Epidemiology of a sexually transmittable herpesvirus, the *Mustelid gammaherpesvirus 1* (MusGHV-1), and its link to reproductive health and physiological stress in European badgers (*Meles meles*)

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Ming-shan Tsai
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Abstract

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Along with virulence and transmissibility, pathogen transmission mode can be decisive on host-pathogen dynamics, where sexually transmitted infections (STIs) are predicted to be more likely to drive vulnerable host population to extinction than infections transmitted mainly through other routes. Despite STIs being common among animals, and often associated with sterility, STIs have been poorly studied in wild animals. As a background introduction to the current state of knowledge about wildlife STIs, in Chapter 1 I reviewed studies reporting STIs in wild musteloids; a group substantially ignored in previous STI reviews. The high proportion of reported cases associated with reproductive diseases and failure, and disproportionally investigation effort in wild populations highlight the need for further research, especially in species of conservation concern. I established the need for systematic wild mammal host-STI models to better understand the actual impact of STIs on wildlife health and population dynamics. In Chapter 2, I laid the foundations for investigating a sexually transmittable pathogen, the *Mustelid gammaherpesvirus 1* (MusGHV-1), in free-ranging European badgers (*Meles meles*), and reported that MusGHV-1 reactivation has a negative effect on female pregnancy success, but not on male sperm abundance. In Chapter 3, I explored influential host risk factors associated with higher MusGHV-1 shedding, such as age, body conditions and sex. In Chapter 4, I explored the effect of MusGHV-1 coinfection with a zoonotic bacterial pathogen, *Clostridium perfringens*. MusGHV-1 reactivation causes a predisposition to *C. perfringens* overgrowth in the gut, especially when infected with a novel MusGHV-1 variant. This led me to further explore the genetic and phenotypic differences between the two MusGHV-1 variants identified in this badger population in Chapter 5. I found social group-biased MusGHV-1 variant frequencies and blood viral load differences, implying different functional virulence between the two variants. In Chapter 6, I identified that weather-related stress experienced during prenatal life exacerbate the likelihood of MusGHV-1 reactivation in adulthood. Collectively, these chapters reveal the potential for host responses to viral STI's to be complex, hinging on individual demographics, coinfection status, environmental conditions, and the viral strain involved; all factors needing proper consideration when attempting to mitigate the role of disease in conservation ecology.

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General introduction

Definition of sexually transmitted/ transmissible infection

According to the World Health Organization (WHO), sexually transmitted diseases (STDs) or sexually transmitted infections (STIs; to include also asymptomatic cases), describe pathogens predominantly transmitted by sexual contacts either horizontally through sexual intercourse or vertically from mother to child (World Health Organization 2014). Human STIs are spread mainly by the horizontal route, such as HIV, chlamydia, gonorrhoea, syphilis, and trichomoniasis (World Health Organization 2018). However, many pathogens of clinical or epidemiological relevance (Bartlett 2004) that are not usually treated as STIs have been documented with occasional sexual transmission, such as infections caused by Ebola and Zika virus (Lee and Nishiura 2019; Mead, Hills, and Brooks 2018). Therefore some may consider it appropriate to designate these pathogens as sexually transmissible to distinguish them from STIs in the stricter sense (Bartlett 2004; Davison 2011). For studies of infections in wildlife, the first term is preferable when the focus is on sexual transmission, as transmission routes of most wildlife pathogens are under-studied, and the venereal route is often neglected (Lockhart, Thrall, and Antonovics 1996). Therefore, in my thesis, STIs in wildlife are confirmed/ suspected to be sexually transmissible, although in most cases without a full investigation of pathological or epidemiological determination of all transmission routes.

Characteristics of STIs

The dynamics of STIs differ from other infections because STI transmission is frequency-dependent rather than density-dependent like most other infectious diseases (Antonovics 2017; Ryder et al. 2005; Thrall, Biere, and Uyenoyama 1995). Thus, STIs can prevail in a population even when the host density is low because intimate contact frequency is independent of host density. This can predict disease dynamics, which are particularly relevant in the context of disease-induced extinction. A comprehensive review of 200 animal STIs by Lockhart and colleagues (Lockhart et al. 1996) compares several disease characteristics between STIs and ordinary infections. They conclude that STIs result in "less mortality" and "are longer-lived in their hosts, are less likely to invoke strong immune responses, have narrower host ranges, show less fluctuation in prevalence over time", and often have "negative effect on host fertility". These characteristics make STIs seem less severe than, for example, many respiratory infections with acute disease or fatal consequences. However, I consider that the far-reaching impacts of STIs may be seriously destructive to vulnerable wild populations and have far-reaching ecological consequences, particularly if STIs result in host sterility.

