankan) and *E. m. borneensis* (Bornean), which faces extinction, and *E. m. sumatranus* (Sumatran) which is critically endangered (Williams et al. 2020).

All Asian elephant subspecies populations are currently decreasing. The overall population has declined by at least 50% over the last three generations (Williams et al. 2020). They face several threats in their range countries such as illegal (trophy) hunting, habitat destruction, and human-conflict because of insufficient sizes of areas set aside as protected habitats and habitat fragmentation (Figure 1). Conservation efforts focus on protecting and increasing the numbers of this important species, as it is an "umbrella species": Because of its large area requirements, its conservation will protect a large number of other species that occupy the same area. It is also a "flagship species" because of its iconic and cultural value and a "keystone species" because of its important ecological role and impact on the environment as a habitat architect (Williams et al. 2020). Yet, another enemy that has coevolved with elephants causes now a "new" threat to their already reduced numbers – the elephant endotheliotropic herpesvirus.

Figure 1. Geographic range distribution of the *Elephas maximus* population and their extant status (Williams et al. 2020).

1.2. The elephant endotheliotropic herpesvirus

1.2.1. The beginning of awareness

"We report the necropsy findings of a juvenile Asian elephant dying peracutely from massive generalized haemorrhage due to lesions in the endothelial cells of the capillaries. The cell nuclei frequently contained inclusion bodies in which herpesvirus particles were demonstrated. This has not been described in elephants before." (Ossent et al., 1990).

In 1988, Lohimi, a 3-year-old female Asian elephant calf died from a haemorrhagic disease. This was the first reported case of elephant endotheliotropic herpesvirus (EEHV) haemorrhagic disease (HD) worldwide. The calf belonged to a circus and presented depression during the morning but ate normally at midday. In the evening, Lohimi was reported to be prostrated and presented a cyanotic and swollen trunk, oedema of the ventral neck, face and around the eyes. Within two hours of symptoms presentation she collapsed and died (Ossent et al., 1990) . This description of the disease course is the typical acute clinical presentation, as described for the majority of the numerous cases reported afterwards.

Since then, several research teams have been expended considerable efforts to improve the understanding of this virus' pathogenic mechanism, possible related risk factors, and to achieve a proper monitoring protocol, a fast treatment, and a protective vaccination for the young calves.

1.2.2. The EEHV variants and serotypes

The first herpesvirus particles found in Asian elephants were confirmed with electron microscopy in 1990, when inclusion bodies were seen in the sinusoidal cells (Ossent et al., 1990). Nine years later, electron microscopy also showed viral capsids morphologically consistent with herpes virions, mainly present in the microvasculature of heart, liver and tongue of nine deadly cases, with a preference for endothelial cells, being this an unusual finding comparing to other previously characterized herpesvirus (Richman et al., 1999).

In elephants, previous herpesvirus resembling viral particles had only been reported in African elephants: 1) in intranuclear inclusion bodies in cells collected from lung nodules of culled elephants (found in 74% of 50 animals from Kruger National Park) in the 1970s (McCully et al. 1971) and 2) in proliferative cutaneous nodular lesions from imported calves from Zimbabwe (Jacobson et al. 1986). Neither lung or skin nodules of this kind have been reported in Asian elephants (Long et al. 2016).

PCR-amplified DNA obtained from ten fatally diseased elephants (eight Asian and two African) revealed proteins encoded to be clearly herpesviruses, however, distinct from any herpesviruses known at the time for other species (Richman et al., 1999). Therefore, due to their highly divergence and unique genetic presentation, it was suggested that the EEHV was an outlier of the mammalian herpesviruses and it should belong to a previously unrecognized subfamily, the Deltaherpesvirinae, within the family Herpesviridae (Richman et al., 1999; Zong et al., 2014). This proposal was not yet adopted, and EEHV is still assigned to the genus Proboscivirus, Betaherpervirinae subfamily.

Additionally, Richman reported that the viral sequences in four fatal infections in Asian elephants were nearly identical. On the other hand, when the sequences achieved for the Asian and African elephants viruses were compared, they presented only 76% of protein identity and 65% identity at a nucleotide level indicating that two different species of herpesviruses were present in this study (Richman et al., 1999). Therefore, the conclusions were that African and Asian elephants may become infected with different species of EEHV, and most probably, the endogenous African elephant herpesvirus was transmitted to susceptible Asian elephant calves by cross-species infection, which could explain the surprisingly severe pathological findings and lethality in the Asian but not in the African species (Richman et al., 1999). It was initially assumed that cross-infection started with the importation of infected African elephants that had contact with or were unnaturally kept in near or in the same enclosures as Asian elephants, for exhibition (Reid et al. 2006). Later this theory was refuted, by the number of cases that appeared in Asian elephant range countries without previous inter-species contact. The first wild case was reported in 2006, in Cambodia (Reid et al. 2006), for a calf that had never been in contact with African elephants. Several more range countries have now reported similar haemorrhagic deaths in their calves, such as India (Barman et al., 2017; Mahato et al., 2019; Stanton et al., 2014; Zachariah et al., 2018; Zachariah et al., 2013), Thailand (Boonprasert et al. 2019; Guntawang et al. 2021; Prompiram et al. 2021; Sripiboon et al. 2017), Laos (Bouchard et al. 2014; Hoornweg et al. 2021; Zachariah et al. 2018), Myanmar (Oo et al. 2020; Zachariah et al. 2018), Malaysia (Lee et al. 2021), Nepal, Sumatra (Long et al. 2016), Singapore, Borneo and Cambodia (Zachariah et al. 2018).

EEHV is a linear double-stranded DNA virus with icosahedral capsids surrounded by a tegument and envelope (eehvinfo.org n.d.; Long et al. 2016; Richman et al. 2014). There are currently seven genotypes (EEHV 1-7) with twelve known variants (EEHV1 A and B; EEHV2; EEHV3 A and B; EEHV4 A and B; EEHV5 A and B; EEHV6; EEHV7 A and B) (Long et al. 2016). Of these, six EEHV species have produced at least one lethal case of HD (no illness reported for EEHV7), and more than 90% of all cases are attributed to the two chimeras (EEHV1A and EEHV1B), specially a very large variety of distinct strains of EEHV1A (Long et al. 2016; Zachariah et al. 2018). Specific EEHV genotypes seem to be present in different host species, where EEHV2, EEHV3A, EEHV3B, EEHV6, EEHV7A, and EEHV7B naturally infect African elephants and EEHV1A, EEHV1B, EEHV4, EEHV5A, and EEHV5B are endemic in Asian elephants (Hoornweg et al. 2021; Long et al. 2016).

Recent efforts that combine PCR detection of asymptomatic animals and assays to access seropositive animals uncovered that the disease was much more widespread than initially

thought (Fuery et al. 2020; Hardman et al. 2012; Hoornweg et al. 2021; van den Doel et al. 2015). Serological assays have been developed to estimate the prevalence of the virus in the healthy captive populations of Asian elephants. An ELISA test, using *E. coli* expressing the EEHV1A glycoprotein B (gB – one of the most common glycoproteins expressed by herpesviruses) as an antigen, reported that 37% of the European captive population were seropositive to EEHV, and was able to achieve nearly 80% of seropositivity for the PCR positive animals to EEHV in the European and North American populations (van den Doel et al. 2015). However, EEHV specific antibodies could not be detected in many other PCR positive animals (24%), and therefore EEHV seropositivity is probably even further underestimated due to the low sensitivity of the assay. In Thailand, a study using this assay presented an antibody seroprevalence of 42.3% (in a total of 994 elephants) for their captive elephants (Angkawanish et al. 2019). Three years later another report of a study combining PCR and an ELISA assay using three peptides based on the gB showed a similar EEHV seroprevalence of 40.1% for the Thai elephant population. The same study also showed that fatal cases were all seronegative to the ELISA, which suggests a primary infection leading to death (Prompiram et al. 2021).

The use of gB, which is relatively well conserved in all herpesviruses, makes it therefore hard to distinguish serological responses between different EEHVs (Fuery et al. 2020). To tackle this, a new assay using the luciferase immunoprecipitation system (LIPS), combined with the genomic sequences of the viruses and the production of antigens using mammalian cells was developed, allowing to distinguish between EEHV1(A and B), EEHV4 and EEHV 5 infections. This study revealed that 100% of the adult animals investigated were seropositive for at least one EEHV genotype (Fuery et al. 2020). For the fatal HD cases, the calves/juveniles were seronegative for the specific EEHV species that caused the illness, providing also evidenced that primo-infection with EEHV1A or 1B was correlated with the lethal disease (Fuery et al. 2020). Re-infections of calves had already been reported, when two calves previously infected with EEHV4 became later viraemic for EEHV1B, suggesting that being infected by one type of EEHV may not protect the calves against other circulating serotypes of this virus (Fuery et al. 2016).

Normally, EEHV-HD cases are reported for animals after one year of age. Therefore, a protective component is likely to be present, such as breast milk before weaning, and most probably by transplacental antibody transfer (Nofs et al. 2013). LIPS serological assay results showed that elephant calves do receive anti-EEHV antibodies transplacentally and there is a decline of maternal antibody titres in juvenile elephants to undetectable levels at around 36 months of age (in one animal a steep decline could be seen at 24 months of age). The absence

of these anti-EEHV antibodies in the age at risk is believed to lead to the development of the lethal HD from primary infection with EEHV1 (1A or 1B) (Fuery et al. 2020).

These results were further supported by a new study using a novel ELISA based on EEHV1A gB and gH/gL (glycoproteins essential for host cell entry). They reported that all Asian elephants sampled in the Laos population (n=69) and all except one calf of the European Asian elephant population (40/41) were seropositive for EEHV, and three lethal cases of HD in Europe presented low (n=2) to undetectable (n=1) EEHV specific antibody levels. This high seroprevalence of adults suggests that the disease is wide-spread within range countries and captive populations and the low or undetectable antibody levels found in the fatal cases of calves is further evidence that young elephants presenting low antibody levels are at risk of dying from this disease and that illness is due to primary infection rather than reactivation of a latent virus (Hoornweg et al. 2021). Such a reactivation was, however, demonstrated in a recent report, which presented a case of a calf dying from a reactivation or re-infection of the viral subtype EEHV1A, which was the same subtype that made the animal viraemic one year before. Therefore, the authors suggest that reactivation of latent status of EEHV should be taken in consideration (Boonprasert et al. 2021).

The ubiquity of the virus is now unquestioned: Both the virus and the disease are evidently widespread, and EEHV is likely to be an ancient infection that has co-evolved and been maintained in elephant herds probably since the beginning of elephants on earth, despite the severity of the disease (Zachariah et al. 2013; Zong et al. 2014). It is now also accepted that African elephant populations are vulnerable to the disease, and although illness is presented normally at an older age, at the sub-adult stage, many EEHV-HD cases in this species are now reported worldwide (Bronson et al. 2017; Fayette et al. 2021; Howard and Schaftenaar 2019; Kongmakee et al. 2015; Latimer et al. 2011; Richman et al. 1999). Healthy elephants intermittently shed EEHV and may naturally shed one or more subtypes (Hardman et al. 2012). Studies on the viral taxonomy report that the virus separated from all other mammalian herpesvirus nearly 100 million years ago, dating back to the ancestors of modern elephants (Richman et al. 2014; Zong et al. 2014). Understanding why this evolutionary partnership still leads to such an aggressive disease and numerous fatal cases is an important task.

1.3. The EEHV haemorrhagic disease

Once displaying symptoms, most elephant calves die with EEHV associated haemorrhagic disease (EEHV-HD) within one hour to seven days, normally presenting one or more of the

following clinical signs: fever, lethargy, bloody diarrhoea, facial oedema or a cyanotic tongue (EAZA 2020a; Garner et al. 2009; Richman et al. 1999; Sharma et al. 2021). Internally, the body experiences massive endothelial destruction and systemic inflammation caused by the EEHV, dysregulating the blood coagulation system and creating severe haemorrhaging and oedema (Guntawang et al. 2021).

Haemostasis is the stopping of a bleeding or haemorrhage, when the blood stops flowing through the walls of a blood vessel or to an organ. It is a dynamic process of maintaining a normal blood fluidity in the body and achieved by complex physiological interactions that regulate the balance between thrombogenic and anti-thrombogenic mechanisms. Once this equilibrium is disrupted, there is a tendency to bleed or to increase clot formation (Fasano and Sequeira 2017; Norris 2003; Palta et al. 2014). After vessel damage, the coagulation process is activated in order to create a clot to seal the lesion (Fasano and Sequeira 2017; Norris 2003; Thornton and Douglas 2010). The platelets start to immediately adhere to the subendothelium to form a plug and a synchronized enzymatic activation of coagulation factors interact to produce fibrin fibres, which in turn will form a mesh over these platelets, thus forming the clot and preventing further blood outflow (Fasano and Sequeira 2017; Norris 2003).

The sequential activation of the coagulation factors is called the “cascade of coagulation” and originates in two pathways (“extrinsic” and “intrinsic” pathways) that converge to a “common pathway”. The extrinsic pathway involves tissue factor and the coagulation factor VII, whereas the intrinsic pathway is represented by the coagulation factors V, VIII, IX and XII (Figure 2). When both pathways converge in the activation of factor X, a final common pathway converts fibrinogen into the final product, fibrin (Adams and Bird 2009; Norris 2003; Palta et al. 2014). The efficacy of the extrinsic pathway can be measured using the prothrombin time (PT) and, for assessing the intrinsic pathway, the activated partial thromboplastin time (aPTT) is measured, and with laboratory test assays we will achieve the time needed to clot formation (Fasano and Sequeira 2017). A schematic representation of the coagulation cascade can be seen in Figure 2.

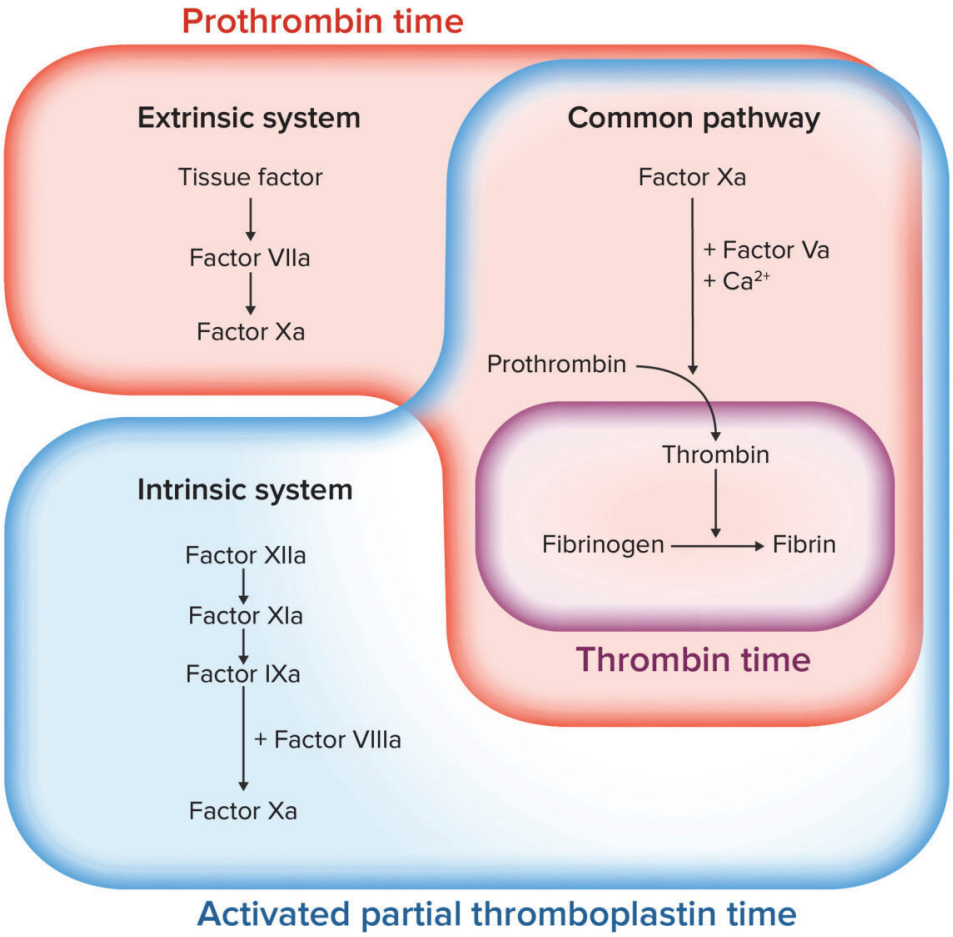

Figure 2. Coagulation cascade diagram showing the intrinsic, extrinsic and common pathways, and the coagulation factors involved. Image courtesy of Lecturio (in https://www.lecturio.com/concepts/coagulation-studies/).

Haematological changes observed in EEHV-HD cases include anaemia, thrombocytopenia, monocytopenia and/or a reduction in plasma protein concentration (Dastjerdi et al. 2016; Richman et al. 1999, 2000). The decrease in platelet counts, heterophilia and monocytopenia, and the presence of clinical signs is normally seen during a rapid increase in viraemia (Dastjerdi et al. 2016; Fuery et al. 2016; Richman et al. 2000).Thrombocytopenia is the only significant haematological parameter present in acute fatal cases, when platelet counts drop within 24 hours prior to death. It is therefore a predictive parameter for acute fatal EEHV-HD (Guntawang et al. 2021). A study showed that EEHV is disseminated in the body by EEHV-infected blood monocytes (Srivorakul et al. 2019). These will then adhere to the endothelia in small or micro blood vessels, allowing the endothelial cells to become infected and serving for replication of EEHV (Guntawang et al. 2021). This process will lead to endothelial cell damage and cause the observed diffused haemorrhagic and oedema of the internal organs

(Guntawang et al. 2021; Perrin et al. 2021a). Affection and destruction of virus-infected cells is believed to lead to what is called the “cytokine storm”: an increased production of inflammatory cytokines. The infection also dysregulates the coagulation system, causing the formation of micro thrombo-emboli in the blood vessels, which supports the presence of disseminated intravascular coagulopathy (DIC) as a contributor to EEHV fatalities (Guntawang et al. 2021; Perrin et al. 2021a). There is not a standardized laboratory diagnosis for DIC in veterinary medicine, and it is not a primary disease but secondary to numerous underlying diseases. These include bacterial, viral or parasitic diseases, heat strokes, burns, neoplasia or severe trauma (Cotter 2019). The underlying disease causes an uncontrolled systemic inflammatory response in the body, characterized by a massive activation and consumption of coagulation factors, endogenous inhibitors, fibrinolytic proteins, and platelets (Cotter 2019). In EEHV-HD, the virus is thought to be the underlying cause of DIC, which further aggravates the bleeding tendencies in sick calves. Therefore, the destruction of small blood vessels with the presence of DIC leads to a diffuse haemorrhage, causing a hypovolemic shock and multi-organ failure, causing the death of the calves (Guntawang et al. 2021; Perrin et al. 2021a).

All genotypes of EEHV-HD cases were reported to present severe oedema, widespread petechial and ecchymotic haemorrhages and thrombosis (Perrin et al. 2021a). However, the degree of thrombocytopenia, the type of affected organs, the severity of the vascular lesions and the viral loads varies among EEHV-infected animals, according to the EEHV genotype(s) present (Guntawang et al. 2021). The heart was reported to be the most consistently and severely affected organ in fatal cases caused by EEHV-1A,1B and EEHV-5, presenting a cardiac haemorrhage score of moderate or severe in 95% of the 27 fatalities analysed (Perrin et al. 2021a). However, no cardiac affection (haemorrhage, inflammation or myofibre degeneration) was noticed in the co-infected fatal case of EEHV1A+EEHV4, despite the high viral load and several intranuclear inclusion bodies observed in the small myocardial vessels (Seilern-Moy et al. 2016). Therefore, different EEHV subtypes can affect calves differently and their specific pathological alterations should be further investigated.

1.4. Coagulation factor VII and F7 gene

Due to the haemorrhagic character of EEHV-HD, the research focus has been directed to understanding the coagulation status of the Asian elephant. Coagulation assessments have been performed with different diagnostic methodologies, either based on human plasma reference (Gentry et al. 1996; Kaye et al. 2016; Lynch et al. 2017) or, more recently, focused on the host blood viscoelasticity via thromboelastography (Flanders et al. 2018; McCann et al.

2019; Perrin et al. 2018). Understanding elephant haemostasis is an important goal, not only to improve general knowledge of elephant health status, but also to improve the understanding of the mechanisms of pathogenesis of EEHV-HD.

With this problem in mind, in Chapter 3 we investigated the coagulation status of healthy Asian elephants, using a fast diagnostic analysis tool which could be used in routine health check-ups performed by caretakers or in a clinical emergency such as EEHV-HD. Chapter 3 focused on the study of a specific coagulation factor – factor VII. Although the deficiency of any of the coagulation factors might cause impaired coagulation, previous reports showed that coagulation factor VII is of particular importance in Asian elephants (Lynch et al. 2017; Molenaar et al. 2016). An Asian elephant bull was reported to have a factor VII deficiency, although without presenting bleeding tendencies, he revealed a very prolonged prothrombin time (PT). Genetic investigation showed that the animal presented a deleterious mutation in the factor VII gene (F7) and that three of his five offspring were carriers of this hereditary coagulopathy (Lynch et al. 2017). The administration of recombinant Factor VII is recommended as part of the treatment of EEHV-HD in hypo-coagulation states. The product has been applied to a sick calf, and although the animal has died with EEHV-HD, the drug presented improvements in his coagulation (Molenaar et al. 2016). How the vascular endothelial damage caused by this virus will affect an elephant with factor VII hereditary coagulopathy is still unknown, and therefore, of interest.

In Chapter 3 we investigate the coagulation status of the healthy Asian elephant and the presence of a genetic coagulation deficiency in factor VII in the Thai and European populations.

1.5. Aim of the study

Aim 1: Understand the impact and prevalence of the elephant endotheliotropic herpesvirus haemorrhagic disease in the captive European Asian elephant population and investigate if hereditability and zoo-associated factors could be involved in the onset of the disease.

To improve our understanding of the impact of EEHV-HD in the European captive Asian elephant population we analysed retrospective data, spanning 35 years of captive breeding. Furthermore, using statistical models, we investigated if whether parental or zoo-associated factors could influence the risk for calves to die with this disease. This aim is addressed in Chapter 2.

Aim 2: Assess the coagulation status of Asian elephants associated with genetic investigation of the F7 gene and the presence of its hereditary coagulation disorder.

We established reference coagulation parameters for measuring PT, aPTT, fibrinogen concentration and platelet counts for the European and Thai Asian elephant populations. We aimed at applying a practical method which allowed for much faster results than other current techniques that is feasible to use under field conditions as well. Furthermore, we investigated the presence of haemophilic animals within our study populations for a specific hereditary coagulopathy previously reported in Asian elephants, by analysing the coagulation factor VII gene (F7). We question whether the presence of mutations in this gene could be correlated to EEHV illness. These topics are covered in Chapter 3.

CHAPTER 2

Elephant Endotheliotropic Herpesvirus Impact in the European Asian Elephant (*Elephas maximus*) Population: Are Hereditability and Zoo-Associated Factors Linked with Mortality?