STIs are more likely to cause sterility (or more typically partial sterility, such as lower fecundity or temporary sterility during active infection) than other categories of infectious diseases. One hypothesis is that pathogen-induced sterility is adaptive (Apari, de Sousa, and Müller 2014). It promotes further sexual transmission, insofar as sterility can prevent females from entering pregnancy and thus prolong the period of mating and, thereby, increase transmission probability (Apari et al. 2014). Similarly, the typically low virulence of STIs could also be adaptive as it likely maintains high mating frequency and may prevent sexual partners from detecting the infection (Ashby et al. 2019; Lockhart et al. 1996). However, host species with promiscuous mating systems and without long-term pair bonds may also suffer from the sterilizing effects of STI, as illustrated by koalas with chlamydia and two-spot ladybirds with mite infection (Hurst et al. 1995; McCallum et al. 2018). It is therefore not clear what evolutionary hypothesis might explain sterility as an adaptive strategy for the pathogen. Alternatively, according to McLeod and Day (2019), sterility virulence can be favoured when infection transmission is density invariant (as it is in STIs) and mortality is density dependent, making small populations particularly vulnerable to extinction due to demographic stochasticity (i.e., the effects of random processes, such as birth/ death/ diseases). Therefore, sterility is arguably a trait of STIs shaped by epidemiological characteristics such as transmission mode or is due to the invasion and deterioration of reproductive organs by the causative agents of STIs, leading to the alteration of their functioning. In human STIs, sterility is evident with severe effects like foetal and neonatal death, infertility, cervical cancer, HIV risk, and poor life quality (World Health Organisation 2016). However, whether STIs can drive population dynamics in wild animals, such as population productivity declines due to host sterility and recruitment decreases due to impaired offspring fitness (Hurst et al. 1995; Tsevat et al. 2017), is still under-studied.

STIs and disease-induced host extinction

The prevalence of infections transmitted by a respiratory or faecal-oral route is generally highly dependent on host population density, e.g., SARS-CoV-2 or the influenza virus. Infections of this kind usually do not drive the host population to extinction because transmission is becoming correspondingly limited when population density has decreased to a certain level due to disease mortality. At that point, the host population will thrive again since the prevalence of infection falls if the population level does not fall below the extinction threshold (i.e., the value below which a population will go extinct) (Society and Press 2017). In contrast, for infections with density-independent transmission modes, such as STIs and vector-borne diseases, reduction of host population density has little effect on infection prevalence (O'Keefe 2005). Theoretical modeling even predicts that adding a limited frequency-dependent transmission component to infection dynamics that are mainly density-dependent may be sufficient to drive host extinction (Ryder et al. 2007). Adding to susceptibility to the mate-finding Allee effect (i.e., when finding a suitable mate is difficult at low population density, with a resulting positive correlation between reproductive fitness and population density or size)(Gascoigne et al. 2009), these may drive

vulnerable host populations towards extinction (Deredec and Courchamp 2006). However, absolute frequency-dependent transmission is unlikely to exist in the natural world. Extremely low density will still limit sexual transmission (Deredec and Courchamp 2006; O'Keefe 2005) because individuals in very low density populations will have a much lower chance to mate thus limiting STI pathogen spread. Whether STIs can actually lead to host extinction is hard to be tested in nature, but well-known wildlife host-STI systems, such as the koala-Chlamydia (Hulse et al. 2019) and ladybirds-mite (Webberley et al. 2006), seem to maintain stabilized population-disease dynamics. A study led by Berec and Maxin (2013), predicts that STIs with a strong sterilizing effect either lead to disease-free persistence or host extinction, depending on the initial prevalence, where high prevalence in the beginning of the epidemic is more likely to cause host extinction while low prevalence results in stable host-pathogen dynamics. Furthermore, limit to demographic loss is also a factor to eliminate the impacts of STIs, as the density-dependent component increases when population density is low. For example, the highly transmissible, pathogenic and sterilizing mite infection in ladybirds in fact has low prevalence in the beginning of the breeding season in spring due to overwintering mortality and restricted mating between the old (widely infected) cohort and the new (naïve) cohort (Ashby et al. 2019). Similarly, the morbidity, mortality and sterility caused by chlamydiosis in koalas are highly dependent on environmentally driven stress factors such as drought or extremely hot weather conditions (Lunney et al. 2012), which result in host population declines, as well as declines in the prevalence of chlamydiosis.

The above real-world examples further emphasize that "mixed" terms of transmission modes are present at different host population density levels (Deredec and Courchamp 2006). This may explain why some pathogens have different strains adopting different transmission modes, such as the herpes simplex virus (HSV) 1 and 2. HSV-1 adopts predominantly a non-genital route while HSV-2 is principally transmitted through venereal or vertical (from mother to child) routes (Davison 2011; Nahmias, Keyserling, and Lee 1989). Furthermore, closely related STI-pathogens can manifest in quite different symptoms. For example, *Treponema pallidum* spp. cause yaws (tropical disease-causing lesions in the skin, skeleton, and joints, spread by direct contact to fluids from an infected person) as well as syphilis (an STD causing lesions of the genitalia) (Šmajs, Norris, and Weinstock 2012). The two *T. palladium* subspecies, however are hard to distinguish, morphologically, and antigenically. Thus, molecular epidemiology is playing an increasingly important role in pathogen variant monitoring and the characterization of transmission modes in host populations, especially in wildlife populations suffering from Allee effects (DeCandia, Dobson, and vonHoldt 2018). Furthermore, anthropogenic stress factors, such as climate change, deforestation, and habitat fragmentation can alter these dynamics in unpredictable ways. It is therefore important to consider genetic, phenotypic and environmental factors in combination when investigating the real impacts of STIs on population dynamics.