(Published article)

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Author Contributions:

S.J. planned and wrote the manuscript and prepared figures and tables; T.B.H. supervised the study and funding acquisition, M.G.D co-supervised the work and had substantial inputs in the data analysis. All authors have read and agreed to the published version of the manuscript.

2.1. Abstract

EEHV is a ubiquitous virus, which most likely has co-evolved with elephants and is shed by healthy individuals and maintained in the herds. Yet, the factors determining calf susceptibility to the virus remain unknown. Here, we explored the impact of EEHV-HD in the European captive Asian elephant population in a retrospective statistical study spanning the last 35 years. We show that EEHV-HD was implicated in more than half of all deaths recorded in calves older than one months old. Moreover, the median age across EEHV-HD fatalities was significantly lower compared to other death causes. Finally, we investigated if heredity and zoo-associated factors could be linked to a higher susceptibility aof calves to this disease. We used a univariable logistic regression model to evaluate if either fathers, mothers, or zoos could, separately, be considered as risk factors to the development of the disease. Afterwards, we used a two multivariable model, combining: (1) fathers and zoos, and (2) mothers and zoos. Overall, we found that two fathers, one mother, and four zoos had three or more times higher risk of their calves becoming sick when compared to all others, pointing us to the presence of a management or environmental element, which can have paternal and maternal influence and leads to calf susceptibility or resistance to EEHV-HD.

Keywords: EEHV; *Elephas maximus*; epidemiology; haemorrhagic disease; hereditary; proboscivirus; zoological institution

2.2. Introduction

Elephant Endotheliotropic Herpesvirus (EEHV) was initially reported in the captive Asian elephant population in 1990 after a three-year-old elephant calf died from an acute haemorrhagic disease (HD) (Ossent et al. 1990). At necropsy, a severe generalized haemorrhagic condition due to vascular endothelial lesions was observed (Ossent et al. 1990). Diseased elephants experience a rapid and systemic spread of the virus, followed by vascular endothelial cell damage associated with an uncontrolled virus replication (Guntawang et al. 2021; Richman et al. 2000). This fulminant disease affects mainly very young calves, often leaving little or no time to provide adequate veterinary treatment (Kendall et al. 2016; van den Doel et al. 2015). Multiple EEHV genotypes and strains have been reported, with EEHV 1 being the most impactful (Boonprasert et al. 2019; Long et al. 2016; Oo et al. 2020; Zachariah et al. 2013). In the European population, 80% of the calves' EEHV-related deaths were reportedly caused by subtype EEHV1a (Perrin et al. 2021b).

EEVH-HD is considered to be an ancient infection among Asian elephants (Zachariah et al. 2013) and is not a disease exclusive of this species as it may also affect African elephants. However, the recorded mortality rate in African elephants is lower, and the animals seem to present symptoms at an older age (EEHV-AG 2019; Fayette et al. 2021; Howard 2019). Currently, the most used antiviral treatment is a human anti-herpetic drug, despite its high costs and reported as presenting unproven efficacy, so far (EAZA 2020a; Hayward 2012; Kendall et al. 2016).

Once thought to be an exclusive zoo disease, fatal cases due to EEHV-HD have been reported in several range countries, such as India (Barman et al. 2017; Zachariah et al. 2013), Thailand (Boonprasert et al. 2019; Guntawang et al. 2021; Sripiboon et al. 2017), Cambodia (Reid et al. 2006), Laos (Bouchard et al. 2014), Myanmar (Oo et al. 2020), Nepal, and Sumatra (Long et al. 2016). The prevalence of EEHV-HD in wild populations is expected to be high, since the medical veterinary teams working in close association with these populations have found evidence of this disease, during necropsies. However, due to a lack of logistic capacities, further investigations have been hampered (Howard and Schaftenaar 2019). In North American zoos, reports show that 53% of deaths since 1980 in their Asian elephant population were caused by EEHV-HD, while in Europe this accounts for 60% of the total deaths since 1995 (Howard and Schaftenaar 2019). Additionally, North American institutions reported that the virus presents a mortality rate of 68% (Howard and Schaftenaar 2019). In 2016, 40% of elephants' deaths in the UK and Ireland were caused by EEHV-HD with an overall population mortality of 21.6% (Kendall et al. 2016), making this the major mortality cause in both continents (Howard and Schaftenaar 2019). In range countries, such as India, a prevalence study showed that at least one of the EEHV variants is present in 35% of their captive Asian elephants (Stanton et al. 2014). Moreover, in Thailand, a seroprevalence of 42% was found (in private, touristic, and logging elephant camps (Angkawanish et al. 2019), showing that EEHV is also maintained within the captive population. Most infectious diseases run a subclinical course and only part of the population will present clinical disease, where the mutual interactions between environment, host, and pathogen genetic factors, influence this ratio (Kimman 2001). To similarity, EEHV-HD must also be influenced by the elephant host genetics and environmental pressures, being the presence and pathogeny of the virus alone, not the only determinant factor.

Even though this disease has been under study for the past three decades, and a significant number of discoveries were recently made on its pathophysiology (Guntawang et al. 2021; Perrin et al. 2021a), the adequate treatment, and the epidemiological impact of it in the overall world elephant population is still not fully understood. Therefore, having a deeper

understanding of the virus' mechanism of action is yet of the highest priority. Moreover, there is an urgent need to identify what risk factors are involved in the onset of the disease, to establish proper actions to protect the calves.

This study aims to assess the impact of EEHV-HD in the European captive Asian elephant population and to explore risk factors linked to a higher prevalence of the disease, such as gender, age, genetic lineage, and location. To address these, we used historical and current data from all captive calves born in Europe from January 1985 to June 2020, conducting the longest, retrospective, and longitudinal observational study so far. The disease seems to affect calves from different genetic backgrounds and breeding facilities at a different rate: while some are profoundly impacted by this haemorrhagic disease, others are minimally or not affected. Therefore, we hypothesize that hereditary (host genetics) and different zoo-associated factors (e.g., management protocols and growing environment) may protect calves against the potentially fatal outcome of the disease.

2.3. Materials and methods

To be able to identify the impact of EEHV-HD, regardless of the virus genotype, in the captive-born Asian elephant population in Europe and investigate the risk factors associated with high mortality, we compiled a dataset of all animals kept in captivity at European zoos, spanning the last 35 years, from January 1985 to June 2020 (n = 330, supplementary materials, Table S2.1—Study population database). This dataset comprises exact birth and death dates, maternal and paternal information, location, and the present status of the elephants (alive, dead by other causes, or dead by EEHV-HD), as well as EEHV infection reports. We collected information from the Asian elephant European Association of Zoo and Aquaria ex situ Programme (EEP, formerly European Endangered Species Programme) Studbook yearly reports, from Zoological Information Management system (ZIMS), from personal contacts with the zoological institutions, from up-to-date registers documented on zoo websites, and from information compiled at elephant large online databases.

2.3.1. Data cleaning, selection and analysis

The starting year of the analysis (1985) was chosen to match the year when the first reported EEHV-HD fatal case was born—Lohimi, a female calf born in a circus, that presented a haemorrhagic syndrome in 1988, which led to her death, at the age of three years (Ossent et al. 1990). Since the population in the study were captive European Asian elephants, only

calves born in captivity were kept in the data set, and all wild-born animals were removed from the study. Thus, non-European captive calves that were translocated to Europe afterwards were also not considered for analysis.

2.3.2. Data collection

Neonatal mortalities and early life deaths accounted for 24.8% of the total deaths due to several causes (e.g., miscarriages, abortions of twinning, stillbirths, surgically removed foetuses, infanticide, rejected by the mother). On this account, a subset of our initial population was created, including only records of successful births and minimal management to ensure a correct adaptation to the first months of life (e.g., proper feeding and non-life-threatening congenital defects). Animals that did not survive to reach two months of age (n = 83; n = 77 under one week and n = 6 dying in their first month of life) were excluded from this dataset. Under this threshold, three animals were mentioned as possible EEHV-HD deaths, presenting low titters of the virus, being stillborn, or having succumbed under 24 h after parturition. These deaths could not be clearly attributed to EEHV-HD and were removed.

The frequencies of births, deaths due to EEHV-HD, and deaths due to other causes per year of study are shown and their distributions were evaluated. We investigated the trends of distribution according to age for each status (status 0 = alive, status 1 = death by EEHV- HD, and status 2 = death by other causes) for all captive-born elephants. Standardized residuals were visually assessed and were not fully normally distributed, therefore, a non-parametric Kruskal–Wallis test was used to compare median ages between groups.

The association of gender with the overall survival time for the entire population in the study and within the EEHV-HD reported cases was investigated using the log-rank (Mantel–Cox) test. Afterwards, a survival analysis (Kaplan–Meier curve) was performed to compare the survival time between the animals that presented EEHV-HD disease (that survived or died) and all others that never presented symptoms.

Finally, to test if hereditary lineage and/or the environment could be potential risk factors to the survival of the elephants in captive populations, we categorized all calves by fathers, mothers, and location during calfhood. The identities of the bulls, dams, and zoos will remain anonymous in our study.

An explorative univariable logistic regression model was performed to separately assess the odds ratio (OR) of EEHV symptomatic calves for individual (I) fathers, (ii) mothers, and (iii) zoos. Parents and zoological institutions included in the analysis were grouped according to

the number of calves produced. Bulls that sired more than ten calves were kept individually while all bulls that sired fewer calves were collapsed into a single group (bulls that sired less than ten offspring). For dams and zoos, the cut-off for keeping them individually was five calves. Fathers, mothers, and zoos with a lower number of calves were considered the baseline for comparison. Afterwards, two multivariable models estimated simultaneously the OR of (i) fathers and zoos as well as (ii) mothers and zoos combination. Results were screened for OR greater than 6.0 which indicates a sixfold higher chance of presenting EEVH sick calves when compared to the baseline category.

All statistical analyses were performed considering an alpha level for significance and tendency of 0.05 and 0.10, respectively. Analyses were conducted using IBM SPSS (IBM SPSS Statistics for Windows, version 24.0, Armonk, New York, NY, USA) predictive analytics software and graphs were produced using GraphPad Prism (version 9, GraphPad Software, San Diego, CA, USA).

2.4. Results

2.4.1. Descriptive analysis

A total of 247 captive-born Asian elephants (females = 116, males = 131) were born between January 1985 and June 2020 and survived more than one month of life. These births occurred in a total of 48 European zoological institutions and animals are now distributed in 68 zoological locations, due to transfers between zoos. A total of 72.1% of the population monitored since 1985 never presented the disease and are still thriving at the moment of writing. We found that 15.8% (n = 39) of the calves were infected and symptomatic for EEHV-HD. Of this percentile, 13.4% were lost to the haemorrhagic disease, and therefore, so far, only 2.4% (n = 6) of the affected calves managed to resist and survive this disease.

A total of 25.5% (n = 63) of the population died within the study period due to several different causes (e.g., foot disease, infectious diseases—including EEHV-HD, tumours, etc.). Accordingly, EEHV-HD is the primary cause of death above one month of age in the European Asian elephant population, producing 52.5% of all reported deaths (n = 33).

We found that only in 1988 no births were registered, and one death was reported, presenting, therefore, a negative balance for that specific year. Moreover, except for 1987, 2015, and 2018 where the number of births and deaths was the same, the number of offspring per year exceeds the number of deceased animals (Figure 3).

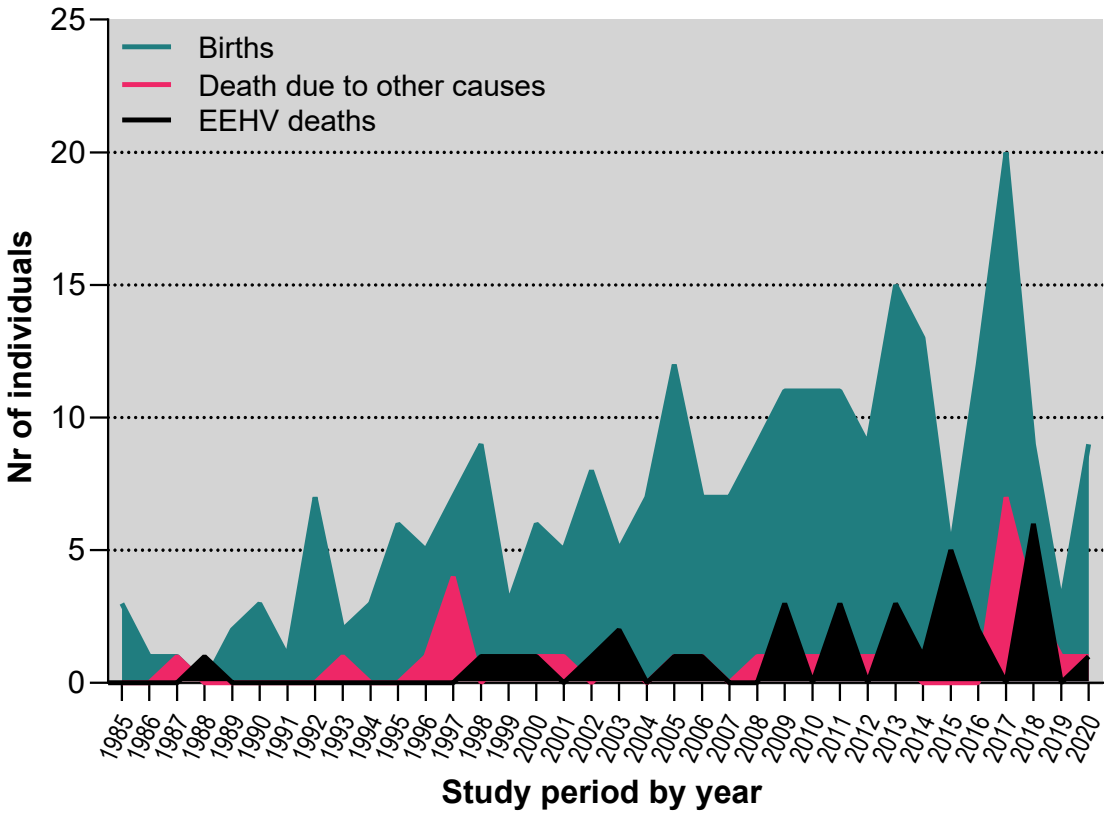

Figure 3. Distributions of the births (green), deaths unrelated (red) to, and related to EEHV-HD (black) of the captive-born Asian elephant calves above one month of age, in the European population from 1985 to 2020.

2.4.2. Survival age and gender relation

There was no impact of gender in the survival time of Asian elephants born after 1985 in Europe ($p = 0.813$) and EEHV-HD fatalities were also not gender related, with an almost 1:1 relationship (females n = 17, males n = 16). Moreover, males (n = 131) were found to be younger than females (n = 116); the overall male median age was around 24 years of age while the female average rounded 30 years.

The animals which died from various non-EEHV-HD-related causes (n = 30), lived between two months and 23 years (median = 8.6 years). For the EEHV-HD fatal cases (n = 33), the earliest related death occurred at 9 months old, and the oldest animal died at 7.6 years of age resulting in a very narrow age range. Additionally, deaths due to this virus occurred at a significantly lower median age (2.7 years old) when compared to the median age of elephants that died due to other causes (8.6 years old) (Figure 4).

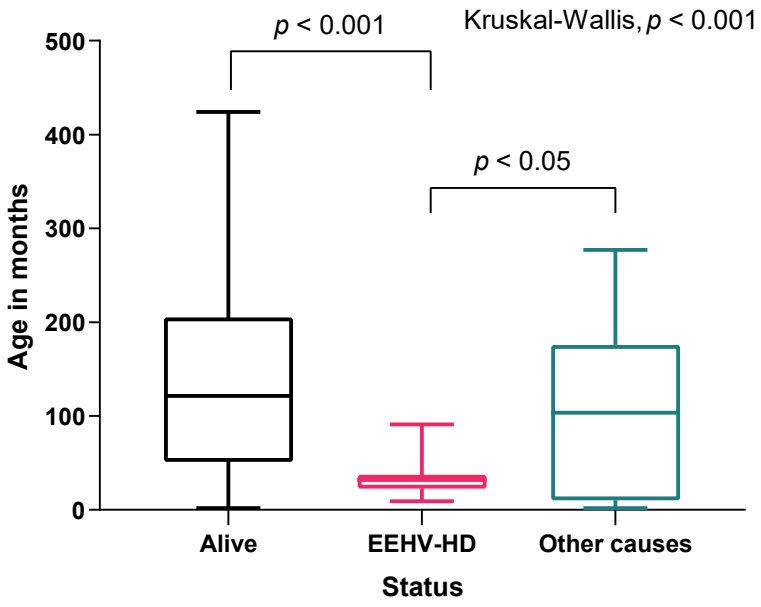

Figure 4. Boxplot of the overall survival time distribution of the calves in months, for the living animals, deaths caused by EEHV-HD, and deaths due to other causes. The box represents the 25th to 75th percentile values of the distribution (interquartile range), the line within the box the median (50th percentile), and the whiskers approximate the 2.5th and 97.5th percentile values.

Pairwise comparisons between EEHV fatal cases and animals that are alive revealed a significantly lower age of life for the diseased animals ($p < 0.001$). The same results were found for the comparison between animals dead due to other causes and those that succumb to EEHV ($p = 0.007$). The median age did not differ between the living animals and those that died due to other causes ($p = 0.057$).

Kaplan–Meier analysis revealed that the survival curve of the animals that presented EEHV-HD and the survival curve of the other individuals that never presented symptoms are significantly different ($p < 0.001$, Figure 5). The median survival age of EEHV-HD symptomatic animals was 35 months, while animals with no reported EEHV-HD presented a median age of 122 months.

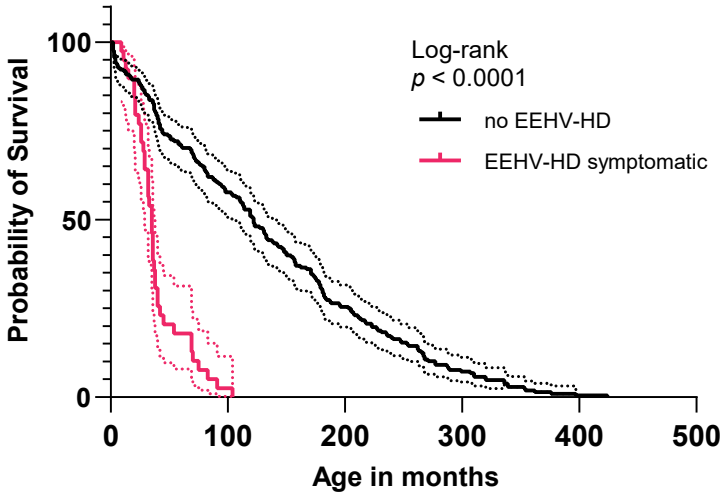

Figure 5. Kaplan–Meier survival curves, distributing the age of infected animals that presented the disease (median 35 months) and the age of the other population in the study (median 122 months). p values obtained using the log-rank test show p < 0.0001.

2.4.3. Father and mother distribution of EEHV-HD fatal cases

When investigating the distribution of the fatal EEHV cases per high breeders, we found that some fathers presented no loss of their offspring due to EEHV-HD (e.g., fathers F2, F3, F4; Figure 6) or minimal loss (e.g., father F8, Figure 4), while others, with nearly the same number of calves, have lost a high percentage of their calves (e.g., fathers F9 with 42% and F7 with 38% of calf loss due to EEHV-HD; Figure 6).

From all the fathers analysed (n = 45), 11 bulls had ten or more calves each. These animals have produced nearly 60% (n = 144) of the entire population present in the study population and were the ones used for the subsequent analysis of parental risk. Calves born to two specific fathers with a high frequency of offspring presented a significant increase associated risk to present EEHV-HD (F7, OR = 3.8, p = 0.03; F9, OR 4.4, p = 0.02) when compared with other sires.

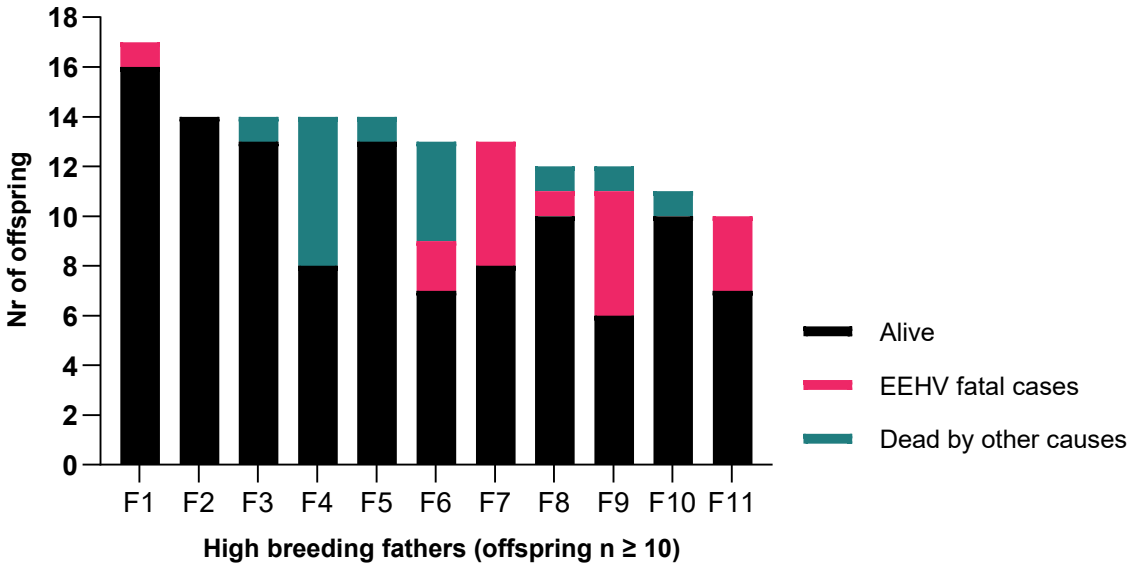

Figure 6. Distribution of the offspring which are still alive, have died due to EEHV-HD, or have died by other causes, per high breeding fathers (n = 11, each producing ten or more calves).

Maternal contribution (n = 97) to the overall deaths of the calves was also investigated; however, there is a very low frequency of births registered per dam when compared with the high offspring number presented by the fathers. One mother presented an increased tendency for her calves to have the disease when compared to all other mothers in the study (OR = 3.8, $p < 0.1$).

2.4.4. Zoo distribution of EEHV-HD fatal cases

Our survival comparisons based on the living location (n = 68 zoos) of the calf showed that high breeding zoos (n = 18) that produced five or more calves conceived a total of 140 calves. The remaining 50 locations presented a lower breeding rate and produced 107 offspring, with the majority of the zoos having produced one or two calves.

Similar to the distribution found for the fathers, we observed that some institutions have suffered high losses. When investigating only the zoos that bred five or more times, we found that some of these locations were not affected at all, while others present an overall offspring loss due to EEHV as high as 50% of the total offspring born at a particular zoo (e.g., zoos Z11 and Z6; Figure 7).

We found that three institutions presented a significantly increased odds ratio, between 8 to 12 times higher, for their calves to present EEHV-HD (Z17, OR 11.8, p = 0.01; Z2, OR 10.6, p < 0.001; Z6, OR 7.9, p = 0.007), than the other zoos in the study.

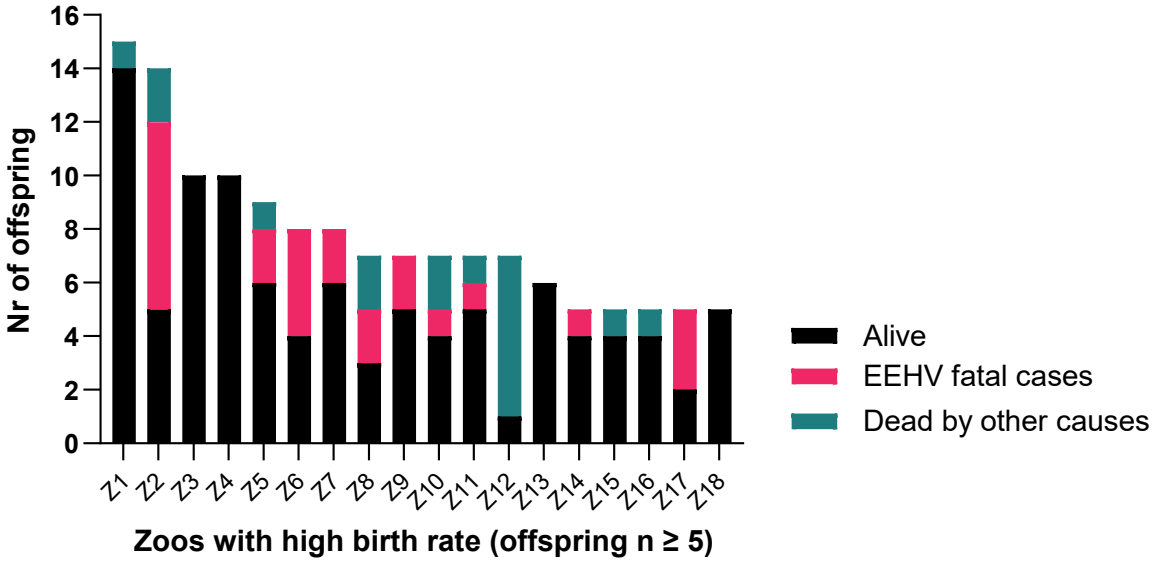

Figure 7. Distribution of offspring that are still alive or died due to EEHV-HD or other causes, by zoos (n = 18) that have produced five or more calves during the study period.

In the multivariable model with fathers and zoos, we found that F9 and Z17 presented a significant increased OR for presenting calves with the disease (OR 6.2, p = 0.04 and OR 19.1, p = 0.016, respectively) and Z9 had an OR > 6.0 (p = 0.086). When combining with mothers, we find that zoos Z2, Z6, and Z17 present a significantly higher probability of reporting calves with EEHV-HD (OR > 6.0, p = 0.012, p = 0.002, and p = 0.013, respectively). On another analysis, a cross-tabulation of all fatal cases caused by EEHV-HD by the respective fathers (n = 18) and locations (n = 18) showed deaths attributed to different sires at the same zoo. Likewise, different calves fathered by the same sire but living in different institutions were also lost (Supplementary Materials, Table S2.2 - Crosstabulation of the distribution of EEHV-HD fatal events per Father and Zoo, for the captive European Asian elephant). At the end of the study, there were 18 calves reaching, or near the age of 2.7 years, the statistical age risk to succumb to EEHV-HD.

2.5. Discussion

In the present study, we compiled all data available since the first detection of a captive Asian elephant with EEHV-HD was detected, making it the most extensive study on the impact of EEHV on the European Asian elephant population to date. Our data showed that EEHV-HD affected calves at around 2.7 years old, which is significantly lower than the median age for other causes of death (8.6 years). These results are in accordance with recent reports from Europe, Thailand, and North American risk ages (Boonprasert et al. 2019; Howard and Schaftenaar 2019; Perrin et al. 2021b). The European Endangered Species Programme's latest report states that birth rates will not replace the loss of the high number of aged females (35–55 years old), of which the majority is considered unable to further reproduce. This will possibly lead to a decrease in female captive elephants in the future. The report also suggested that female elephants should become pregnant for the first time at 8 years of age and that ideally, there is an interbirth interval of 7 years (Schmidt and Kappelhof 2019). Since EEHV-HD deaths occur at a significantly lower and narrower age range than other causes, killing mainly youngsters before sexual maturity is reached will, therefore, reduce the possibility of these calves substituting the elder ones, as well as reducing the overall number of possible future breeders. Consequently, this affects the breeding efforts made by the zoos on keeping a reproductive group to maintain a healthy and sustainable captive population.

After removing all premature deaths, EEHV-HD alone was responsible for 52% of fatalities, nearly the same amount as reported for North American institutions (53%, Howard & Schaftenaar, 2019). This mortality rate for EEHV deaths in Europe is slightly lower than the previous study published for the continent (57%—for calves surviving the first day of life, data until 2017; K. L. Perrin et al., 2021) and is most likely a reflection on the increased number of survivors and the outstanding birth rate that year (20 new births in 2017). Despite the similarities between different countries, when we compare mortalities, we found that EEHV-HD presented a higher mortality rate in the European population (85%), compared to the one reported for North America (68%; Howard & Schaftenaar, 2019) or Thailand (nearly 69%; Boonprasert et al., 2019). There are no indications that a more virulent serotype of the virus is present in Europe, therefore it is most likely that this mortality rate difference is related to the management of the disease. Due to their tradition of elephant training under the guidance of the mahouts, Thailand has facilitated veterinary access to these animals, to perform medical check-ups, and to treat very young calves. In the North American captive population, although in a protective contact system (where elephant keepers must not share the same unrestricted space with elephants), their management allows for direct training of young calves up to 24 months of age (AZA 2020). This allows for regular monitoring, as well as prompt and more

effective prevention or veterinary treatment of calves once symptoms are present. In Europe, all EAZA members must also comply with the protected contact handling policy as it will become effective from 2030 on (EAZA 2020b). European zoos are also encouraged to start training their calves from the age of 4 months, and several behaviours facilitating medical support are expected to be achieved by the age of one year, for all breeding European institutions (EAZA 2020a). Hence, the lower mortality rates presented by Thailand and North America most probably reflect the substantial amount of survival cases due to effective treatment when compared to the population of this study. Nevertheless, the numbers of survivors in Europe have risen in the past few years, and it is expected to improve due to a higher awareness of the disease and the positive outcome that early monitoring and fast medical intervention can have.

In this study, Europe presented lower EEHV-HD morbidity (15.8%) than North America, where one in every four calves (25%) has presented the disease (Howard and Schaftenaar 2019). This finding suggests that the European captive-born calves, although also exposed to the virus, become ill with EEHV-HD less often. However, it is most likely that this is related to under-detected or subclinical cases during the past years in Europe.

Bennet (2018) has performed a genogram on the Asian elephant captive populations living in Europe and North America to assess the possibility of a family link and found that EEHV-HD-related deaths appeared to be grouped into clusters. However, since the elephants in that study were originally located at the same institution, it remained unclear whether the clustering was due to genetic or environmental pressure (Bennett 2018). Our study supports this indication of clustering of cases in certain zoos and accepts potential effect modification by either mothers or fathers. Combining a multivariable model, fathers and zoos revealed a higher risk for two specific zoos and one father to have their calves developing the disease. In the model using mothers and zoos, we also find a significantly higher risk for three specific zoos. Together, these findings indicate the possibility of a multifactorial disease, where a zoo-associated component must be assumed to be involved and a hereditary predisposition might be expressed under the influence of certain environmental pressures. This highlights the importance of collecting relevant risk factor information for all calves (retrospectively and prospectively) for more detailed analyses on risk factors. As an example, a hereditary coagulation disorder has been reported in an Asian elephant herd, where a breeding bull, although asymptomatic, presented a prolonged prothrombin time (one of the tests used to assess coagulation capability). This coagulopathy was caused by a specific mutation, leading to a lack of activity of one important clotting factor (coagulation factor VII) which led to an increase in bleeding time. Three of his five offspring were reported to be carriers of this

mutation (Lynch et al. 2017). How the body of a calf, carrier of this hereditary coagulopathy, would react to the vascular endothelial damage caused by EEHV is unknown.

One can debate whether the initial year of the study (1985) may be considered pre- mature since, in the ’80s, diagnostic techniques for EEHV were not sufficiently developed or accurate. Part of the diagnostic gaps in the early years of the study period have been addressed by performing retrospective analyses with qPCR in frozen samples. These samples were tested to detect and quantify the virus, giving us a better idea of possible past cases that might have been overlooked (Latimer et al. 2011; Reid et al. 2006; Zachariah et al. 2013).

The narrow age range of EEHV-HD deaths found also implies that there might be an essential element that debilitates the Asian elephant calves at this specific age of their life. Therefore, a stressful element may play a part in triggering the virus, but also, there might be protective factors helping the calves that survived this risk age to overcome this period and thrive. Therefore, another worthwhile line of research would be to focus on finding what are the protective factors, especially at this sensitive young age.

EEHV should not stop breeding programs at zoological institutions due to several reasons, including the continuous decrease of the global population of Asian elephants and its endangered status to face extinction. Lethal cases of this disease are found worldwide, and reports show that EEHV is ubiquitous and that elephants are the natural host and co-evolved with EEHV (van den Doel et al. 2015; Zachariah et al. 2013). It is essential to gain a better knowledge of the disease’s pathophysiology and risk factors, to support the development of vaccination, and to improve treatment. All these research efforts to deepen this virus’ investigations can only be undertaken at a global scale and they are of extreme importance to halt EEHV-HD.

At the end of this study, 80 Asian elephant calves were at the age of the previously reported fatal cases. Therefore, routine monitoring of these young calves and preparedness to tackle this disease is crucial to favor a positive outcome of the disease, while efforts to find more epidemiological risk elements of this haemorrhagic disease should be under investigation.

Finally, this is an observational study, and therefore it is not possible to prove causality. Nevertheless, it guides us on the importance of follow-up studies to assess management conditions and to find the factors that protect or place the calf at a higher mortality risk. It is important to mention that the most meaningful and novel findings of this statistical study come from the updating and continuous analysis of a long-life living being, with a very long gestation time and inter-generational gap, enlightening the importance of longitudinal studies in

elephants. Therefore, we suspect that more fathers, mothers, and institutions will be considered as related risk factors in the future and suggest that the starting period of this study should be used as a “milestone” for further studies.

2.6. Conclusions

This longitudinal epidemiological study investigates the elephant endotheliotropic herpesvirus impact in European zoological institutions, using the largest up-to-date dataset on captive Asian elephants.

Our findings support previous studies, showing that EEHV is the primary cause of death among Asian elephants, besides neonatal mortality. Calves with EEHV-HD died at a very young age, around 2.7 years old (median age), which is a significantly younger age at death than that for other causes. Nevertheless, it is important to keep monitoring for EEHV until a later age of at least 8 years old (the oldest animal died with EEHV at 7.6 years of age).

The results of this study suggest the involvement of zoo-associated factors, which might in part be related to management, and which can be influenced by either father or mother (or a combination of both), on the onset of EEHV-HD. Indeed, in total, two fathers, one mother, and four zoos presented a higher risk for their calves to develop the disease, when compared to all others in the study, hinting at the involvement of one or more environmental and triggering elements, with possible genetic associations.

More focus needs to be placed on the underlying factors of this disease, in particular, the study of management differences between zoos with a higher risk of fatal outcomes due to EEHV-HD and low-risk zoos could inform Studbook breeding decisions.

CHAPTER 3

Assessing Coagulation Parameters in Healthy Asian Elephants (*Elephas maximus*) from European and Thai Populations.

(Published article)

Jesus, S.A.; Schmidt, A.; Fickel, J.; Doherr, M.G.; Boonprasert, K.; Thitaram, C.; Sariya, L.; Ratanakron, P.; Hildebrandt, T.B. Assessing coagulation parameters in healthy Asian Elephants (*Elephas maximus*) from European and Thai Populations**.**

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Conceptualization, S.A.J., T.B.H., and J.F.; methodology, S.A.J., T.B.H., J.F., and C.T.; C.T. and P.R. provided access to samples; K.B. had substantial contribution to sample collection; S.A.J. planned and wrote the manuscript and prepared figures and tables; T.B.H. supervised the study and funding acquisition; A.S., J.F., and L.S. processed genetic material; M.G.D. had substantial inputs in the data analysis. All authors have read and agreed to the published version of the manuscript.

3.1. Abstract

The Asian elephant population is continuously declining due to several extrinsic reasons in their range countries, but also due to diseases in captive populations worldwide. One of these diseases, the elephant endotheliotropic herpesvirus (EEHV) haemorrhagic disease, is very impactful because it particularly affects Asian elephant calves. It is commonly fatal and presents as an acute and generalized haemorrhagic syndrome. Therefore, having reference values of coagulation parameters, and obtaining such values for diseased animals in a very short time, is of great importance. We analysed prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen concentrations using a portable and fast point-of-care analyser (VetScan Pro) in 127 Asian elephants from Thai camps and European captive herds. We found significantly different PT and aPTT coagulation times between elephants from the two regions, as well as clear differences in fibrinogen concentration. Nevertheless, these alterations were not expected to have biological or clinical implications. We have also sequenced the coagulation factor VII gene of 141 animals to assess the presence of a previously reported hereditary coagulation disorder in Asian elephants and to investigate the presence of other mutations. We did not find the previously reported mutation in our study population. Instead, we discovered the presence of several new single nucleotide polymorphisms, two of them being considered as deleterious by effect prediction software.

Keywords: coagulation; Asian elephant; EEHV; factor VII; F7 gene; prothrombin; activated PTT; fibrinogen

3.2. Introduction

The world Asian elephant population faces several threats, especially in their range countries, including hunting, logging, loss of habitat, and consequent human–elephant conflict. According to the IUCN Red List, the number of Asian elephants has declined by ~50% over the last three generations (Williams et al. 2020). Captive and wild elephant health status research is therefore paramount to aid the conservation efforts for this species. Elephant blood analytical examinations most often focus on blood biochemistry and hemogram tests. Assessment of coagulation parameters is virtually never used as part of a normal clinical check-up, and very rarely are they tested before surgery or other similar invasive interventions, due to the need of specific instruments and specialized operators.

In addition to a complete blood count, coagulation time results and fibrinogen concentrations can be used as valuable and easily accessible health indicators, because stress, illness, injury, medications, and surgery affect coagulation parameters (Zoetis 2019). Coagulation times provide information in a large variety of clinical ephemerons alterations, such as sepsis, hepatic disfunction, decrease in vitamin K, shock, trauma, embolism, platelet bleeding disorders, coagulation factory deficiency, and disseminated intravascular coagulation (DIC) (Fasano and Sequeira 2017; Palta et al. 2014; Zehnder et al. 2011; Zoetis 2019). Liver disfunction may affect the coagulation cascade in several ways since this organ produces most of the coagulation factors and affects vitamin K absorption. Therefore, any illness affecting the liver, such as inflammation, neoplasia, biliary statis, and the use of chronic medication, may lead to coagulation deficiency. Infectious diseases, severe systemic diseases, or immune-mediated diseases can also alter normal coagulation times. Due to this panoply of factors that may affect coagulation, it is suggested that coagulation times should be accessed as a pre-surgical test for any animal, regardless of age (Zoetis 2019). Fibrinogen is used as a specific and sensitive marker for inflammation in humans (Davalos and Akassoglou 2012) and in horses, for example, and its early recognition has been shown to be essential for the diagnosis of diseases and proper treatment planning. In horses, fibrinogen serial testing provides information regarding treatment efficacy in length and prognosis in several infectious or inflammatory conditions, such as pleuropneumonia, abdominal abscess, endometritis, and endocarditis (Zoetis 2019). Coagulation is a process activated after a vessel damage, when the body reacts in order to stop the haemorrhage, locally creating a viscous and thick material—a clot—to seal this lesion (Fasano and Sequeira 2017; Norris 2003; Thornton and Douglas 2010). Platelets start to adhere to the subendothelium, forming a plug, and sequentially activated intervening factors (coagulation factors) start interacting in a so-called “cascade”, in order to produce fibrin. Fibrin fibres form a mesh over the platelets, creating a seal at the injury site to stop further blood loss (Fasano and Sequeira 2017; Norris 2003). The regulation of this process is fine-tuned in order to control the growth of the clot and to prevent the aggregation of a thrombus, which can lead to complications such as stenosis or embolism (Fasano and Sequeira 2017; Norris 2003). Therefore, coagulation is a dynamic process between coagulation-promoting mechanisms and those that stop it from expanding beyond the injury site. Such maintenance of haemostasis is essential to avoid both continuous bleeding and thrombosis (Norris 2003; Palta et al. 2014).

The “cascade model of coagulation” is the model most traditionally used to explain the complex process of clot formation. According to this model, a stepwise enzymatic conversion of zymogens (precursors that circulate in an inactive form in the plasma) leads to the final product, a fibrin clot. This synchronized enzymatic activation along the coagulation cascade

splits into two main pathways: the extrinsic pathway (after vessel wall damage, it includes tissue factor and factor VII) and the intrinsic pathway (involving contact with a negatively charged surface and coagulation factors V, VIII, IX, XI, and XII). Both pathways then converge in the activation of factor X, leading to a final common pathway where fibrinogen is converted into fibrin (Adams and Bird 2009; Norris 2003; Palta et al. 2014). The time needed for clot formation can be measured using the prothrombin time (PT) for the extrinsic pathway and using the activated partial thromboplastin time (aPTT) for the intrinsic pathway (Fasano and Sequeira 2017).

In humans, numerous genetic mutation(s) of the F7 gene, the gene encoding coagulation factor VII, are known to cause deficiency and reduced activity of this factor, leading to an overall reduction in the coagulation efficiency. The condition can be inherited or acquired, transmitted with autosomal recessive inheritance, and is among the rare congenital bleeding disorders—it is the most commonly present (Mariani and Bernardi 2009). The most common type of mutation is point mutation (single-nucleotide polymorphism, SNP), which can either be silent (i.e., synonymous) when the coded amino acid (aa) sequence stays the same, or it can be a missense variant (i.e., non-synonymous) when the change causes an alteration in the aa sequence of the encoded protein. In humans, 221 unique variants have been reported so far for the F7 gene (Giansily-Blaizot et al. 2020; McVey et al. 2020). People with factor VII deficiency may experience prolonged and uncontrolled bleeding episodes with initial onset and bleeding severity varying greatly among people. While some individuals are asymptomatic, others may develop mild, moderate, or even severe life-threatening complications as early as in infancy (Mariani and Bernardi 2009). As in humans, factor VII deficiency has also been reported several times in dog breeds, such as Beagles, English Bulldogs, Alaskan Malamutes, Boxers, and also in mixed-breeds (Cotter 2019). Like humans, the symptoms of this deficiency also vary in dogs, as there the disease is normally not accompanied by spontaneous bleeding, although some animals present bruises and prolonged bleeding after surgical intervention (Cotter 2019). In 2017, a factor VII deficiency was also detected in an Asian elephant bull, and, although the animal did not have a bleeding tendency, it demonstrated a prolonged PT time. After further investigation, a deleterious mutation on the F7 gene was detected that was also passed onto his offspring (Lynch et al. 2017). Therefore, we know that factor VII deficiency is also present in Asian elephants, but the degree of its distribution in the population is unknown. Such knowledge is particularly important as Asian elephant can be struck by elephant endotheliotropic herpesvirus haemorrhagic disease (EEHV-HD), which causes acute generalized haemorrhagic diathesis due to capillary endothelial lesions (Ossent et al. 1990). How this vascular endothelial damage caused by the virus affects an elephant carrier of factor VII hereditary coagulopathy is still

unknown; therefore, it is important to investigate. EEHV-HD have been intensely studied in the past two decades, especially in Asian elephants (Fickel et al. 2001, 2003; Long et al. 2016; Ossent et al. 1990; Reid et al. 2006; Richman et al. 1999; Schaftenaar et al. 2010). The disease is responsible for a high fatality rate in very young calves worldwide, reaching more than 50% of all captive born deaths above one day of life, for the US and European zoos (Howard and Schaftenaar 2019; Jesus et al. 2021; Perrin et al. 2021b). Therefore, due to the impact of this disease and its haemorrhagic characteristics, research teams have dedicated more attention to the coagulation status of this species. Several coagulation assessment studies of Asian elephants have recently been published using different diagnostic methodologies based on human plasma as reference (Gentry et al. 1996; Kaye et al. 2016; Lynch et al. 2017). Additional studies focused on host blood viscoelasticity via thromboelastography (Flanders et al. 2018; McCann et al. 2019; Perrin et al. 2018). Understanding elephant haemostasis has become a very important goal, both to improve the general knowledge base for elephant health status assessments, and to decipher the mechanisms by which EEHV-HD acts.

With this study we aim at increasing the knowledge of some of the most common coagulation parameters in a practical way and to obtain results in just a few minutes. Furthermore, we wanted to look at the possible genetic involvement of a hereditary factor VII deficiency on this disease onset and outcome. Although other coagulation factor deficiencies might be present, this investigation focuses only on the detection of a previously reported hereditary disorder involving factor VII in Asian elephants. For this, we have investigated the presence of mutations on the F7 gene in calves that have survived the disease, calves that have died with EEHV-HD, and other elephants that never presented symptomatology of EEHV-HD.

3.3. Materials and methods

A total of 167 Asian elephants were assessed in our study. According to the specific parameter analysed, the sample size varies, due to several reasons, because, for example, animals tested using stored frozen samples from past EEHV-HD fatalities were not assessed for coagulation times due to the impossibility to collect fresh blood. Fresh blood samples were collected from 127 Asian elephants in 21 zoos in Europe and in 10 Asian elephant touristic camps or farms in Thailand (Supplementary Materials Table S3.1). Samples were collected by blood draw during routine check-up examinations, without sedation of the animals. Blood was either collected by natural flow, by using a butterfly needle or was drawn with a syringe attached to a needle with an adequate gauge size to avoid mechanical haemolysis. Most of

the samples were collected by venipuncture of the ear vein, the rest were drawn from the saphenous vein in the hind leg.

3.3.1. Coagulation time and fibrinogen measurements—clinical haemostasis evaluations

After collection of the sample to a syringe, blood was distributed to a 2 mL ethylenediaminetetraacetic acid (EDTA) anticoagulating tube, and to sodium citrate tubes of 1.3 mL (3.2–3.8% concentration; KABE Labortechnik GmbH, Nümbrecht-Elsenroth, Germany), or, exceptionally, 2.5 mL citrate tubes were used. To avoid further haemolysis, the needle was detached before transferring the blood from the syringe to the tubes. Samples collected to EDTA tubes were stored in −20 °C or −80 °C. Specific sodium citrate-coated tubes were used immediately to measure the following three coagulation parameters: prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen concentration. Whenever possible, PT and aPTT were performed immediately in the first 20–30 min after blood collection using the portable coagulation diagnostic analyser VetScan® VSpro Specialty Analyzer (ABAXIS Europe GmbH, Griesheim, Germany). Equipped with test specific cartridges, the analyser allows in vitro determination of PT and aPTT times using cat and dog as reference species (Coagulation Cartridge, Abaxis Inc., Union City, CA, USA) and fibrinogen concentration (Fibrinogen Test Cartridge, Abaxis Inc., Union City, CA, USA) using horse as a validated species. The analyser as also been used in smaller species and it is validated for lower volume of samples (Condrey et al. 2020); therefore, it is also applicable to use in small wildlife species. A combined PT/aPTT single test measurement offers a rapid quantitative result. A microcapillary designed test aspirates the citrated whole blood from a reservoir. By traveling through two parallel capillary paths, the blood is in contact with activators for coagulation. A light system detects when these microcapillaries blood flow stops, being this the test endpoint and the final quantitative coagulation time (Hyatt and Brainard 2016).

The fibrinogen test was measured by thrombin-mediated enzymatic conversion to fibrin, being applicable to other species (Condrey et al. 2020).

All measurements were performed according to the manufacturer’s protocol. Analysis of fibrinogen were not always achieved for all individuals due to presence of haemolysis in the plasma samples. Cartridge loading with blood samples was performed very carefully to avoid haemolysis and foaming, both of which could lead to erroneous test results.

3.3.2. Platelet counts

Blood smears were performed immediately after blood collection, using one drop of blood collected into the EDTA-coated tubes. These smears were then stained with Diff-Quick (Medion Diagnostics AG, Düdingen, Switzerland). The remaining EDTA blood was stored at −20 °C or −80 °C until further investigation. The stained smears were used to count the platelets under microscopic oil immersion objective observation. A total of ten fields were counted, and the final platelet count was obtained by calculating the average of these fields multiplied by 15,000.

3.3.3. Sample collection for the analysis of the coagulation F7 Gene

For the analysis of coagulation F7 we used frozen blood samples collected into EDTA tubes and stored at −20 °C or −80 °C, in order to preserve DNA content. Tissue samples (liver, myocardium, tongue, etc.) from dead elephants were also analysed, including samples from calves that died due to EEHV-HD (Supplementary Material Table S3.1).

3.3.4. DNA extraction

For blood samples (200 µL EDTA-blood) from European animals, we used the “DNA blood extraction kit”, while for tissue samples, we applied the “Tissue DNA Mini extraction kit” (both peqLab Biotechnology, Erlangen, Germany). Thai Asian elephant DNA was extracted from blood samples, using Genomic DNA Mini Kit (Geneaid, New Taipei city, Taiwan). All extraction procedures followed the respective manufacturer protocols. DNA concentrations were measured using a NanoDrop™ One/One^{C} spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). DNA solutions were stored at −20 °C degrees.

All Asian elephant samples from European zoos were processed at the Leibniz-Institute for Zoo and Wildlife Research (IZW, Berlin, Germany) and analysed for molecular characterization of the F7 gene. Asian elephant samples collected in Thailand were processed at the Faculty of Veterinary Science at Mahidol University, Bangkok, and sequenced externally (U2Bio, Sequencing Service, Bangkok, Thailand).

3.3.5. Amplification and sequencing of DNA

Primer sequence information and amplification conditions were obtained from a previous study (Lynch et al. 2017), and optimized with minor modifications to cycle number and primer combination (Table 1).

PCRs were performed in a final volume of 25 µL consisting of 2 µL of DNA extract, 0.5 µL **(**final concentration of 0.2 µM**)** of each primer (Biolegio, Nijmegen, The Netherlands), 12,5 µL DreamTaq MasterMix, and completed with 9.5 µL nuclease-free water (both Thermo Scientific, Vilnius, Lithuania).

Table 1. Names and sequences of the forward and reverse primers (5′–3′) used to amplify the eight exons of the F7 gene.

F7e1_F	**GAGCAGCTGAGGAACTTAGC**	**F7e1_R**	**CCCACTTTCCAGATTTGAGG**
F7e2_F	TACAAGCCAGGAGAAGGAGC	F7e2_R	ATGGACTCCAGGAGACATGG
F7e3_F	TCTGTGGCTGACTTGTTTGC	F7e3_R	AGAAGGGGGTGAGGTAGGG
F7e4_F	AACTCACCGCCATCTCTCC	F7e4_R1	TCAACACTCTCAGATTGGAAGG
F7e5_F	CTGTACCAGCTGCTTTTCCC	F7e5_R1	TCAGTAAAGGTTATGCCCGC
F7e6_F	AGCTCAGGCAGATGTAACCC	F7e6_R1	GCTGACCTGCCATTTTTCTC
F7e7_F	GCCAGATAAGAGGGCAGTTG	F7e7_R1	CGATAGCAGAGAGGTTTGCC
F7e8_F1	TGACAGGCCAAAGACACAAC	F7e8_R1	GTCCCATCCAGGTAGCCAG
F7e8_F2	ACGTAGTGCCCCTCTGTTTG	F7e8_R2	GCAGCAGCAGCTTTATTTCC
F7e8_F3	TCTCCCGGTACATTGAGTGG	F7e8_R3	GACGTCCATCTCTCTCAGCC

In (Lynch et al. 2017), exons 3 and 4 were amplified with two different primer pairs. From these, we only used the 2nd pair for exon 3 and from exon 4 information we used the forward primer 1 in combination with reverse primer 2 to amplify the final exon 4.

PCRs were performed on G-STORM GS1 thermocycler (Gene Technologies Ltd., Somerton, UK). Cycling conditions for all exons but exon 4 were: 95 °C 3 min, 35 × (95 °C 30 s, 58 °C 30 s, 72 °C 1 min), final extension at 72 °C 7 min, followed by eternal 20 °C. For exon 4 we applied an annealing temperature of 53 °C. Presence of PCR products was visualized by electrophoresis on 1% agarose gels.

Prior to the subsequent sequencing excess primers and dNTPs were removed using the ExoFastAP Purification Kit (Thermo Scientific™, Schwerte Germany). Amplified F7 exons

were then Sanger sequenced bidirectionally using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems – Thermo Fisher Scientific, Waltham, MA, US). Terminated fragments were separated on an ABI 3130*xl* Genetic Analyzer and visualized using the Sequencing Analysis Software v5.2 (both Applied Biosystems®, USA). After removal of primer sequences F7 exon fragments were mapped to using Geneious (v8.0.5, https://www.geneious.com, San Diego, CA, USA), and *Loxodonta africana* F7 was used as a comparative reference (Genbank acc. No. NM_001330481.1). Finally, we used the software SeqMan Pro (DNASTAR Lasergene package v11.2.1, Madison, WI, USA) to generate the assembly of the gene.

Amplifications and sequencing were performed for 65 European elephants (from a total captive population of 307 individuals (Schmidt and Kappelhof 2019)). Only six of the Thailand population had the full gene sequenced. The other 70 were only sequenced for the exons which we determined to present missense (non-synonymous) mutations (exons 2, 4, and 5), due to cost restriction. Nevertheless, the samples obtained in Thailand were amplified using the same PCR protocol and same primer pairs. Successful amplicons were sent to U2Bio (Bangkok, Thailand) Sequencing Service for DNA sequencing, and the obtained sequences were sent to IZW, Berlin, to be added to the Asian elephant F7 data set for analysis in Geneious (v8.05).

3.3.6. Data selection and analysis

Average coagulation times were estimated per study region (Thailand, Europe) and then estimated by age class for each gender. We assigned animals to 1 of 5 age classes: class (1) from birth until four years old, class (2) from five to nine years old, class (3) from ten to 19 years old, class (4) from 20 to 34 years old, and class (5) older than 35 years. Two foetuses (one male and one of unknown gender) were sequenced and analysed for the F7 gene and belong to age class 0; therefore, these are not represented in the coagulation parameters analysed with fresh blood. The category of EEHV-HD status separated healthy individuals that never presented EEHV symptoms and calves which were diseased with EEHV-HD. Database can be found in Supplementary Materials Table S3.1.

Univariate analysis of variance (UNIANOVA) and tests of between-subject effects were used to evaluate the effects of the study region and EEHV-HD status on overall coagulation times, fibrinogen concentration, and platelet count values. The same statistical tests were used to investigate the influence of gender and age class in the mean results of PT and aPTT times, fibrinogen concentration, and platelet counts. To account for multiple comparisons, we performed multiple-comparison post hoc statistical tests (Tukey-HSD and Bonferroni).

Single nucleotide polymorphisms (SNPs) were evaluated for possible impact on the factor VII protein structure in comparison with complete F7 gene, which, currently, is only available for *Loxodonta africana*, as carried out by the reference study (Lynch et al. 2017), and using the web-based protein variation effect predicting software packages SIFT (Vaser et al. 2016) and PROVEAN v1.1.3 (Choi and Chan 2015). For the mutations considered to be deleterious and not tolerated, a Kruskal–Wallis test was applied to evaluate the impact of the SNP in the PT time of coagulation. To compare the genotypes, more specifically, to assess the differences between SNPs causing missense mutation, between region (Thailand and Europe) and between different EEHV-HD status (regardless of the region), we used Fisher exact test and chi-square tests.

Data analysis using UNIANOVA, tests of the between-subject effects and post hoc multi comparison tests were conducted using IBM SPSS Statistics (version 24.0, Armonk, New York, NY, USA) predictive analytics software. Missense mutation analysis using Fisher's exact test, chi-square tests and drawing of graphs were performed using GraphPad Prism (v9, GraphPad Software, San Diego, CA, USA). Statistical significance was designated at $p \leq 0.05$. Unless stated otherwise, results in the text are presented as means.

3.4. Results

3.4.1. Overview of the study population

A total of 167 Asian elephants (n = 76 Thai elephants and n = 91 European), were analysed. Females (n = 104) were on average 25 years old (SD 14), while males (n = 37) were on average 18 years old (SD 16). This age difference was significant (p = 0.013) for both regions, but not significantly different within regions (p = 0.149).

For the Thai population, we found that both females and males were, on average, 21 years of age. On the other hand, European female elephants in the study presented a mean age of 28 years old, while the males were younger, at around 17 years of age.

3.4.2. Influence of location and EEHV-HD status on coagulation time, fibrinogen concentration, and platelet counts

Results presenting means, SD, and population sample size used for each of the following tested parameters are presented in Table 2.

Table 2. Estimated mean and SD of prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and platelet counts for different groups of individuals, sorted according to study region, gender, age class, and known presence or absence of EEHV-HD.

		PT (s)			aPTT (s)			Fibrinogen (mg/dL)			Platelet Count (×10^3/µL)		
EEHV	**REGION**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
No EEHV-HD	Thailand	17.13	0.86	57	143.80	18.68	57	467	112	57	540	274	49
	Europe	17.51	1.23	62	126.10	17.31	62	601	179	54	604	173	58
	Total	17.33	1.08	119	134.58	19.98	119	530	167	111	575	226	107
EEHV-HD survivors	Thailand	17.45	0.54	6	123.40	22.14	6	481	162	6	701	218	6
	Europe	18.70	0.71	2	109.15	2.90	2	560	.	1	281	198	2
	Total	17.76	0.79	8	119.84	19.87	8	492	151	7	596	278	8
Total between Groups	Thailand	17.16	0.84	63	141.86	19.77	63	468	116	63	558	271	55
	Europe	17.55	1.23	64	125.57	17.30	64	601	111	54 *	594	182	60
	Total	17.36	1.07	127	133.65	20.22	127	530	132	117	576	229	115
Gender	**AGE class**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
F	1	16.88	0.74	4	128.43	2.08	4	526	153	4	433	215	3
	2	17.12	1.40	9	125.61	21.56	9	423	103	9	693	408	6
	3	17.46	0.91	11	133.43	16.45	11	555	135	11	500	167	10
	4	17.46	1.13	37	132.18	18.67	37	560	116	35	627	216	36
	5	17.26	1.14	24	130.42	17.20	24	661	247	22	522	181	24
	Total	17.34	1.11	85	130.97	17.66	85	570	176	81	577	225	79
M	1	18.00	1.03	4	118.25	10.66	4	597	35	3	555	336	4
	2	16.98	0.43	4	133.05	17.02	4	578	119	3	615	58	4
	3	17.38	0.68	9	141.07	15.36	9	503	140	8	646	249	7
	4	17.53	1.99	6	130.13	35.49	6	533	57	4	448	116	4
	5	17.28	0.51	4	137.90	12.53	4	470	162	4	394	210	3
	Total	17.43	1.08	27	133.60	20.99	27	525	119	22	553	223	22
Total between "age classes"	1	17.44	1.03	8	123.34	8.95	8	556	116	7	503	276	7
	2	17.08	1.17	13	127.90	19.88	13	462	123	12	662	308	10
	3	17.43	0.80	20	136.87	16.03	20	533	136	19	560	211	17
	4	17.47	1.25	43	131.89	21.19	43	558	111	39	609	215	40
	5	17.27	1.07	28	131.49	16.63	28	632	244	26	508	185	27
	Total	17.36	1.10	112	131.60	18.45	112	561	166	103	572	223	101

Age classes: class (1) 0–4 years old, class (2) 5–9 years old, class (3) 10–19 years old, class (4) 20–34 years old, and class (5) > 35 years old. F—female; M—male; SD—standard deviation of mean; N—number of individuals in the respective group. * After removal of outlier animal "122" (Supplementary Materials Table S3.1). EEHV-HD—elephant endotheliotropic herpesvirus haemorrhagic disease.

Coagulation times

A total of 127 elephants (63 from Thailand and 64 from Europe) were assessed for their prothrombin (PT) and activated partial thromboplastin time (aPTT). PT time was significantly lower in the Thai group then in the European group (p = 0.026). Thai elephants had an average PT of 17.16 ± 0.58 s, while European elephants had an average of 17.55 ± 1.23 s. In contrast

average aPTT was significantly lower ($p < 0.0001$) in the European group (125.57 ± 17.30 s), than in the Thai group (141.86 ± 19.77 s; Figure 8).

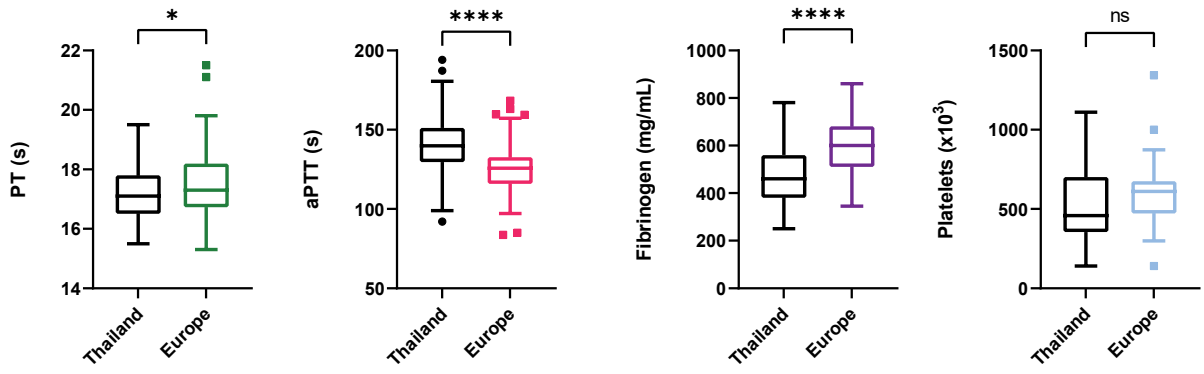

Figure 8. Boxplot of the PT, aPTT, fibrinogen, and platelets values grouped by study region. The box represents the 25th to 75th percentile values of the distribution (interquartile range), the line within the box represents the median (50th percentile), and the whiskers approximate the 2.5th and 97.5th percentile values. Stars indicate significance threshold. *: $p < 0.05$, ****: $p < 0.0001$; ns—not significant.

Concerning EEHV-HD, no significant difference in PT time ($p = 0.158$) was found between the group of calves having survived the disease ($n = 8$) and the rest of the population ($n = 119$). In contrast, aPTT was significantly different between these calves and the rest of the population ($p = 0.004$).

Fibrinogen

One individual from Europe was removed as outlier from the comparison due to its extremely high fibrinogen concentration (1633 mg/dL). Therefore, we investigated fibrinogen concentration in a total of 117 elephants and a highly significant difference between the two groups was found ($p < 0.0001$). The Thai elephant group ($n = 63$, had a lower mean value (468 ± 116 mg/dL), than the European group ($n = 54$, 601 ± 111 mg/dL). Fibrinogen values of calves that had survived EEHV-HD ($n = 7$) did not significantly differ ($p = 0.902$) from animals that never had the disease (Figure 8).

Platelet Counts

Although Asian elephants from European zoos had, on average, higher overall platelet counts, that difference was not significant ($p = 0.376$) in comparison to the Thai elephants and a

minimal difference on the platelet counts separate them; Europe: n = 60, mean = 594 × 10^3/µL ± 182; Thailand: n = 55, mean = 558 × 10^3/µL ± 271). Platelet counts found for EEHV-HD survival calves were also not significant (p = 0.708) (Figure 8).

3.4.3. Influence of gender and age class on coagulation time, fibrinogen concentration, and platelet counts

Although not significant, males had a slightly prolonged coagulation time, a lower platelet count, and lower fibrinogen concentration than females (Table 2).

Coagulation times

No effects associated with age (p = 0.816) or sex (p = 0.700) were found to influence the overall prothrombin time. An overall average of 17.36 s was found (±1.10) for the Asian elephants in the study (Table 2).

In similarity with the results of PT times, we found that for aPPT no age (p = 0.442) or gender (p = 0.504) was associated with difference in values, and on average, we found that this coagulation route lasts 131.60 s (±18.45) (Table 2).

Fibrinogen

For the 103 elephants analysed for fibrinogen concentration (females n = 81, males n = 22), an average of 561 mg/dl (±166) was found (Table 2). Sex did not prove to have an impact on the total fibrinogen concentration (p = 0.419). However, although not significant, the first two age groups— (1) calves until four years of age and (2) young elephants from five to nine–years old—presented an overall lower value of fibrinogen, compared with elephants of older ages, independent of the region. Furthermore, between the age group (2) (from 5–9 years old) and group (5) (older than 35 years), there was a borderline effect for the younger ages to present a lower average fibrinogen concentration (p = 0.057).

Platelets

No significant difference on the platelet counts was recorded between the different age groups (p = 0.231), or gender (p = 0.665), and an average of 572 × 10^3/µL was found for the elephants in our study (Table 2).

3.4.4. Coagulation factor VII gene (F7)

Analysis of F7 gene sequences

The alignment of sequences from 141 individuals were compared with the available *Loxodonta africana* reference and showed ten polymorphic positions (Table 3) distributed in exons 2, 4, 5, and 8. Of these, six were silent (synonymous), but four caused missense (non-synonymous) mutations. These SNPs were present in exons 2 (C193G), 4 (C332T), and 5 (T437A and G509A).

Table 3. Single nucleotide polymorphisms (SNPs) found in four exons of the coagulation factor F7 gene in the Asian elephants evaluated in this study. SNPs are listed according to their position, alteration in the codon, and type of mutation.

Exon	SNP Position	Codon Change	Type of Mutation
2	C142G	CTG > GTG	missense
4	C281T	CCG > CTG	missense
	G294C	GGG >GGC	silent
	G300C	CTG > CTC	silent
5	T386A	CTG > CAG	missense
	G458A	CGA > CAA	missense
	T489C	GAT > GAC	silent
8	C870T	CGC > CGT	silent
	C975T	AGC > AGT	silent
	T1161C	AGT > AGC	silent

Positions refer to the *Loxodonta africana* F7 cDNA without 5`untranstlated region (Genbank acc.no NM_001330481.1). The actual position of the SNP in the triplet is underlined.

Distribution of missense SNPs in the European and Thai populations

The distribution of polymorphisms causing missense mutations was significantly different between study regions only in exon 2 ($p < 0.0001$), exon 4 ($p = 0.47$), and exon 5 (T386A $p = 0.59$ and G458A $p = 0.89$; positions of the SNPs detected in the F7 gene can be found in the Supplementary Materials Table S3.2).

For the biallelic SNP C142G on exon 2, we sequenced 136 animals of which 80 were homozygous for this SNP (C/C *n* = 67, G/G *n* = 13) and 56 were heterozygous. When comparing exon 2 SNP allele distribution by study region, we found a significant difference between the regions. In the Thai elephant population analysed (*n* = 76), the majority of individuals (66%) carried the homozygous C/C wild-type genotype (i.e., the allele from the *Loxodonta africana* reference sequence), while among the Asian elephants analysed from European zoos (*n* = 60), the majority (57%) carried the heterozygous C/G genotype (*n* = 34) (Table 4). The exon 2 “G”-allele of the F7 gene will cause a substitution of leucine by Valine (Leu48Val) in the factor VII protein. Both protein effect prediction software packages (SIFT, PROVEAN) considered such amino acid exchange to be tolerable and likely to have a neutral effect (Table 4).

Table 4. Distribution of the missense mutations found, by region, by non-EEHV symptomatic elephants and EEHV-HD symptomatic calves. Prediction of aa substitution and its impact on the biological function of the protein tested are presented for both PROVEAN and SIFT software.

Missense SNP	Amino Acid	SIFT	PROVEAN	State	Thailand	Europe	Total	No EEHV-HD	EEHV-HD	Total
exon2, C142G										
C				wild-type *	50	17	67	62	5	67
C/G	Leu48Val	Tolerated	Neutral	Heterozygous	22	34	56	50	6	56
G	Leu48Val			Homozygous different	4	9	13	11	2	13
Total					76	60	136	123	13	136
exon4, C281T										
C				wild-type	70	49	119	108	11	119
C/T	Pro94Leu	Not Tolerated	Deleterious	Heterozygous	6	2	8	7	1	8
Total					76	51	127	115	12	127
exon5, T386A										
T				wild-type	0	0	0	0	0	0
A	Leu129Gln			Homozygous	75	63	138	126	12	138
A/T	Leu129Gln	Not Tolerated	Deleterious	Heterozygous	1	2	3	3	0	3
Total					76	65	141	129	12	117
exon5, G458A										
G				wild-type	75	63	138	126	12	138
G/A	Arg153Gln	Tolerated	Neutral	Heterozygous	0	2	2	2	0	2
A	Arg153Gln	Tolerated	Neutral	Homozygous	1	0	1	1	0	1
Total					76	65	141	129	12	141

* wild-type here indicates matching to the F7 gene of *Loxodonta africana* (GenBank acc.no. NM_001330481.1); EEHV: elephant endotheliotropic herpes virus; EEHV-HD: EEHV haemorrhagic disease.

There were two of the three SNPs detected in F7 exon 4 which were synonymous, while the 3rd (C281T) was non-synonymous. The exon 4 “T”-allele causes an amino acid substitution from proline to leucine (Pro94Leu; Table 4). The majority of both Thai elephants (*n* = 70) and of Asian elephants from European zoos (*n* = 49) were homozygous for the wild-type “C”-allele encoding proline at that position. A total of eight animals (Thailand *n* = 6, European zoos *n* = 2) were heterozygous and none were homozygous for the “T”-allele. The substitution of proline by leucine was predicted to be deleterious for the structural integrity of factor VII protein (Table 4). Out of the six heterozygous individuals that had been tested for PT time, five had a significantly higher PT time than the population mean ($p = 0.017$).

In F7 exon 5, we detected one silent mutation and two missense mutations. For the first one of the two missense mutations, no animal was homozygous for the wild-type (*Loxodonta africana*) “T”-allele and only three elephants were heterozygous (T/A). The majority of the population (across both “study regions”) was homozygous for the “A”-allele. This allele leads to a substitution of leucine by glutamine (T386A; Leu129Gln), which was predicted to be deleterious for protein integrity. Heterozygous elephants “A/T” were very rare (*n* = 3). For the second SNP having a missense allele (G458A), we only detected three animals to carry the non-synonymous “A”-allele (in a total of 141 sequenced individuals). One of them was homozygous (A/A) and two heterozygous (G/A). The “A”-allele will cause a substitution of arginine by glutamine (Arg153Gln). However, this alteration was predicted to be tolerated and neutral (Table 4).

Distribution of missense SNPs between non-EEHV and EEHV symptomatic cases

We found no association between the distribution of any of the detected missense SNPs, neither being heterozygous nor homozygous, and a previous symptomatology of EEHV-HD. Thus, none of the missense mutations detected in this study could be associated with the chance of developing EEHV-HD ($p > 0.05$, for all exons).

3.5. Discussion

In the present study, we analysed the coagulation time (PT and aPTT), fibrinogen concentrations, and platelet count of Asian elephants from 10 camps in Thailand and 21 European zoos with a new and fast results method. The large dataset presented here, which gathered data from a broad range of age classes, gives us good reference values for coagulation parameters in the Asian elephant population. To the best of our knowledge, this is the first study using a VSPro, a very fast diagnostic point-of-care analyser, specifically for

measuring coagulation time and fibrinogen concentration in elephants. Furthermore, we investigated the presence of genetic mutations in the coagulation F7 gene, and their possible connection to hereditary coagulation disorder.

3.5.1. Fast diagnostic analyser (VSPro)

The materials and methods used in this study to obtain coagulation times and fibrinogen concentration were designed to minimize procedure times. The VSPro analyser has been previously used and compared with other traditional laboratory methods in dogs [23], where it yielded reliable results for detecting abnormalities in PT and aPTT (Dixon-Jimenez et al. 2013). Similar to these previous results, in the present study, we obtained a readout of a PT/aPTT coagulation time as fast as 3 min. Together with its simplicity of use, the VSPro analyser proved to be of advantage when analysing these parameters in elephants.

The fastest result for fibrinogen concentration obtained in our study was at 10 min after the initiation of the protocol. Therefore, our method is so far the fastest technique for PT/aPTT measurement and fibrinogen concentration evaluation in elephants, which will be essential in emergency situations, as for example during an EEHV-HD outbreak. Our approach also allowed us to drastically reduce the possibility of laboratory work associated errors and divergences that would derive from sending the blood samples for analysis to different laboratories, potentially even using different methods. The manufacturer's protocol recommends the equipment to be used between 15 °C and 30 °C. These recommendations could not always be followed during fieldwork, as it was necessary to analyse some samples at temperatures < 15 °C (winter in Europe) and > 30 °C (summer in Thailand). There was only one day with ambient temperatures > 40 °C when the VSpro stopped functioning properly (displaying an overheating alert). On all other out-of-recommended-range temperature days the device worked normally.

Venipuncture and blood drawing are part of a routine veterinary procedure to check an animal's health status. It is also considered a minimally invasive method for sample collection. Therefore, when combined with regular check-ups, no additional stress or pain was caused to the animals sampled for this study. Additionally, this procedure did not require sedation, which could affect the blood coagulation cascade. Low stress is also important to prevent the risk of spleen contraction, which could increase cell count, platelet count, and aggregation, and alter several coagulation factors levels, including fibrinogen. This would influence the coagulation cascade and bias the PT and aPTT measured in this study.

In most extinction-threatened species, coagulation is still rarely investigated. We hope with this research to provide practitioners and researchers with a quick and simple tool that can be easily implemented to further explore coagulation research in zoo and wildlife species.

3.5.2. Coagulation times

We found that neither gender and nor age influenced PT and aPTT times, fibrinogen concentrations, and platelet counts. Although the difference was not significant, males had in general a prolonged coagulation time, a lower platelet count, and a lower fibrinogen concentration than females. Unfortunately, our data set consisted of almost three times as many females than males (85 vs. 27). So, this result needs to be further investigated in a better gender-balanced study.

A significant difference, however, was found in PT times between Asian elephants from European zoos and Thai camp elephants. Due to our large sample size, we were able to even detect a small difference of just 0.39 s. However, such small difference will not cause any biological effect of clinical significance in the coagulation capacity, and therefore no therapy is advised. Our PT results (*n* = 127, mean = 17.36 s, SD = 1.07 s) were higher than previous studies reported for Asian elephants using much smaller sample sizes and different measuring methods (*n* = 7, PT-simp: mean = 9.6 s, SD = 0.7 s and PT-inn: mean = 10.3 s, SD = 1.1 s (Gentry et al. 1996); *n* = 6, PT median = 14.74 s (range 11.6–20.9 s) (Kaye et al. 2016); *n* = 23, PT: median = 11.0 s (range 9.7–14.9 s) (Perrin et al. 2018). This suggests that each measuring method will give different results for healthy elephants rendering the comparison of results from different methods impossible without reference samples.

For aPTT, we also found a statistically significant difference between elephants from the two study regions, with lower times for the European zoo elephant population. We do not expect this result to translate into biological effects in the coagulation capacity. Nevertheless, we found a big range of aPTT values in our study population (*n* = 127; min = 84 s, max = 194 s) when compared with other species also analysed with VSPro, where a smaller range of time is reported (manufacturer reference ranges: dogs 71–105 s, cats 86–137 (Zoetis 2019), other studies: *n* = 109 guinea-pigs 61–84 s (Condrey et al. 2020), *n* = 14 wallabies 71–84; although, PT could not properly be measured using this POC for this species (Nevitt et al. 2016)). In a normal human population, aPTT is known to also vary greatly between individuals, and this wide reference range interval is affected by several causes, such as biological variability, instrumentation and reagent variability, and physiological changes, such as pregnancy, physical stress, or trauma.(Levy et al. 2014)

Previous tests to determine aPTT values in elephants used different methods and were based on human plasma as reference for comparison (Gentry et al. 1996; Kaye et al. 2016; Perrin et al. 2018). Their results differ greatly in scale from the values measured in our study. A coagulation deficiency is reported to become evident when PT or aPTT is greater than 1.5× above the upper end of the reference range (Condrey et al. 2020). Assuming that this applies as well for other mammalian species and specifically to elephants, this supports the notion that the PT and aPTT differences found between “study regions”, even though they were statistically significant, do not bear clinical relevance. Accordingly, and combined with the healthy status of the animals sampled and all values being within the manufacturers range limit, we assume that all animals in our study had a normal aPTT coagulation time. The combined results from PT and aPTT measurements suggest that these values should define a new reference value for practitioners using this method on Asian elephants and will allow to stop hitherto applied comparisons with several different techniques. Having a large data set composed of Asian elephants from different regions and from a wide age range, we consider our values reliable and reproducible.

Regarding the findings on the EEHV-HD status, no significant difference was found for PT time between the surviving calves and the rest of the population ($p = 0.158$). In contrast, aPTT varied significantly between these groups ($p = 0.004$). However, both results could be due to the difference between sample sizes ($n = 8$ survivors; $n = 119$ non EEHV-HD cases), where the number of survivors might be too small to detect a difference.

3.5.3. Fibrinogen

Although not significant, we found a tendency for the younger age classes (0–9 years of age) to have a lower fibrinogen concentration than the older elephants (especially those older than 35 years), independent of the study region.

In our Asian elephant sample set the mean fibrinogen concentration was 561 ± 166 mg/dl, higher than reported in previous studies (Gentry et al. 1996; Kaye et al. 2016; Perrin et al. 2018; Salakij et al. 2005; Silva and Kuruwita 1993). As these studies had used measuring methods differing from ours, we assume that this may be a method related difference.

We found an outlier in our initial study population, presenting more than three times the average concentration found for the sampled population in the study. This animal was sampled during the process of foetal mummification. Foetal retention in elephants is not an uncommon phenomenon and there are several reports of interrupted parturition with retention of up to 84

months (Hermes et al. 2008; Schaftenaar 2013; Thitaram et al. 2006). This finding emphasizes the importance of fibrinogen measurement, which can be used as a useful diagnostic tool for health routine check-up.

3.5.4. Platelets

An average of 572 × 10^3 platelets/μL was found for the elephants in our study, which is in accordance with previous Asian elephant haematology studies (Niemuller et al. 1990; Pich et al. 2016; Salakij et al. 2005) and lower than one reported study (Lewis 1974). Several outliers were found in the platelet count analysis, presenting values reaching up to 1343 × 10^3 platelets/μL. No disease was diagnosed at the time of sampling for these animals, so we cannot attribute these results to any sickness or health compromised status.

3.5.5. Genetic analysis of F7 gene

A previous study in the F7 gene of Asian elephants reported a deleterious mutation in a single nucleotide position (SNP A202G) which was attributed to prolong PT time (Lynch et al. 2017). Although the animals investigated in our study did not carry this SNP, we found ten new point mutations—six were considered to be synonymous or silent, and four non-synonymous or missense. Two of these missense mutations were predicted by SIFT and PROVEAN to be tolerated or to have a neutral impact in the protein structure. The other two non-synonymous variants correspond to Pro94Leu (exon 4, C281T) and Leu129Gln (exon 5, T386A), and they were both predicted to be not tolerated and to cause deleterious changes in the protein. Proline has a cyclic structure and since it is the wild-type protein, we assume that there is a bending in the structure of factor VII at that location. According to SIFT predictions, Proline cannot be substituted by any other aa. Therefore, having a leucine (aliphatic and open chain structured) at that point would alter the protein structure and invalidate its coagulation functioning. However, there were no homozygous individuals in our study with the mutant type, meaning all Asian elephants have at least one wild-type functioning allele. From the six heterozygous elephants with this variant, five had higher coagulation PT times (which is influenced by factor VII activity) than the average in the study. However, these individuals had only a prolongation of nearly one second and have a lower mean that the upper quadrant. Therefore, no reliable conclusion on their predisposition to have a coagulation deficiency can be made and they were considered healthy.

In exon 5, at position T386A, the F7 gene from *Loxodonta africana* (used as reference here) has a “T”, the triplet thus coding for a Leucin. None of the *Elephas maximus* present in the

study presented this homozygous wild-type nucleotide. Our Asian elephant population is 97% homozygous with a mutant-type allele (A/A), causing a shift to glycine, and only three individuals were heterozygous (A/T). The change from leucine (non-polar and hydrophobic aa) to glycine (polar and hydrophilic aa) was predicted as deleterious. We predict that this amino acid resides in a position of the protein, which is not actively involved in the coagulation process, because PT times for animals that were homozygous or heterozygous for this mutation were with the normal range.

Although factor VII deficiency is a rare disease and more than 200 genetic variants have been reported in humans so far (Giansily-Blaizot et al. 2020; McVey et al. 2020), some of these mutations seem to be recurrent and a few with relatively high frequency (Mariani and Bernardi 2009). We found only one SNP to be significantly different between the two regions (exon 2, C142G, Leu48Val), with the majority of the Asian elephant Thai population being homozygous wild-type and the majority of the Asian elephants from European zoos heterozygous for the mutation. This significant distribution of genetic variance was not accompanied by a difference in PT times. This mutation was also considered as tolerable or neutral by the predicting software, therefore we cannot assume that the integrity of factor VII and consequently the extrinsic coagulation pathway will be affected by the presence of this SNP.

3.6. Conclusions

Knowing the physiological status of the coagulation of Asian elephants is of great importance, as it provides a baseline of normal ranges to compare with, when facing diseased situations, such as an EEHV-HD outbreak. This study was performed in Asian elephants living in Thailand and in Europe and it give us a reference range of normal values of coagulation parameters—PT, aPTT times and fibrinogen concentrations—discriminated between different age groups, genders, and regions. Samples were, for the first time, processed using a very practical point-of-care analyser (VSPro) and most results were achieved under 20 min, making it a suitable diagnostic method for emergency cases, and in the field of Asian elephant range countries.

Although we have found significant differences in the coagulation times between the European and Thailand populations, the time gaps reported were very low; therefore, they were not expected to cause any biological effect.

With this study, we have improved the knowledge of F7 gene variation in Asian elephants. We found ten intraspecies variations that can be used as reference for future F7 gene analysis in Asian elephants. Findings on coagulation F7 gene revealed several single nucleotide position

mutations in the population that did not translate to a significant alteration in the coagulation time of the individuals with the mutations.

Due to lack of financial, time, logistical, and human resources, it was not possible to run all the validation tests during this investigation. However, as a future perspective, we believe it would be an important topic for further research. Nevertheless, the large sample size used in this study and the results obtained are good indicators that this POC can be used in Asian elephants. These preliminary results are important for future clinical practice comparisons.

CHAPTER 4

General Discussion

In this thesis, the impact of EEHV-HD in the captive European Asian elephant population was investigated using a retrospective analysis on available data starting with the first reported EEHV-HD fatal case in 1985. More than half of the calves born in zoos since the beginning of the study period have died from EEHV-HD at a very young age of around 2.7 years. For the purpose of evaluating the involvement of possible hereditary and zoo-associated factors in the outcome of this disease, we used a univariate logistic regression model to examine the risk of specific high breeding (produced high number of offspring) fathers, mothers, and zoos. The analysis was extended by combining a multivariate model grouping fathers and zoos and mothers and zoos. We found that four zoos, two fathers and one mother presented an odds ratio three or more times higher than others in the study, meaning that their calves were at a higher risk of becoming sick with EEHV-HD. The findings of this investigation suggest the involvement of zoo-associated factors with possible sire or dam (or a combination of both) influence for calves to manifest the disease. These results are also in agreement with a previous study, where the genogram (family relationship diagram) of the Asian elephant captive populations suggested clustering of fatal cases in certain zoos and a possible family link (Bennett 2018). Together, these findings indicate the possibility of a multifactorial disease, supporting the theory that other agents or cofactors might contribute to the disease (Zachariah et al. 2013), when a zoo-associated component must be assumed to be involved and a hereditary predisposition might be expressed under the influence of certain environmental pressures. The study reported on Chapter 2 is to-date the most extensive study on the impact of this disease ever done in Europe.

In Chapter 3 we established a standard for the normal coagulation status of the Asian elephant population, which can later be used as reference values for further studies. To achieve this, we analysed the coagulation time (PT and aPTT), fibrinogen concentrations and platelet count on a large sample size (n= 127) of healthy Asian elephants from 10 camps in Thailand and 21 European zoos. The methodology chosen allowed us to obtain fast results and it proved to be feasible also in field work conditions. A readout of PT/aPTT coagulation times was obtained as fast as 3 min and fibrinogen concentration was obtained as fast as 10 min after the initiation of the protocol. This method is so far the fastest technique to measure these coagulation parameters in elephants, which could be essential in emergency situations, as for example

during an EEHV-HD outbreak. We achieved a new reference range of normal values for Asian elephants' PT, aPTT times and fibrinogen concentrations. The results are summarized in Table 2 (Chapter 3), and discriminate between different regions, gender and age groups. Significant differences in the coagulation times between the European and Thailand populations were found, although the time gaps were very short and therefore are not expected to cause any biological effect.

We also investigated whether the Thai and European Asian elephant populations presented a specific hereditary coagulation disorder associated with factor VII of the coagulation cascade. To assess this, we sequenced the F7 gene of 141 animals. Although we did not find the previously reported mutation in our study population, we discovered ten new single nucleotide polymorphisms, two of which are predicted to be deleterious, and would therefore cause deleterious changes in coagulation factor VII. For one of these mutations (C281T, Table 4), there were no homozygous individuals with the mutant type found in our study, being the majority homozygous with the wild-type or heterozygous, which means that all Asian elephants have at least one wild-type functioning allele. Six of these heterozygous individuals were also assessed for coagulation times, where five individuals presented higher coagulation PT than the study average. Nevertheless, this difference represents nearly one second and is not considered of biological importance. For the other deleterious mutation (T386A, Table 4), the wild-type nucleotide is "T". None of the Asian elephants in our study population presented only this nucleotide in that position. Our population is 97% homozygous for the mutant-type allele (A/A), and only three individuals were heterozygous (A/T). This result shows that there is a shift from production of leucine (wild-type) present in the *Loxodonta africana* of reference, to glycine in the *Elephas maximus* in the study. Nevertheless, for this mutation, PT times for the homozygous or heterozygous animals were within the normal range of the total studied population. Therefore, although this amino acid change is predicted to be deleterious, we assume that the shift occurs in a position of the coagulation factor VII protein that is not directly involved in the coagulation process.

Regarding the EEHV-HD cases in the study, a link between the presence of this deleterious mutations and the disease was not present, and we can therefore assume that animals presenting these missense mutations are not at a higher risk of succumbing to the disease. Nevertheless, our results contribute to a better knowledge of the F7 gene intraspecies variation in Asian elephants, which could be of further use for comparison with future coagulation gene studies.

Consequences of a high rate of early age deaths to the EEP breeding program

Our findings revealed that EEHV-HD affects calves at an average age of 2.7 years, which is significantly lower than the elephants dying due to other causes (8.6 years). These results were in agreement with previous reports on risk ages from Europe, Thailand, and North American (Boonprasert et al. 2019; Howard and Schaftenaar 2019; Perrin et al. 2021b). We show that EEHV-HD alone was responsible for 52% of all reported fatalities. This is also in accordance with North American population of Asian elephants (53%, Howard & Schaftenaar, 2019). The European population presented a higher mortality risk (85%) than the North American one (68%; Howard & Schaftenaar, 2019) or the Thailand one (nearly 69%; Boonprasert et al., 2019). The different continents differ in the management of the disease and access to the sick calves. In general, Thailand and North America present more direct contact training and veterinary access of very young calves, which is of great aid for monitoring and treating a calf suffering from EEHV-HD. We therefore attribute these lower mortalities presented by Thailand and North America to reflect most likely the substantial number of surviving calves in these countries because of effective and timely treatment. The number of EEHV-HD survivors in the European population has increased in the past few years, and is expected to improve further.

The European Endangered Species Programme's latest report states that birth rates will not replace the loss of the high number of aged females (35–55 years old). The report also suggested that female elephants should start reproducing at 8 years of age (Schmidt and Kappelhof 2019). Our results show that EEHV-HD deaths occur at such lower and narrower age range that many of the youngsters are dying before reaching sexual maturity, reducing therefore the possibility of these calves to substitute the elders and the overall number of possible future breeders. Therefore, EEHV-HD further aggravates European breeding efforts made to keep a healthy reproductive group and a sustainable captive population.

With this problem, all efforts are being done to keep every calf alive and halt this disease. To achieve this, the zoological community is dedicating much effort to research to develop a better treatment and to create a vaccine which could protect the calves from suffering the haemorrhagic disease.

Treatment and vaccination against EEHV-HD

The treatment of EEHV-HD is based on early and aggressive therapies which includes several of the following options: Anti-herpetic drugs, pain relief, antibiotics, intravenous fluids and blood and/or plasma transfusions (EAZA 2020a). Currently, human anti-herpetic drugs are used as antiviral treatment against EEHV, such as Famciclovir, Ganciclovir or Acyclovir, but despite its high costs there is still no proof of its efficacy (Dastjerdi et al. 2016; EAZA 2020a; Hayward 2012; Kendall et al. 2016). Despite several reports of successful treatments by including these drugs in the overall treatment (Richman et al. 1999, 2000; Schaftenaar et al. 2010), many others have also reported that the survival rates obtained with using the drug did not alter the outcome for severely diseased animals (Dastjerdi et al. 2016; Kendall et al. 2016; Yun et al. 2021).

Intravenous transfusion of whole blood and/or plasma in Asian elephant populations has been progressively developing in zoos, as it was an essential component in the successful treatment of many recently ill elephant calves (Artis Zoo 2018; ChesterZoo 2019; Guevara et al. 2017; Schaftenaar and Zoo 2018). Blood or plasma transfusions can replace the coagulation factors and platelets consumed or depleted in the body of a calf with EEHV-HD, and is now recommended in several treatment protocols by advisory groups (EAZA 2020a; Houston Zoo 2015; Molenaar 2019; Oklahoma Zoo 2017; Wiedner 2019).

As for other herpesviruses, the best approach would be to vaccinate calves early with attenuated or dead virus vaccines. Since the virus has still not been successfully cultured until today, this will not be feasible (Zong et al. 2014). Ossent and colleagues made the first attempt to isolate the virus, using triturated skeletal muscle from a diseased animal into different cell cultures: primary bovine embryo lung cells, bovine MDBK, chicken embryo fibroblasts and rabbit RK13. Virus isolation was unsuccessful in any of the attempts (Ossent et al. 1990). In 1999, Richman and colleagues also attempted to cultivate the virus from fatal cases using Vero and MARC African green monkey kidney cells, embryonating chicken eggs, baby hamster kidney cells, rabbit kidney-13 cells, equine dermal cells, human foreskin fibroblasts, and Asian and African elephant fibroblasts but was also unsuccessful (Richman et al. 1999). Recently, another attempt was made to isolate EEHV using a continuous cell culture system with U937 cells (cell line derived from a human myeloid leukaemia). The viral replication in these cells only occurred in the early passages, without being able to produce a stable culture of EEHV (Photichai et al. 2020). Therefore, an alternative method using the Modified Vaccina Ankara (MVA) with recombinant virus vector is currently under study in North America (Clinton et al. 2022). Very recently, this research team has successfully generated an MVA

recombinant expressing EEHV-gB and purified recombinant gB protein from mammalian cells. In their preclinical studies done in mice, they showned that MVA-gB or gB subunit of vaccinated mice created robust gB-specific antibodies and obtained polyfunctional T cells (CD4+ and CD8+) responses, after homologous prime-boosts (Clinton et al. 2022). Also recently, Chester Zoo has announced that they have started a vaccine trial in elephants, using the same type of vaccine which is normally administrated in elephants against cowpox disease – also an MVA, but advancing no further information on the study and date of expected outcome (Gill 2022; Mills 2002).

Importance of assessing coagulation times and fibrinogen measurements

Coagulation times provide us with information about a large variety of clinical ephemeron alterations such as sepsis, hepatic disfunction, decrease of vitamin K, shock, trauma, embolism, platelet bleeding disorders, coagulation factory deficiency and disseminated intravascular coagulation (DIC) (Fasano and Sequeira 2017; Palta et al. 2014; Zehnder et al. 2011; Zoetis 2019). For example, liver dysfunction may affect the coagulation cascade in several ways, since this organ produces most coagulation factors and affects vitamin K absorption (vitamin K is a cofactor for the synthesis of several coagulation factors). Infectious diseases, severe systemic diseases or immune-mediated diseases are also known to alter normal coagulation times. Coagulation times are therefore recommended to be assessed as a pre-surgical test for any animal, regardless of age (Zoetis 2019). Fibrinogen, for example, is used as a specific and sensitive marker for inflammation in humans (Davalos and Akassoglou 2012) and in horses, where fibrinogen serial measurements provide valuable information on treatment efficacy and prognosis in several ephemerons status as pleuropneumonia, abdominal abscess, endometritis, endocarditis (Jacobsen 2007; Nolen-Walston and Sweeney 2009; Tomlinson et al. 2015; Zoetis 2019).

In comparison with other studies in Asian elephants, our PT results presented longer times. However, those previous reports were performed using much smaller sample sizes and different measuring methods (Gentry et al. 1996; Kaye et al. 2016; Perrin et al. 2018). Regarding aPTT, our results also differ greatly in scale from the previous reports in elephants, which again used different methods that were based on human plasma as a reference for comparison (Gentry et al. 1996; Kaye et al. 2016; Perrin et al. 2018). Likewise, our Asian elephant study population presented a different (higher) mean fibrinogen concentration than previously reported (Gentry et al. 1996; Kaye et al. 2016; Perrin et al. 2018; Salakij et al. 2005; Silva and Kuruwita 1993). Since for all these studies the measurement methods differed from

ours, therefore we assume that this may be a method related difference and it suggests that each assay will give different results for healthy elephants, rendering the comparison of results impossible. Combining the healthy status of the animals sampled and all values obtained being within the manufacturers range limit, we assume that all animals in our study had a normal coagulation time, and together with the large data set composed of Asian elephants from different regions and from a wide age range, we consider our values reliable and reproducible and should constitute a strong basis as reference values for future studies employing similar methodologies.

Disseminated intravascular coagulation is often diagnosed based on the presence of a predisposing disease and three or more abnormal haemostatic parameters such as aPTT, PT, fibrinogen, d-dimer, platelet count, and RBC morphology (Cotter 2019). Knowing that viral induced DIC is proposed to play a role in the aggravation of the EEHV-HD disease progression in fatal cases (Guntawang et al. 2021; Perrin et al. 2021a), it would be important to include these parameters during monitoring alongside the viremia, treatment and post symptomatic recovery. There are no reports about the continuous surveillance of coagulation alterations during an EEHV-HD episode using serial sampling to measure coagulation times. Daily to weekly blood monitoring allows us to detect a rise in the virus genomic equivalents (VGE) at least two weeks before the symptomatology (Long et al. 2016). Normally, the recognition of high titers of 5000VGE/mL or above initiates treatment (Edwards et al., 2021; Houston Zoo, 2015). Continuous measurements of coagulation parameters could possibly detect abnormalities in the coagulation status in advance of an EEHV-HD outbreak and plasma transfusion could be started earlier to support the coagulation system by providing coagulation factors.

Additionally, coagulation testing could not only evaluate vascular disbalance when the high titers are obvious but also alongside treatment to help prognosis, and even after treatment to assess recovery. Based on novel reports, DIC and a gross vascular alteration are generally present, with severe destruction of the microvasculature visualised for example by a cyanotic tongue and oedema of the face and limbs (Guntawang et al. 2021; Perrin et al. 2021a) and therefore, PT and aPTT are expected to take long to recover to normal values in EEHV-HD surviving cases. Long lasting coagulation effects of the disease in the survival cases are still to be studied. However, we expect the timeframe from a DIC scenario to a complete recovered status to cover at least several weeks or even months.

In chapter 3, when analysing the coagulation times of EEHV-HD surviving calves, which recovered for months to years before sampling, no significant difference in PT between this

groups and the rest of the population was found. In contrast, aPTT differed significantly. Nevertheless, both results could be attributed to the result of the substantial difference in sample sizes (*n* = 8 survivors; *n* = 119 non EEHV-HD); the number of survivors might be too small to obtain a reliable difference for PT or aPTT. This finding warrants the need for further investigation and continuous monitoring of EEHV-HD surviving elephants.

One other interesting finding, although not significant, is that our male sample presented in general a more prolonged coagulation time, lower platelet counts, and lower overall fibrinogen concentration than females. In contrast, human females present a more prolonged coagulation time and bleeding time than human males (Adhana et al. 2018; Roy et al. 2011). Unfortunately, our data set was not gender-even, having three times as many females as males (85 vs. 27). These results would be of interest to further investigate in a better gender-balanced study.

Conclusions

At the end of this study, we can conclude that EEHV-HD had a very high impact on the breeding population being kept under human care in Europe, and that it is important to keep monitoring young Asian elephants for EEHV until the age of 8 years old. Furthermore, our findings on specific zoos, fathers and mothers presenting a higher risk for their calves to develop EEHV-HD highlights the presence of a management or environmental element, of possible paternal and maternal influence, leading to higher susceptibility of these calves to the disease. Together with the findings of a young and narrow age range of the fatalities, we must assume that there are essential elements playing a role in triggering the virus, and therefore, understanding what these underlying factors are would be of great importance to halt the disease and protect the calves.

A baseline of coagulation parameters normal ranges using a practical analyser is now available for future comparison, as coagulation testing should be included in routine monitoring of Asian elephant calves, since it could give us an idea of the vascular disbalance not only during severe illness, but also alongside treatment to help prognosis and even post treatment to assess recovery.

Our genetic study of the coagulation factor VII gene showed that Asian elephants present several intraspecies mutations and findings are available for further clinical studies. Investigation of other coagulation factors and haemostatic contributors would be of interest to improve our understanding of the coagulation system of this species.

Future perspectives

Finalizing this study, we realize that although a substantial knowledge on the EEHV infection and haemorrhagic disease, its diagnostics and treatment has been accumulated in the past three decades, questions for further investigations seem to exponentially increase.

Knowing that this is an ancient virus that has coevolved with elephants, it would be expected that latent states are prolonged and casualties rare. Would it have been possible for wild elephant populations to have had this number of fatalities in the past and still survive until today? Could we have overlooked the haemorrhagic disease and misdiagnosed it for decades? Could the host become now more vulnerable to this species-specific herpesvirus? And if so, what led to this increase in calf susceptibility to the disease? Should we consider all adult elephants as survivors to this disease and if so, what allowed them to overcome the risk age and which protective elements helped them to survive and thrive? Also, from a genetic point of view, why do some calves seem to be less vulnerable to the disease, and could maternal antibodies be the only reason protecting them? Understanding what the triggering factors might be present that led to such a severity of disease and number of deaths should be further investigated.

One important thing to remember is that elephants are long-lived species and present a very long gestation period of 22 months. Since the first reported EEHV-HD case, only 35 years have passed, which means only one full generation (25 years generational gap for elephants, Williams et al., 2020) has been analysed. Therefore, our findings highlight once more the importance of continuation of these longitudinal studies.

Another worthwhile line of research would be to focus on identifying what the possible protective factors are, especially at the sensitive young age. The vaccine might still take many years to be properly effective in elephants, and therefore certain management decisions and additions of protective elements at the risk age might be paramount to stop disease development once a calf is infected. Therefore, investigation of management differences between zoos with higher EEHV-HD case number and low to none fatal cases zoos, combining with investigations of the genetic background of progeny, should be further studied in the near future.

One factor of obvious impact on mortality rates is the handling of calves. In Europe, all zoological institutions should be in protected contact handling system by 2030 and it is

encouraged to start training calves from the age of 4 months onwards (EAZA 2020a, 2020b). The capacity to regularly monitor and reach the calf to start treatment is still not a reality in many European zoos. Improving access to calves is extremely important to increase survival to EEHV-HD.

Also, as EEHV-HD greatly misbalances such an important system as the coagulation cascade, a prolonged vascular recovery period is expected. Long lasting coagulation effects of this disease process is still to be studied and understood.

Additionally, in most endangered species, coagulation is very rarely investigated. Using the quick and simple method presented here, it could be easily implemented to expand coagulation research in zoo and wildlife species, which would give us a broader knowledge on what a healthy coagulative status of different threatened species looks like.

In general, to prepare and fight against EEHV-HD, and help the worldwide Asian and African elephant populations, it is essential to invest in what is already an extreme global effort: the continuous gain of a better knowledge on the disease's pathophysiology and risk factors, to support the development of an effective vaccination, and to improve treatment.

Summary

Elephant endotheliotropic herpesvirus in *Elephas maximus* - epidemiology, risk factors and coagulation parameters

The Asian elephant (*Elephas maximus*) is an endangered species, suffering a continuous decline in their population numbers. Elephant Endotheliotropic Herpesvirus haemorrhagic disease (EEHV-HD) is the primary cause of calf mortality of Asian elephants worldwide. The disease is presented as an acute haemorrhagic syndrome caused by vast endothelial destruction and disseminated intravascular coagulation, leading to sudden death.

In this thesis, we investigated 1) the impact of EEHV-HD in the European captive Asian elephant population, 2) the presence of hereditary or zoo-associated factors as a risk to develop the disease in Europe, 3) the coagulation status of healthy Thai and European Asian elephant populations, 4) the presence of genomic mutations in coagulation factor VII that could lead to a hereditary coagulopathy in Thai and European Asian elephants.

Our findings reveal that more than half of the captive born fatalities were caused by EEHV-HD alone and suggest the involvement of zoo-associated factors with a possible sire and/or dam influence on the onset of the disease. Using a specific fast point-of-care analyser we have stablished reference values for coagulation parameters, such as coagulation times (PT and aPTT) and fibrinogen concentration in healthy elephants, detailed by gender, age, regions at study and EEHV status (survivors of EEHV-HD or animals that never present the disease). Our methods and results regarding coagulation assessment, can be used and compared for future routine health check-ups or in emergency, such as during an EEHV-HD outbreak. Furthermore, we report the finding of several new single point mutations in coagulation F7 gene, found in *Elephas maximus* from Thailand and Europe.

Overall, our findings highlight the importance of doing continuous retrospective epidemiological studies and stresses the need to further investigate the underlying risks or protective factors that make calves especially susceptible or resistant to the onset and outcome of EEHV-HD.

Zusammenfassung

Endotheliotropes Elefantenherpesvirus bei *Elephas maximus* - Epidemiologie, Risikofaktoren und Gerinnungsparameter

Der Asiatische Elefant (*Elephas maximus*) ist eine vom Aussterben bedrohte Tierart, deren Bestand kontinuierlich abnimmt. Die hämorrhagische Erkrankung durch das Endotheliotrope Elefantenherpesvirus (EEHV-HD) ist die Hauptursache für die Kälbersterblichkeit bei asiatischen Elefanten weltweit. Die Krankheit äußert sich als akutes hämorrhagisches Syndrom, das durch eine weitgehende Zerstörung der Endothelien und eine disseminierte intravaskuläre Gerinnung verursacht wird und zum plötzlichen Tod führt.

In dieser Arbeit untersuchten wir 1) die Auswirkungen von EEHV-HD in der europäischen Population asiatischer Elefanten in Gefangenschaft, 2) das Vorhandensein erblicher oder zoo-assoziierter Faktoren als Risiko für die Entwicklung der Krankheit in Europa, 3) den Gerinnungsstatus gesunder thailändischer und europäischer asiatischer Elefantenpopulationen, 4) das Vorhandensein genomischer Mutationen im Gerinnungsfaktor VII, die zu einer erblichen Koagulopathie bei thailändischen und europäischen asiatischen Elefanten führen könnten.

Unsere Ergebnisse zeigen, dass mehr als die Hälfte der Todesfälle bei in Gefangenschaft geborenen Elefantenkälbern auf EEHV-HD zurückgeführt werden können, und deuten auf die Beteiligung von Zoo-assoziierten Faktoren mit einem möglichen Einfluss des Vaters und/oder der Mutter auf das Auftreten der Krankheit hin. Mit Hilfe eines speziellen schnellen Point-of-Care-Analysegeräts haben wir Referenzwerte für Gerinnungsparameter wie Gerinnungszeiten (PT und aPTT) und Fibrinogenkonzentration bei gesunden Elefanten ermittelt, die nach Geschlecht, Alter, Untersuchungsregionen und EEHV-Status (überlebende EEHV-HD-Tiere oder Tiere, die nie erkrankt sind) aufgeschlüsselt sind. Unsere Methoden und Ergebnisse zur Beurteilung der Blutgerinnung können bei künftigen Routineuntersuchungen oder in Notfällen, z. B. bei einem EEHV-HD-Ausbruch, verwendet und verglichen werden. Darüber hinaus berichten wir über die Entdeckung mehrerer neuer Einzelpunktmutationen im F7-Gen für die Blutgerinnung, die bei *Elephas maximus* aus Thailand und Europa gefunden wurden.

Insgesamt unterstreichen unsere Ergebnisse die Bedeutung kontinuierlicher retrospektiver epidemiologischer Studien und betonen die Notwendigkeit, die zugrunde liegenden Risiko- oder Schutzfaktoren weiter zu untersuchen, die Kälber besonders anfällig oder resistent für den Ausbruch und die Folgen von EEHV-HD machen.

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Supplementary material

Supplementary Tables of Chapter 2:

Table S2.1. Study population database, from January 1985 until June 2020

EEHV	Surv	Status	Name	Father	Mother	Sex	Death Date	Birth Date	Status_Age	Birth_Zoo	Actual_Zoo
0	0	0	1	F25	M116	F		31-Jan-1985	424	B42	Z1
0	0	2	2	F37	M45	F	18-Feb-1985	18-Feb-1985	0	B19	Z24
0	0	2	3	F18	M84	F	12-Mar-1985	11-Mar-1985	0	B22	Z29
0	0	2	4	F32	M77	F	12-Apr-1985	12-Apr-1985	0	B25	Z35
1	0	1	5	F23	M22	F	21-Jul-1988	24-May-1985	38	B10	Z12
0	0	2	6	F9	M10	M	1-Dec-1987	1-Dec-1985	24	B23	Z62
0	0	2	7	F12	M17	F	21-Sep-2003	22-Jan-1986	212	B12	Z14
0	0	2	8	F19	M124	F	16-Mar-1986	18-Feb-1986	1	B31	Z42
0	0	2	9	F23	M20	F	22-May-1986	22-May-1986	0	B52	Z70
0	0	2	10	F35	M15	F	21-Apr-1987	28-Mar-1987	1	B37	Z48
0	0	0	11	F25	M116	F		15-May-1987	397	B42	Z74
0	0	2	12	F18	M84	F	21-Nov-1987	20-Nov-1987	0	B22	Z29
0	0	2	13	F8	M87	F	8-Dec-1987	8-Dec-1987	0	B40	Z53
0	0	0	14	F23	M19	F		13-Feb-1989	376	B52	Z44
0	0	2	15	F35	M15	M	25-Sep-1997	28-Feb-1989	103	B37	Z59
0	0	2	16	F35	M50	M	19-Jun-2000	31-Mar-1990	122	B37	Z49
0	0	0	17	F30	M124	F		5-Apr-1990	362	B44	Z33
0	0	2	18	F25	M65	F	15-Jun-1990	15-Jun-1990	0	B42	Z55
0	0	2	19	F8	M120	F	24-Jun-1990	24-Jun-1990	0	B40	Z53
0	0	2	20	F8	M120	F	22-Jul-1990	22-Jul-1990	0	B40	Z53
0	0	2	21	F21	M46	M	22-Nov-1990	22-Nov-1990	0	B51	Z69
0	0	0	22	F32	M41	F		25-Nov-1990	354	B46	Z15
0	0	0	23	F25	M116	F		6-Jan-1991	353	B42	Z43
0	0	2	24	F12	M38	F	28-Oct-1991	28-Oct-1991	0	B12	Z14
0	0	0	25	F26	M36	F		1-Mar-1992	339	B14	Z17
0	0	0	26	F17	M108	F		23-Mar-1992	339	B18	Z45
0	0	2	27	F35	M15	M	29-Oct-2009	27-Mar-1992	211	B37	Z10

0	0	0	28	F47	M118	M		25-May-1992	336	B18	Z10
0	0	2	29	F26	M122	M	1-Jul-1993	1-Jun-1992	13	B14	Z16
0	0	0	30	F23	M22	M		13-Jun-1992	336	B10	Z33
0	0	2	31	F38	M114	M	1-Apr-1996	29-Sep-1992	42	B34	Z21
0	0	2	32	F32	M86	F	4-Mar-1993	4-Mar-1993	0	B46	Z59
0	0	2	33	F8	M27	M	21-Jun-1993	21-Jun-1993	0	B40	Z53
0	0	2	34	F11	M105	F	9-Sep-1993	9-Sep-1993	0	B9	Z11
0	0	0	35	F35	M50	F		1-Oct-1993	320	B37	Z37
0	0	0	36	F25	M116	M		23-Oct-1993	320	B42	Z6
0	0	2	37	F23	M58	unknown	15-Dec-1993	15-Dec-1993	0	B52	Z70
0	0	2	38	F26	M54	M	30-May-1994	29-May-1994	0	B14	Z16
0	0	0	39	F25	M7	M		19-Aug-1994	310	B42	Z63
0	0	0	40	F26	M36	M		23-Aug-1994	310	B14	Z52
0	0	0	41	F23	M19	M		14-Nov-1994	307	B52	Z37
0	0	2	42	F21	M46	F	23-Jan-1995	23-Jan-1995	0	B29	Z39
1	0	1	43	F32	M41	F	13-Jul-1998	1-Mar-1995	40	B46	Z59
0	0	2	44	F18	M84	F	12-Sep-2008	2-Mar-1995	162	B22	Z72
0	0	0	45	F28	M83	M		8-Jul-1995	299	B31	Z62
0	0	2	46	F13	M1	F	1-Dec-2018	26-Oct-1995	277	B26	Z52
0	0	2	47	F11	M105	F	25-May-1997	19-Dec-1995	17	B9	Z11
0	0	2	48	F17	M108	M	1-Jan-2001	25-Dec-1995	60	B18	Z52
0	0	2	49	F17	M13	M	9-Jan-1996	9-Jan-1996	0	B18	Z23
0	0	2	50	F12	M38	M	9-Feb-2006	15-Mar-1996	119	B12	Z51
0	0	2	51	F35	M30	F	17-Mar-1996	17-Mar-1996	0	B3	Z4
0	0	2	52	F5	M85	M	29-Mar-1996	29-Mar-1996	0	B40	Z53
0	0	0	53	F25	M116	M		24-May-1996	289	B42	Z28
0	0	0	54	F17	M118	F		4-Jun-1996	288	B18	Z45
0	0	0	55	F17	M90	F		1-Jul-1996	287	B18	Z23
0	0	2	56	F35	M30	M	1-Jul-1996	1-Jul-1996	0	B3	Z4
0	0	2	57	F13	M70	M	25-Mar-2005	7-Jul-1996	104	B26	Z47
0	0	2	58	F17	M95	M	17-Oct-1996	17-Oct-1996	0	B18	Z23
0	0	2	59	F12	M23	M	19-Jun-1997	17-Feb-1997	4	B12	Z14
0	0	0	60	F21	M46	F		6-May-1997	277	B4	Z47
0	0	2	61	F25	M7	F	21-Jul-1997	16-May-1997	2	B42	Z55

1	0	1	62	F23	M19	M	20-Nov-1999	8-Sep-1997	26	B52	Z70
0	0	0	63	F26	M122	M		9-Nov-1997	271	B14	Z31
0	0	2	64	F26	M106	M	9-Apr-2017	23-Dec-1997	231	B14	Z60
0	0	2	65	F11	M105	F	7-Sep-2018	31-Dec-1997	248	B9	Z11
0	0	2	66	F2	M52	M	17-Jan-1998	17-Jan-1998	0	B5	Z7
0	0	0	67	F26	M101	M		8-Feb-1998	268	B14	Z57
0	0	0	68	F32	M55	M		13-Feb-1998	268	B46	Z17
0	0	0	69	F32	M121	M		1-Mar-1998	267	B46	Z47
0	0	0	70	F26	M37	F		8-Mar-1998	267	B14	Z20
0	0	2	71	F11	M42	M	25-Apr-1998	25-Apr-1998	0	B9	Z11
0	0	0	72	F35	M50	M		4-May-1998	265	B37	Z56
0	0	2	73	F26	M54	M	9-Jul-1998	9-Jul-1998	0	B14	Z16
0	0	0	74	F38	M114	F		19-Jul-1998	263	B34	Z31
0	0	2	75	F2	M66	F	31-Jul-1998	31-Jul-1998	0	B5	Z7
0	0	0	76	F11	M111	F		6-Aug-1998	262	B48	Z8
0	0	2	77	F8	M85	M	26-Jul-1999	20-Aug-1998	11	B40	Z53
0	0	0	78	F11	M75	F		27-Aug-1998	262	B48	Z68
1	0	1	79	F1	M14	M	12-Jan-1999	11-Jan-1999	0	B33	Z45
0	0	0	80	F32	M56	F		5-Apr-1999	254	B1	Z2
0	0	0	81	F26	M36	F		2-May-1999	253	B14	Z17
0	0	0	82	F17	M22	F		27-Nov-1999	247	B10	Z56
0	0	2	83	F32	M29	M	26-Jan-2000	26-Jan-2000	0	B46	Z59
1	0	1	84	F20	M82	M	28-Dec-2000	5-Apr-2000	9	B6	Z6
0	0	2	85	F25	M117	F	7-May-2000	9-Apr-2000	1	B42	Z55
0	0	0	86	F25	M116	F		1-May-2000	241	B42	Z43
1	0	1	87	F23	M19	M	15-Oct-2003	10-Jun-2000	40	B52	Z70
0	0	0	88	F11	M42	M		18-Jul-2000	239	B9	Z10
0	0	0	89	F11	M105	M		7-Oct-2000	236	B9	Z9
0	0	0	90	F1	M41	F		28-Nov-2000	235	B46	Z59
1	0	1	91	F12	M38	M	6-Oct-2003	12-Feb-2001	32	B12	Z14
0	0	2	92	F13	M35	M	18-May-2001	18-May-2001	0	B26	Z36
0	0	0	93	F13	M1	M		16-Jun-2001	228	B26	Z2
0	0	0	94	F26	M54	M		16-Jul-2001	227	B14	Z11
0	0	0	95	F2	M52	F		2-Nov-2001	223	B5	Z44

0	0	0	96	F13	M70	F		5-Nov-2001	223	B26	Z9
0	0	2	97	F17	M108	M	9-Nov-2001	9-Nov-2001	0	B18	Z23
1	0	1	98	F1	M14	M	20-Dec-2002	20-Jan-2002	11	B46	Z59
0	0	2	99	F14	M67	unknown	2-Feb-2002	2-Feb-2002	0	B50	Z68
0	0	2	100	F26	M106	M	6-Apr-2017	4-Mar-2002	181	B14	Z60
0	0	0	101	F24	M112	M		5-Apr-2002	218	B28	Z38
0	0	2	102	F8	M63	F	12-May-2002	12-May-2002	0	B42	Z55
0	0	0	103	F23	M39	F		14-May-2002	217	B52	Z70
0	0	2	104	F26	M122	M	11-Apr-2017	27-May-2002	178	B14	Z60
1	0	1	105	F21	M55	M	12-Aug-2005	30-Jun-2002	37	B40	Z53
0	0	2	106	F14	M4	unknown	1-Jul-2002	1-Jul-2002	0	B50	Z68
0	0	0	107	F25	M116	M		18-Aug-2002	214	B42	Z27
0	0	2	108	F26	M37	M	7-Apr-2017	5-Sep-2002	175	B14	Z60
0	0	0	109	F10	M72	F		2-Feb-2003	208	B19	Z24
0	0	0	110	F10	M53	F		20-Mar-2003	207	B19	Z10
0	0	0	111	F1	M29	F		13-May-2003	205	B46	Z59
0	0	0	112	F17	M118	F		14-May-2003	205	B18	Z23
0	0	2	113	F17	M64	M	22-Jun-2003	22-Jun-2003	0	B18	Z23
0	0	0	114	F1	M119	F		26-Jul-2003	203	B46	Z15
0	0	2	115	F16	M92	M	17-Aug-2003	17-Aug-2003	0	B53	Z73
0	0	0	116	F1	M41	M		21-Feb-2004	196	B46	Z20
0	0	2	117	F11	M105	M	5-Mar-2004	5-Mar-2004	0	B9	Z11
0	0	0	118	F42	M98	F		7-Mar-2004	195	B9	Z11
1	0	1	119	F14	M51	F	17-Dec-2006	16-Mar-2004	33	B50	Z68
0	0	2	120	F14	M28	M	1-Jul-2004	1-Jul-2004	0	B49	Z66
0	0	2	121	F14	M9	M	2-Apr-2017	25-Sep-2004	150	B50	Z60
0	0	2	122	F11	M42	M	30-Oct-2017	10-Oct-2004	156	B9	Z26
0	0	0	123	F38	M114	M		17-Oct-2004	188	B34	Z65
0	0	0	124	F17	M108	M		26-Nov-2004	187	B18	Z70
0	0	2	125	F15	M81	F	25-Jan-2005	25-Jan-2005	0	B32	Z44
0	0	0	126	F2	M80	M		14-Feb-2005	184	B5	Z32
0	0	0	127	F2	M25	F		3-Apr-2005	183	B5	Z58
0	0	0	128	F21	M55	F		11-Apr-2005	182	B40	Z50
0	0	0	129	F23	M19	F		3-May-2005	182	B52	Z70

0	0	0	130	F2	M52	M		8-May-2005	181	B5	Z58
1	0	1	131	F21	M121	M	28-May-2005	28-May-2005	0	B40	Z53
0	0	0	132	F26	M36	M		6-Jun-2005	180	B14	Z5
1	0	1	133	F44	M82	F	5-Apr-2011	15-Jun-2005	70	B6	Z6
0	0	2	134	F21	M109	F	12-Jul-2005	12-Jul-2005	0	B40	Z53
0	0	0	135	F23	M39	M		24-Jul-2005	179	B52	Z59
0	0	0	136	F1	M107	F		2-Aug-2005	179	B2	Z3
0	0	0	137	F30	M76	M		7-Aug-2005	178	B14	Z5
0	0	0	138	F10	M53	M		28-Oct-2005	176	B19	Z25
0	0	0	139	F14	M61	M		11-Dec-2005	174	B21	Z22
0	0	2	140	F13	M70	M	31-Jan-2006	21-Jan-2006	0	B26	Z36
0	0	2	141	F13	M35	F	10-Feb-2006	24-Jan-2006	1	B26	Z36
0	0	0	142	F13	M1	F		27-Jan-2006	173	B26	Z36
0	0	0	143	F25	M63	F		11-Mar-2006	171	B42	Z55
0	0	0	144	F12	M38	M		20-Mar-2006	171	B12	Z36
0	0	0	145	F30	M54	F		30-Mar-2006	171	B11	Z13
0	0	2	146	F30	M101	M	15-Apr-2017	23-May-2006	130	B14	Z60
0	0	0	147	F25	M116	M		22-Jul-2006	167	B42	Z22
1	0	1	148	F42	M105	M	23-Jul-2009	12-Nov-2006	32	B9	Z11
1	0	1	149	F14	M51	F	17-May-2009	19-Jan-2007	28	B50	Z68
0	0	2	150	F33	M40	M	3-Apr-2007	3-Apr-2007	0	B1	Z2
0	0	0	151	F17	M64	F		11-Apr-2007	158	B18	Z23
0	0	0	152	F48	M110	M		16-Apr-2007	158	B11	Z68
0	0	0	153	F1	M14	F		7-May-2007	157	B13	Z15
0	0	0	154	F8	M106	F		9-May-2007	157	B11	Z14
0	0	0	155	F30	M36	F		8-Aug-2007	154	B14	Z17
0	0	2	156	F15	M81	M	7-Oct-2007	7-Oct-2007	0	B32	Z44
0	0	2	157	F1	M41	F	28-Dec-2007	28-Dec-2007	0	B46	Z59
0	0	0	158	F7	M71	M		30-Dec-2007	150	B47	Z18
1	0	1	159	F14	M9	M	3-May-2009	17-Jan-2008	15	B50	Z68
0	0	0	160	F1	M119	M		17-Feb-2008	148	B13	Z71
0	0	0	161	F30	M37	M		25-Feb-2008	148	B14	Z41
0	0	2	162	F30	M101	unknown	7-Mar-2008	7-Mar-2008	0	B14	Z16
0	0	2	163	F8	M97	M	11-Mar-2008	11-Mar-2008	0	B11	Z13

1	0	1	164	F11	M78	F	29-Nov-2015	4-May-2008	91	B27	Z37
0	0	0	165	F10	M53	M		6-May-2008	146	B19	Z61
0	0	0	166	F16	M92	M		19-Jul-2008	143	B16	Z50
0	0	0	167	F30	M76	M		4-Sep-2008	142	B14	Z41
0	0	0	168	F17	M118	M		21-Nov-2008	139	B18	Z61
0	0	0	169	F2	M52	F		13-Dec-2008	138	B5	Z7
0	0	2	170	F27	M107	M	11-Jan-2009	10-Jan-2009	0	B2	Z3
0	0	2	171	F1	M29	F	17-Dec-2013	10-Feb-2009	58	B46	Z54
1	0	1	172	F44	M82	F	27-May-2011	15-Mar-2009	26	B6	Z6
0	0	0	173	F28	M83	F		22-Apr-2009	134	B31	Z42
0	0	2	174	F11	M50	M	11-May-2009	11-May-2009	0	B27	Z37
0	0	0	175	F1	M55	F		17-May-2009	133	B3	Z50
0	0	0	176	F30	M68	M		23-May-2009	133	B14	Z17
0	0	0	177	F17	M108	F		3-Jul-2009	132	B18	Z38
0	0	0	178	F14	M51	F		23-Jul-2009	131	B50	Z68
0	0	0	179	F8	M8	M		27-Jul-2009	131	B11	Z25
1	0	1	180	F14	M79	M	13-Apr-2011	6-Aug-2009	20	B48	Z64
0	0	2	181	F2	M80	F	26-Aug-2009	26-Aug-2009	0	B5	Z7
0	0	0	182	F1	M40	F		10-Nov-2009	127	B1	Z2
0	0	2	183	F11	M78	unknown	27-Nov-2009	27-Nov-2009	0	B27	Z37
0	0	2	184	F15	M81	F	14-Jun-2010	21-Dec-2009	6	B32	Z44
0	0	2	185	F30	M101	M	17-Feb-2010	17-Feb-2010	0	B14	Z16
0	0	0	186	F30	M36	M		9-Mar-2010	123	B14	Z17
0	0	0	187	F2	M25	M		15-Mar-2010	123	B5	Z60
0	0	0	188	F14	M49	M		12-Apr-2010	122	B50	Z30
0	0	0	189	F27	M94	F		7-May-2010	122	B19	Z10
0	0	0	190	F27	M32	M		11-May-2010	121	B19	Z67
1	0	1	191	F42	M98	M	29-Jul-2013	18-Jul-2010	36	B9	Z11
0	0	0	192	F40	M11	F		20-Jul-2010	119	B46	Z59
0	0	0	193	F27	M18	M		25-Jul-2010	119	B19	Z67
0	0	0	194	F27	M72	M		6-Aug-2010	119	B19	Z67
0	0	2	195	F11	M78	unknown	27-Oct-2010	27-Oct-2010	0	B27	Z37
0	0	0	196	F27	M53	F		9-Dec-2010	114	B19	Z10
0	0	0	197	F2	M21	F		22-Dec-2010	114	B45	Z58

1	0	1	198	F42	M105	F	3-Jul-2013	22-Jan-2011	29	B9	Z11
0	0	0	199	F46	M12	F		5-Feb-2011	113	B20	Z27
0	0	0	200	F30	M37	M		6-Feb-2011	113	B14	Z51
0	0	2	201	F10	M115	M	6-May-2011	11-Mar-2011	2	B36	Z47
0	0	0	202	F34	M110	M		8-Apr-2011	111	B11	Z13
0	0	0	203	F10	M46	F		12-Apr-2011	110	B36	Z47
0	0	0	204	F15	M103	M		6-May-2011	110	B32	Z25
1	0	1	205	F27	M107	F	7-Dec-2015	18-Jun-2011	54	B2	Z3
0	0	0	206	F30	M76	M		8-Aug-2011	107	B14	Z51
1	1	0	207	F14	M9	M		18-Oct-2011	104	B50	Z30
0	0	2	208	F15	M81	F	22-Jan-2012	28-Oct-2011	3	B32	Z44
0	0	2	209	F31	M26	unknown	24-Mar-2012	24-Mar-2012	0	B51	Z69
1	0	1	210	F31	M102	F	25-Mar-2012	25-Mar-2012	0	B48	Z64
0	0	2	211	F26	M34	M	9-Apr-2012	9-Apr-2012	0	B28	Z38
0	0	0	212	F17	M64	M		13-Apr-2012	98	B18	Z4
0	0	2	213	F26	M91	F	20-Apr-2012	20-Apr-2012	0	B18	Z23
0	0	0	214	F2	M80	M		8-May-2012	98	B5	Z40
0	0	0	215	F2	M52	F		21-May-2012	97	B5	Z7
0	0	2	216	F30	M36	M	11-Jun-2012	22-May-2012	1	B14	Z16
1	0	1	217	F3	M35	F	24-Jun-2013	29-May-2012	13	B39	Z52
0	0	0	218	F8	M97	F		25-Jul-2012	95	B11	Z13
0	0	0	219	F44	M82	F		12-Aug-2012	94	B6	Z6
0	0	0	220	F1	M40	M		1-Nov-2012	92	B1	Z67
1	0	1	221	F42	M99	M	27-Oct-2015	25-Nov-2012	35	B9	Z11
0	0	0	222	F27	M32	F		24-Dec-2012	90	B19	Z10
1	0	1	223	F42	M98	F	15-Sep-2015	21-Jan-2013	32	B9	Z11
0	0	0	224	F40	M29	F		11-Feb-2013	88	B41	Z46
1	0	1	225	F4	M3	F	24-Nov-2018	14-Feb-2013	69	B7	Z9
1	0	1	226	F12	M60	M	24-Nov-2014	25-Feb-2013	21	B12	Z14
1	0	1	227	F16	M92	M	15-Mar-2016	2-Mar-2013	36	B30	Z19
0	0	2	228	F29	M5	unknown	5-Mar-2013	5-Mar-2013	0	B8	Z10
0	0	0	229	F27	M53	F		13-Mar-2013	87	B19	Z10
0	0	0	230	F30	M37	M		15-Jul-2013	83	B14	Z67
1	1	0	231	F31	M16	M		27-Jul-2013	83	B24	Z34

0	0	0	232	F25	M62	F		2-Aug-2013	83	B42	Z55
0	0	0	233	F40	M113	F		10-Aug-2013	82	B46	Z59
0	0	2	234	F12	M100	F	26-Aug-2013	26-Aug-2013	0	B12	Z14
0	0	0	235	F25	M63	F		2-Oct-2013	81	B42	Z55
1	0	1	236	F14	M49	M	4-Jun-2015	12-Oct-2013	20	B50	Z68
0	0	0	237	F27	M94	F		27-Oct-2013	80	B19	Z10
0	0	0	238	F29	M93	F		1-Nov-2013	80	B43	Z56
0	0	0	239	F27	M72	M		31-Dec-2013	78	B19	Z61
1	0	1	240	F10	M115	F	23-Jan-2016	4-Feb-2014	24	B36	Z47
0	0	0	241	F31	M79	F		4-Mar-2014	76	B48	Z8
0	0	0	242	F27	M18	F		17-Mar-2014	75	B19	Z24
1	1	0	243	F11	M78	M		19-Mar-2014	75	B27	Z37
0	0	0	244	F30	M76	M		16-May-2014	73	B14	Z17
0	0	0	245	F23	M39	F		17-Jun-2014	72	B52	Z70
0	0	0	246	F42	M119	M		17-Jul-2014	71	B13	Z15
0	0	0	247	F30	M68	M		20-Jul-2014	71	B14	Z17
0	0	0	248	F42	M2	M		19-Aug-2014	70	B13	Z15
0	0	0	249	F14	M9	M		16-Sep-2014	69	B50	Z4
0	0	0	250	F42	M14	F		17-Sep-2014	69	B13	Z15
1	1	0	251	F31	M26	F		24-Sep-2014	69	B51	Z69
0	0	2	252	F1	M89	unknown	1-Oct-2014	1-Oct-2014	0	B33	Z45
0	0	2	253	F34	M69	unknown	7-Oct-2014	7-Oct-2014	0	B11	Z13
0	0	0	254	F46	M12	F		13-Oct-2014	68	B20	Z27
0	0	2	255	F34	M73	M	8-Nov-2014	8-Nov-2014	0	B11	Z13
0	0	2	256	F1	M24	F	7-Jan-2015	7-Jan-2015	0	B33	Z45
0	0	2	257	F26	M34	F	31-Mar-2015	25-Mar-2015	0	B28	Z38
0	0	2	258	F11	M74	M	21-May-2015	22-Apr-2015	1	B38	Z50
0	0	0	259	F27	M32	F		20-May-2015	61	B8	Z10
1	0	1	260	F11	M55	F	28-May-2018	16-Jun-2015	35	B38	Z50
1	0	1	261	F15	M118	F	13-Jun-2018	13-Jul-2015	35	B18	Z23
1	0	1	262	F6	M105	F	25-Oct-2018	20-Aug-2015	38	B9	Z11
0	0	0	263	F40	M11	M		20-Aug-2015	58	B46	Z59
0	0	2	264	F12	M100	M	4-Sep-2015	4-Sep-2015	0	B12	Z14
0	0	0	265	F2	M52	M		1-Jan-2016	54	B5	Z7

1	0	1	266	F15	M48	M	6-Jun-2018	11-Jan-2016	29	B18	Z23
0	0	2	267	F15	M108	F	28-Jan-2016	28-Jan-2016	0	B28	Z38
0	0	2	268	F15	M108	M	28-Jan-2016	28-Jan-2016	0	B28	Z38
0	0	0	269	F8	M97	M		15-Mar-2016	51	B11	Z13
0	0	0	270	F24	M43	M		5-Apr-2016	51	B41	Z54
0	0	2	271	F25	M62	F	14-May-2016	14-May-2016	0	B42	Z55
0	0	0	272	F14	M49	F		10-Jun-2016	49	B50	Z68
0	0	2	273	F29	M8	M	1-Aug-2016	31-Jul-2016	0	B8	Z10
1	1	0	274	F42	M6	F		19-Sep-2016	45	B13	Z15
0	0	0	275	F2	M104	M		7-Oct-2016	45	B41	Z54
0	0	0	276	F43	M80	F		12-Oct-2016	44	B30	Z40
0	0	0	277	F24	M107	F		16-Oct-2016	44	B2	Z3
1	1	0	278	F6	M99	F		16-Dec-2016	42	B9	Z11
0	0	0	279	F27	M123	M		22-Dec-2016	42	B19	Z10
0	0	0	280	F27	M18	F		23-Dec-2016	42	B19	Z24
1	0	1	281	F6	M98	M	25-Oct-2018	17-Jan-2017	21	B9	Z11
0	0	0	282	F49	M21	F		18-Jan-2017	41	B45	Z58
0	0	0	283	F27	M72	F		19-Jan-2017	41	B19	Z24
0	0	0	284	F34	M69	M		26-Jan-2017	41	B11	Z14
0	0	0	285	F39	M31	F		25-Feb-2017	40	B52	Z70
0	0	0	286	F42	M14	F		13-Mar-2017	40	B13	Z15
0	0	0	287	F34	M73	M		20-Mar-2017	39	B11	Z13
0	0	0	288	F43	M25	F		21-Mar-2017	39	B30	Z40
1	0	1	289	F22	M40	F	7-Apr-2020	25-Mar-2017	36	B1	Z2
0	0	0	290	F27	M94	M		5-May-2017	38	B19	Z10
0	0	0	291	F42	M119	M		15-May-2017	37	B13	Z15
0	0	0	292	F12	M60	M		17-May-2017	37	B12	Z14
0	0	0	293	F28	M83	M		25-May-2017	37	B31	Z42
0	0	0	294	F34	M110	M		8-Jun-2017	37	B11	Z13
0	0	2	295	F34	M59	M	18-Jun-2017	12-Jun-2017	0	B11	Z13
0	0	0	296	F2	M29	M		4-Jul-2017	36	B35	Z46
0	0	0	297	F10	M115	M		8-Jul-2017	36	B36	Z47
0	0	2	298	F15	M91	F	9-Sep-2017	3-Sep-2017	0	B18	Z23
0	0	0	299	F29	M53	M		19-Sep-2017	33	B8	Z10

0	0	0	300	F22	M57	M		25-Sep-2017	33	B1	Z2
0	0	2	301	F41	M16	F	3-Oct-2017	3-Oct-2017	0	B24	Z34
0	0	0	302	F4	M3	M		8-Nov-2017	32	B7	Z9
0	0	2	303	F39	M39	unknown	7-Dec-2017	7-Dec-2017	0	B52	Z70
0	0	0	304	F11	M74	F		25-Dec-2017	30	B38	Z50
0	0	0	305	F11	M47	F		13-Jan-2018	29	B38	Z50
0	0	0	306	F42	M2	M		10-Feb-2018	29	B13	Z15
0	0	2	307	F3	M35	M	21-Feb-2018	21-Feb-2018	0	B39	Z52
0	0	2	308	F26	M108	unknown	15-Mar-2018	15-Mar-2018	0	B28	Z38
0	0	0	309	F36	M37	F		27-Mar-2018	27	B17	Z20
0	0	0	310	F24	M76	M		2-Apr-2018	27	B15	Z17
0	0	2	311	F11	M55	M	26-Jun-2018	11-Apr-2018	2	B38	Z50
0	0	0	312	F15	M96	M		5-May-2018	26	B18	Z23
0	0	0	313	F6	M105	M		17-May-2018	25	B9	Z11
0	0	0	314	F24	M68	M		26-Jun-2018	24	B15	Z17
0	0	0	315	F15	M64	M		24-Dec-2018	18	B18	Z23
0	0	2	316	F31	M33	F	16-Jan-2019	12-Jan-2019	0	B50	Z68
0	0	2	317	F26	M34	M	30-Sep-2019	25-Jan-2019	8	B28	Z38
0	0	0	318	F29	M8	F		26-Feb-2019	16	B8	Z10
0	0	0	319	F29	M32	F		6-Jun-2019	13	B8	Z10
0	0	0	320	F45	M88	M		11-Jan-2020	6	B28	Z38
0	0	0	321	F39	M39	M		5-Feb-2020	5	B52	Z70
0	0	0	322	F6	M99	F		26-Feb-2020	4	B9	Z11
0	0	0	323	F43	M80	M		8-Mar-2020	4	B30	Z40
0	0	0	324	F41	M16	M		17-Mar-2020	3	B24	Z34
0	0	0	325	F25	M63	M		18-Mar-2020	3	B42	Z55
0	0	0	326	F2	M104	F		27-Mar-2020	3	B41	Z54
0	0	2	327	F39	M31	F	5-Apr-2020	5-Apr-2020	0	B52	Z70
0	0	0	328	F27	M107	M		4-May-2020	2	B2	Z3
0	0	0	329	F2	M43	F		9-May-2020	2	B41	Z54
0	0	0	330	F8	M97	F		18-Jun-2020	0	B11	Z13

Table S2.2. Crosstabulation of the distribution of EEHV-HD fatal events per Father and Zoo, for the captive European Asian elephant

Zoo/Father	F42	F6	F1	F32	F44	F20	F11	F14	F23	F12	F16	F22	F15	F27	F10	F3	F21	F4	Total
Z11	5	2																	7
Z59			1	1															2
Z6					2	1													3
Z37							1												1
Z50							1												1
Z64								1											1
Z68								4											4
Z12									1										1
Z70									2										2
Z14										2									2
Z19											1								1
Z2 Z23												1	 2						1 2
Z3														1					1
Z47															1				1
Z52																1			1
Z53																	1		1
Z9																		1	1
Total	5	2	1	1	2	1	2	5	3	2	1	1	2	1	1	1	1	1	33

Supplementary Tables of Chapter 3:

Table S3.1. Study population database.

EEHV Status	Group	Code	Location	Sex	Age	Age Class	E2_ C142 G	E4_ C281 T	E5_ T386 A	E5_ G458 A	PT (sec)	aPTT (sec)	Fibrinogen (mg/dL)	Platelet count (x10^3)
0	0	1	1	F	1	1	C	C	A	G	16.7	126.2	580	
0	0	2	1	F	2	1	C	C	A	G	15.9	127.1	620	309
0	0	3	1	F	4	1	C	C	A	G	17.4	130.3	298	
0	0	4	1	F	5	2	C/G	C	A	G	17.0	136.0	510	

0	0	5	1	F	6	2	C	C/T	A	G	18.1	129.6	580	
0	0	6	1	F	7	2	C/G	C	A	G	16.6	138.1	380	333
0	0	7	1	F	8	2	C	C/T	A	G	16.9	134.4	350	354
0	0	8	1	F	8	2	C	C	A	G				
0	0	9	1	F	9	2	C	C	A	G	15.5	108.5	330	
0	0	10	1	F	10	3	C/G	C	A	G	18.1	146.2	400	396
0	0	11	1	F	15	3	C/G	C	A	G	16.5	159.0	290	408
0	0	12	1	F	15	3	C/G	C	A	G				
0	0	13	1	F	30	4	C	C	A	G				
0	0	14	1	F	32	4	G	C	A	G	17.5	138.4	380	1023
0	0	15	1	F	35	5	C	C	A	G				
0	0	16	1	F	40	5	C	C	A	G				
0	0	17	1	F	40	5	G	C	A	G	16.8	147.9	410	660
0	0	18	10	F	20	4	C	C	A	G	18.0	148.3	550	
0	0	19	10	F	21	4	C/G	C	A	G	16.5	170.2	440	458
0	0	20	10	M	30	4	C	C/T	A	G	18.6	126.3	490	378
0	0	21	10	M	30	4	C/G	C	A	G	15.8	187.2	610	
0	0	22	10	M	30	4	C	C	A	G	16.8	149.7	540	349
0	0	23	10	F	30	4	C	C	A	G	17.2	129.3	470	400
0	0	24	10	F	30	4	C	C	A	G	16.3	153.5	480	447
0	0	25	10	F	35	5	C	C	A	G	16.0	130.7	680	140
0	0	26	10	F	40	5	C	C	A	G	16.1	146.5	590	363
0	0	27	10	F	51	5	C	C/T	A	G	17.7	147.6	430	300
0	0	28	2				C	C	A	G	16.4	176.9	348	756
0	0	29	2				C/G	C	A	G	16.3	149.9	330	510
0	0	30	2				C	C	A	G	17.6	142.6	350	1110
0	0	31	2				C/G	C	A	G	16.2	140.7	420	603
0	0	32	2				C	C	A	G	17.7	175.9	380	471
0	0	33	2				G	C	A	G	18.1	194.0	400	501
0	0	34	2				C	C	A	G	18.6	143.6	410	582
0	0	35	2				C/G	C	A	G				
0	0	36	2				C	C	A	G				
0	0	37	2				C	C	A	G	16.4	118.9	340	
2	0	38	2			1	C/G	C	A	G	17.1	156.8	320	285

2	0	39	2			1	C	C	A	G	17.3	106.7	480	879
0	0	40	3		3	1	C/G	C	A	G	16.7	162.6	360	450
0	0	41	3				C	C	A	G	17.8	151.2	390	387
0	0	42	3				C	C	A	G				
0	0	43	3				C/G	C	A	G	18.3	172.8	460	228
0	0	44	3				C/G	C	A	G				
0	0	45	3				C	C/T	A	G				
0	0	46	3				C	C	A	G				
0	0	47	3				C	C	A	G	17.0	98.9	390	1110
0	0	48	3				C	C	A	G	18.2	142.5	470	669
0	0	49	3				C	C	A	G				
0	0	50	3				C	C	A	G				
2	0	51	4	M	4	1	C	C	A	G	17.9	128.1	600	837
0	0	52	4	F		4	C	C	A	G	17.1	119.6	540	348
0	0	53	4	F		4	C	C	A	G	18.4	124.2	650	1074
2	0	54	5	F	4	1	C	C	A	G	17.5	130.1	605	681
2	0	55	6	M	3	1	C/G	C	A	G	16.7	126.6	630	819
2	0	56	7	F	4	2	C	C/T	A	G	18.2	92.1	250	702
0	0	57	8	M	12	3	C/G	C	A	G	18.4	143.6	570	1041
0	0	58	8	M	13	3	C	C	A	G	17.8	125.7	360	381
0	0	59	8	M	14	3	C	C	A	G	17.0	146.3	510	411
0	0	60	8	F	24	4	C/G	C	A	G	16.9	135.5	520	201
0	0	61	8	F	24	4	C	C	A	G	17.9	147.6	410	360
0	0	62	8	F	42	5	C	C	A	G	17.0	132.2	410	183
0	0	63	8	F	44	5	C	C	A	G	16.1	134.9	680	279
0	0	64	8	M	52	5	G	C	A	G	17.8	135.0	250	339
0	0	65	9	M	6	2	C	C	A	G	17.1	136.0	450	552
0	0	66	9	M	10	3	C/G	C	A	G	16.5	164.4	480	684
0	0	67	9	M	13	3	C/G	C	A	G	16.6	140.4	440	435
0	0	68	9	F	13	3	C/G	C	A	G	16.9	139.8	580	381
0	0	69	9	F	20	4	C/G	C	A	G	17.2	155.4	670	1080
0	0	70	9	F	21	4	C	C	A	G	17.4	139.2	780	600
0	0	71	9	F	21	4	C/G	C	A	G	16.3	131.4	490	936
0	0	72	9	F	22	4	C	C	A	G	15.8	138.3	520	690

0	0	73	9	F	23	4	C	C	A	G	16.2	180.5	560	984
0	0	74	9	F	25	4	C	C	A	G	17.6	164.3	370	906
0	0	75	9	F	26	4	C	C	A	G	19.5	124.4	420	684
0	0	76	9	M	60	5	C	C	A/T	A	17.6	156.3	480	216
0	1	77	11	F	34	4								578
0	1	78	12	M	13	3					17.7	163.1		
0	1	79	13		0	0		C	A	G				
0	1	80	13	F	22	4					16.6	121.6	830	477
0	1	81	13	F	32	4	C/G	C	A	G	17.3	85.1	620	698
0	1	82	13	F	42	5	C	C	A	G	17.6	116.1	670	501
0	1	83	14	M	22	4	C		A	G	16.2	83.7	490	609
0	1	84	14	F	28	4	C	C	A	G	17.1	122.3	610	597
0	1	85	14	F	41	5	C	C	A	G	16.8	100.1	640	729
0	1	86	14	F	42	5					18.5	117.9	610	686
0	1	87	14	F	45	5					18.2	135.8	750	492
0	1	88	15	F	18	3	C/G	C	A	G				
0	1	89	15	F	21	4	G	C	A	G	19.4	115.7	710	443
0	1	90	15	F	41	5		C	A	G				
0	1	91	15	M	58	5	C/G	C	A	G				
1	1	92	16	M	3	1	C/G	C	A	G				
0	1	93	16	F	48	5	C/G	C/T	A	G				
1	1	94	17	M	2	1	C/G	C						
1	1	95	17	F	3	1	C	C	A	G				
0	1	96	17	F	10	3		C	A	G	16.3	105.9	530	792
0	1	97	17	F	14	3	C/G	C	A	G	17.0	139.6	650	717
0	1	98	17	F	21	4	C/G	C	A	G	17.3	117.1	600	653
0	1	99	17	F	38	5	C/G	C	A	G	17.0	129.5	860	668
0	1	100	17	F	50	5		C	A	G	17.8	133.4	590	704
0	1	101	17	F	51	5	C	C	A	G	16.3	114.4	650	512
0	1	102	17	M	4	1	C		A	G				
0	1	103	18	M	6	2	C/G		A	G	16.5	112.7	600	590
0	1	104	18	F	10	3	C/G	C	A	G	17.0	121.8	530	566
0	1	105	18	F	23	4	C		A	G	17.4	129.6	490	633
0	1	106	18	F	27	4	C		A	G	17.3	109.9	640	453

0	1	107	18	F	29	4	C/G	C	A	G	16.1	126.1	510	624
0	1	108	18	F	33	4	G	C	A	G	17.3	143.2	480	566
0	1	109	18	F	37	5		C	A	G				
2	1	110	19	M	3	1	G		A	G	18.2	111.2	560	422
1	1	111	19	F	7	2	C/G	C	A	G				
0	1	112	19	M	19	3	C/G	C	A	G	17.0	120.0	345	732
0	1	113	19	F	24	4	G	C	A	G	21.5	126.5	500	639
0	1	114	19	F	33	4	C/G	C	A	G	17.8	121.5	440	629
0	1	115	19	F	45	5	C/G	C/T	A	G	19.6	117.6	570	635
0	1	116	20	M	27	4	C/G		A	G				
0	1	117	20	F	1	1								309
0	1	118	20	F	8	2	C/G	C	A	G	15.3	137.4	480	1001
0	1	119	20	M	13	3	C	C	A	G				
0	1	120	20	F	32	4	C/G	C	A	G	17.0	135.0	690	653
0	1	121	20	F	35	5	C/G		A	G				
0	1	122	20	F	43	5	G	C	A	G	17.7	168.3	1,633	848
0	1	123	21	M	5	2					17.5	129.6		632
0	1	124	21	M	13	3	C/G	C	A	G	17.2	130.3	790	839
0	1	125	21	M	25	4	C/G	C	A	G				
0	1	126	22	F	21	4	C				17.5	117.7	703	768
0	1	127	22	F	25	4	C/G	C	A	G	19.2	129.6	540	873
0	1	128	22	M	39	5	C/G		A	G	17.0	128.7	510	626
0	1	129	22	F	50	5	C/G		A	G	18.7	130.6	760	458
0	1	130	23	M	9	2					16.8	153.9	685	687
1	1	131	24	F	2	1	G	C	A	G				
0	1	132	24	F	8	2	C	C	A	G	16.7	97.2	450	1343
0	1	133	24	M	29	4					21.1	108.6		
0	1	134	24	F	47	5					15.5	154.4		651
0	1	135	25	M	0	0	C/G	C	A	G				
0	1	136	25	M	7	2	C/G	C	A	G				
0	1	137	25	F	7	2					19.8	157.2	480	428
0	1	138	25	F	22	4	C	C	A	G	16.1	159.4	420	608
0	1	139	25	F	35	5			A/T	A/G	16.7	108.6	580	339
0	1	140	25	F	53	5	C		A	G	17.8	159.7	650	470

0	1	141	26	F	36	5		C	A	G	15.3	117.4		593
0	1	142	27	F	22	4					16.1	107.5	660	612
0	1	143	27	M	23	4					16.7	125.3		455
0	1	144	28	F	31	4					18.0	132.2	563	477
0	1	145	29	F	12	3	G	C	A	G	18.9	126.5	760	299
0	1	146	29	F	13	3	C	C	A	G	17.1	131.0	730	350
0	1	147	29	F	41	5	G	C	A	G	18.4	126.8	480	617
0	1	148	29	F	58	5	C/G	C	A	G	17.7	121.8	680	479
0	1	149	30	F	14	3	C/G		A	G	18.5	130.9	530	465
0	1	150	30	F	17	3	C/G	C	A	G	18.7	112.8	590	627
0	1	151	30	F	47	5	C/G		A	G	19.0	106.2	750	560
0	1	152	31	F	22	4	C	C	A	G	17.5	132.9	690	626
0	1	153	32	M	18	3		C						
0	1	154	33	F	32	4	C/G	C	A	G				
1	1	155	34	F	2	1	C/G	C	A	G				
0	1	156	34	F	20	4	C/G		A	G				
0	1	157	34	F	24	4	G		A	G				
0	1	158	34	F	24	4	C	C	A/T	A/G				
2	1	159	35	M	3	1					19.2	107.1		141
0	1	160	35	F	23	4					18.5	118.1		326
0	1	161	35	F	25	4					18.2	119.7		455
0	1	162	35	F	38	5								678
0	1	163	36	F	12	3					17.1	154.2	510	
0	1	164	36	M	13	3					18.2	135.8	530	
0	1	165	36	F	31	4					17.0	119.8	670	
0	1	166	36	F	42	5					16.1	131.8	480	
0	1	167	36	M	48	5					16.7	131.6	640	

* EEHV_Status 0) Never presented EEHV-HD symptoms, 1) fatal case from EEHV-HD, 2) Survival case from EEHV-HD; Group 0) Thailand, 1) Europe; Age class 1) 0-4 years old, 2) 5-9 years old, 3) 10-19 years old, 4) 20-34; years old, 5) >35 years old; E2_C142G, E4_C281T, E5_T386A, E5_G458A are missense mutations and here is shown the nucleotide present for each individual tested for each SNP.

Table S3.2. Positions of the SNPs detected in the F7 gene of Asian elephants from Thailand and from European zoos.

```
ATG GCT TCC CAT TCC CGC GGG CTC GCC CTT CTC TGC TTT CTG CTC   45
Met Ala Ser His Ser Arg Gly Leu Ala Leu Leu Cys Phe Leu Leu   15

GGG TTT CAG CAC CCT CTG ACA GCA GTC TTC ATG AAC CAG GAG GAA   90
Gly Phe Gln His Pro Leu Thr Ala Val Phe Met Asn Gln Glu Glu   30

GCC AAC AGC GTC TTA CAC AGG CAA AGG CGA GCC AAC AGT TTC TTC   135
Ala Asn Ser Val Leu His Arg Gln Arg Arg Ala Asn Ser Phe Phe   45

        G
GAA GAA CTG AGG TCA GGG TCA CTG GAG AGA GAG TGC AAG GAA GAA   180
Glu Glu Leu Arg Ser Gly Ser Leu Glu Arg Glu Cys Lys Glu Glu   60
        VAL
                              *
CAG TGC TCG TTC GAG GAA GCC AGG GAG ATC TTC AAG AGC ACT GAG   225
Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Ser Thr Glu   75

AGG ACT AGG CAG TTC TGG GTG GCT TAT ACC GAT GGA AAC CAG TGC   270
Arg Thr Arg Gln Phe Trp Val Ala Tyr Thr Asp Gly Asn Gln Cys   90

             T
ACC TCA AAC CCG TGC CAG AAT GGG GGC CTG TGT GTG GAC CAG CTC   315
Thr Ser Asn Pro Cys Gln Asn Gly Gly Leu Cys Val Asp Gln Leu   105
            Leu

CAG TCT TAC ATT TGC TTC TGC CTT GAT GAT TTT GAG GGT CGG AAC   360
Gln Ser Tyr Ile Cys Phe Cys Leu Asp Asp Phe Glu Gly Arg Asn   120

                                  A
TGT GAG ACA AAC AAA AAC AGC CAG CTG ATC TGT CTG AAT GAA AAC   405
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*Cys Glu Thr Asn Lys Asn Ser Gln **Leu** Ile Cys Leu Asn Glu Asn* 135

Gln

GGA GGC TGT GAA CAG TAC TGC AGT GAC AAC GCA GAG ACC AAG CGT 450

Gly Gly Cys Glu Gln Tyr Cys Ser Asp Asn Ala Glu Thr Lys Arg 150

A

*TCC TGC **CGA** TGT CAT GAC GGC TAC ACG CTC ATG GCT **GAT** GGA GTG* 495

*Ser Cys **Arg** Cys His Asp Gly Tyr Thr Leu Met Ala **Asp** Gly Val* 165

Gln

TCC TGC ACG CCC ACA GTT GAA TAT CCG TGT GGA AAA ATA CCT GTT 540

Ser Cys Thr Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Val 180

CTG GAA AAA AGA AAT GAC AAC ATC CCC CAA GGC CGA ATT GTG GGT 585

Leu Glu Lys Arg Asn Asp Asn Ile Pro Gln Gly Arg Ile Val Gly 195

GGC AGG TTG TGT CCC AAA GGG GAG TGT CCA TGG CAG GCT GTG ATA 630

Gly Arg Leu Cys Pro Lys Gly Glu Cys Pro Trp Gln Ala Val Ile 210

AAG CTG CAG GGG ACT CTG CTG TGT GGG GGA TCT CTG CTT GAC GCC 675

Lys Leu Gln Gly Thr Leu Leu Cys Gly Gly Ser Leu Leu Asp Ala 225

ACC TGG GTG GTC TCC GCA GCC CAC TGT TTC AAC AAA CCC GGC ATC 720

Thr Trp Val Val Ser Ala Ala His Cys Phe Asn Lys Pro Gly Ile 240

CTC AGG AAC TGG GAG AAT ATA ACA GTG GTG TTG GGT GAG CAC GAC 765

Leu Arg Asn Trp Glu Asn Ile Thr Val Val Leu Gly Glu His Asp 255

TTT AGT GAC GAG GAC GGC GAT GAA CAA GAA CGG CGA ATT GCT CAG 810

Phe Ser Asp Glu Asp Gly Asp Glu Gln Glu Arg Arg Ile Ala Gln 270

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ATC ATA ATC CCT GAC AAG TAT GTG TCA GGC AAG ACC GAC CAC GAC   855
Ile Ile Ile Pro Asp Lys Tyr Val Ser Gly Lys Thr Asp His Asp   285

ATT GCC CTG CTG CGC CTG AGA ACG CCG GTG AAC TTC ACT GAC TAC   900
Ile Ala Leu Leu Arg Leu Arg Thr Pro Val Asn Phe Thr Asp Tyr   300

GTA GTG CCC CTC TGT TTG CCT GAC AAG AGA TTC TCA GAG CAA ACA   945
Val Val Pro Leu Cys Leu Pro Asp Lys Arg Phe Ser Glu Gln Thr   315

CTC GCC TTC ATC CGT TTC TCC TCC GTG AGC GGC TGG GGC CAG CTT   990
Leu Ala Phe Ile Arg Phe Ser Ser Val Ser Gly Trp Gly Gln Leu   330

CTC GAC AGG GGC GCC ACA GCC CTC GAG CTC ATG ACT ATA GAC GTG   1035
Leu Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Thr Ile Asp Val   345

CCC AGG CTG ATG ACC CAG GAC TGT AAT GAG CAA ATG CAA AGG ACC   1080
Pro Arg Leu Met Thr Gln Asp Cys Asn Glu Gln Met Gln Arg Thr   360

GCC AAC TCC CCA GTG GTG ACC GAG AAC ATG TTC TGT GCT GGC TAC   1125
Ala Asn Ser Pro Val Val Thr Glu Asn Met Phe Cys Ala Gly Tyr   375

CTG GAT GGG ACC AAG GAT GCC TGC AAG GGT GAC AGT GGG GGC CCT   1170
Leu Asp Gly Thr Lys Asp Ala Cys Lys Gly Asp Ser Gly Gly Pro   390

CAT GCC ACC AAG TAC CGA AAC ACA TGG TAC CTG ACA GGA ATT GTC   1215
His Ala Thr Lys Tyr Arg Asn Thr Trp Tyr Leu Thr Gly Ile Val   405

AGC TGG GGT GAG GGC TGT GCA GCC GTG GGC CAC GTT GGG GTG TAC   1260
Ser Trp Gly Glu Gly Cys Ala Ala Val Gly His Val Gly Val Tyr   420

ACC AGG GTC TCC CGG TAC ATT GAG TGG CTG AAC AGG CTC ATG GAC   1305
Thr Arg Val Ser Arg Tyr Ile Glu Trp Leu Asn Arg Leu Met Asp   435
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TCG AAC CCG AGC CCA GGC CGT TTC CTG TCA GCC CGT TTT CCC TAG 1350

Ser Asn Pro Ser Pro Gly Arg Phe Leu Ser Ala Arg Phe Pro End 450

Triplets containing SNPs are labelled in bold together with the amino acid coded by that triplet. The actual position of the SNP is underlined. Grey boxes indicate SNPs for which one allele is causing a missense (non-synonymous) mutation. Nucleotides of the alternative allele are given on top of the respective SNP, amino acid changes are indicated below the SNP. Numbers at the right indicate position of the last nucleotide (top line) or the last amino acid (lower line). Nucleotide and amino acid positions are based on the African elephant (*Loxodonta africana*) cDNA of the F7 gene coding for coagulation factor VII (without 5' untranslated region). * Previously reported mutation found in Asian elephants (Lynch et al. 2017), but not present in our European and Thai elephant study population.

List of publications

Published publications

Jesus S A, Doherr M G, Hildebrandt T B (2021): Elephant endotheliotropic herpesvirus impact in the European Asian Elephant (*Elephas maximus*) population: Are hereditability and zoo-associated factors linked with mortality? *Animals*.; 11(10): 2816. doi.org/10.3390/ani11102816

Jesus S A, Schmidt A, Fickel J, Doherr M G, Boonprasert K, Thitaram C, Sariya L, Ratanakron P, Hildebrandt T B (2022): Assessing coagulation parameters in healthy Asian Elephants (*Elephas maximus*) from European and Thai Populations. *Animals*, *12*, 361**.** doi.org/10.3390/ani12030361

Oral Presentations

Fontes S (2020): Conservation Heroes – Zoo elephant research and contribution to their wild cousins. Berlin Science Week 2020. November 1-10, 2020, Berlin, Germany. Available at: https://www.youtube.com/watch?v=i4iZ4-vV13s

Jesus S A, Hildebrandt T B (2019): Elephant endotheliotropic herpesvirus haemorrhagic disease – The impact on the European captive population of Asian elephant (*Elephas maximus*). 12th DRS Doktorandensymposium 2019, September 27, 2019, Berlin, Germany.

Jesus S, Hildebrandt TB (2019): Doença hemorrágica causada pelo herpesvirus endoteliotrópico dos elefantes – o seu impacto na população captiva de elefantes Asiáticos (*Elephas maximus*) na Europa. 5th Scientific Reunion of the Iberian Association of the EAZWV 2019, November 16-17, 2019, Lisbon, Portugal.

Fontes S A J, Fickel J, Schmidt A, Hildebrandt T (2018): Elephant endotheliotropic herpesvirus (EEHV) infection in Asian elephants (Elephas maximus) possible correlated hereditary coagulation disorder. 13th European Wildlife Disease Association 2018, August 27-31, 2018, Larissa, Greece.

Fontes SJ, Hildebrandt TB (2018): Understanding the fatal elephant endotheliotropic herpesvirus (EEHV) infection – possible correlated hereditary disorder and protective factors. Wildlife Group of the SAVA Congress 2018, March 1-3, 2018, Johannesburg, South Africa.

Jesus S, Hildebrandt T (2018): Elephant Endotheliotropic Herpesvirus haemorrhagic disease and the impact of this disease on the European captive population of Asian elephant (*Elephas maximus*). Joint EAZWV/AAZV/Leibniz-IZW Conference 2018, October 6-12, 2018, Prague, Czech Republic.

Fontes S, Schmidt A, Fickel J, Hildebrandt T (2017): Factor de coagulação VII e seu relacionamento com a infecção fatal por herpesvirus endotelial em elefantes Asiáticos. 4th Scientific Meeting of the Iberian Section of Zoo and Wildlife Veterinarians 2017, November 3-4, 2017, Madrid, Spain.

Fontes S, Fickel J, Hildebrandt TB (2017): Genetic analysis of coagulation factor VII in fatalities caused by EEHV-HD in Asian elephants (*Elephas maximus*). 11th International EEHV workshop 2017, May 15-17, 2017, London, UK.

Poster Presentations

Jesus S, Pluháčková J, Bolechová P, Hildebrandt T (2019): Hand-rearing in Asian elephants (*Elephas maximus*) - a case report, Joint Leibniz-IZW/EAZWV/ECZM Conference 2019, June 12-15, 2019, Kolmården, Sweden.

Fontes S J, Schmidt A, Fickel J, Hildebrandt T B (2017): Genetic analysis of coagulation factor VII and its correlation with elephant endotheliotropic herpesvirus in Asian elephants (*Elephas maximus*). 11th International Conference on Behaviour, Physiology and Genetics of Wildlife 2017, October 4-7, 2017, Berlin, Germany.

Fontes SJ, Hildebrandt TB, Fickel J (2017): Understanding immunity against fatal elephant endotheliotropic herpesvirus (EEHV) infection. International Zoo and Wildlife Conference 2017, May 24-27, 2017, Berlin, Germany.

Magazine interviews

Tavares A (2020): Herpesvírus endoteliotrópico – Há uma investigadora Portuguesa a estudar o herpesvírus dos elefantes. *Veterinária Actual*, May 2020; 18-24. Available at: https://www.flipsnack.com/7999EADEFB5/va-maio-revista-digital/full-view.html

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Interessenskonflikte

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch in Anspruch genommen habe.

I hereby confirm that I have written this thesis independently. I certify that I have used only the sources and aids indicated.

Berlin, den 13. Juni 2022 Sónia Alexandra de Jesus Fontes

“The question is, are we happy to suppose that our grandchildren may never be able to see an elephant except in a picture book?” – David Attenborough

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