Current status and challenges of STI studies in wild mammals

Investigating infections in wildlife that are primarily asymptomatic/ with no obvious sign of disease, as is the case for most STIs, is technically difficult and logistically challenging. It often requires sedation of the animal and invasive sampling (Kent et al. 2018), which is even more challenging when the studied population is threatened by extinction. Furthermore, sterility – seemingly a primary sign of disease of STIs - is extremely hard to investigate in wildlife. It requires long-term surveillance of identifiable individuals and their reproductive success. Such measures include pedigree analysis (Annavi et al. 2014), examination of pregnancy status using ultra-sound in females during gestation (Domingo-Roura et al. 2003), sperm analysis for males during mating seasons (Gomendio et al. 2007), reproductive hormone analysis (Sugianto et al., 2018) or postmortem examination of pregnancy in females (Strand, Skogland, and Kvam 1995). The practical challenges are compounded by the low mortality rate characteristic of STIs. Only when there is a sense of urgency are resources dedicated to studying STIs in the wild, such as the sexually transmitted chlamydia infections in koalas that have been partly responsible for the recent population declines in Australia (Gonzalez-Astudillo et al. 2017). Systematic investigation of STIs in wild mammals should raise the conservation agenda regarding the theoretical and increasing empirical evidence that STIs can pose extinction risks to vulnerable wildlife populations.

The need for studying STIs in mammals using new model species

Although existing theories of STIs provide a solid foundation for understanding human STI dynamics, it is unknown how readily this can be applied to wild species and substantial variation in STI dynamics between host-pathogen systems is to be expected (Lockhart et al. 1996). An essential contributor to this variation is the epidemiology of the pathogens. For example, some STIs, such as herpesvirus infection have no recovery stage (Grinde 2013a), which means elimination of the virus from the host is not possible; and STI infections tend to recur after recovery due to the minimal immune response, which were linked to the less severity of disease (Lockhart et al. 1996). A further complication is that co-infection of STIs with other pathogens can enhance/ suppress the pathogenesis of the STI. For example, herpesvirus infection is a risk factor of chlamydia infection in koalas (Stalder et al. 2015); in contrast, a human STI, GB virus C (formerly known as hepatitis G virus), can inhibit HIV virulence and vertical transmission (Xiang et al. 2001). Furthermore, STI dynamics are also subject to factors related to host mating, such as mate choice, promiscuity, mating system (monogamous or polygynandrous), or mating dominance. These factors make it difficult to apply theoretical STI dynamic models developed on one host-pathogen system to another. Although the ladybird-mite STI system (see above) has provided well-established empirical tests of STI dynamic theories in natural settings (Ashby et al. 2019), comparable studies for mammalian systems are generally lacking. It is also surprising that herpesviruses (i.e., members of the *Herpesviridae* family, a ubiquitous pathogen family in the animal kingdom widely associated with sexual transmission), are little studied beyond

human genital herpes (HSV-2) (Fatahzadeh and Schwartz 2007; Foss et al. 2009). On the basis of the well-documented impacts on reproduction by herpesvirus infections in livestock animals like horses, cattle, pigs (Smith 1997; Thiry et al. 1989) and experimental animals like mice (Cardenas et al. 2011), I consider it essential to explore new model species to understand the STI dynamics of herpesviruses in non-human mammalian wildlife species. There are numerous species, such as koalas (Stalder et al. 2015), elephants (Wellehan et al. 2008) or some cetaceans like dolphins or sea lions (Deming et al. 2021; Van Elk et al. 2009), which suffer well-documented herpesvirus infections. However, these host species have study limitations (e.g., due to conservation status or logistical difficulties in sampling the whole population in the field, difficulties to obtain demographic data on the individual level such as sex, precise age, genealogy, etc). To eliminate these restrictions, I used the European badger (*Meles meles*) as a model species to investigate sexually transmittable herpesvirus dynamics in wildlife. I conducted my research in a relatively closed and stable badger population in Wytham Woods (hereafter referred to as the Wytham Woods population), which has established long-term population data from more than 30 years ago, an individual identification system, and has frequently served as a model to gain understanding of wildlife ecology and behaviour as evidenced by many successful studies (reviewed in Macdonald et al., 2015).

European badgers as a wildlife model to study STIs

European badgers belong to the Musteloidea, order Carnivora. They have a polygynandrous and highly promiscuous mating system (Dugdale, Griffiths, and Macdonald 2011) and are evolutionarily distinct in regard to their reproductive strategies, which involve delayed implantation (blastocysts floating in the uterus for up to 330 days after fertilization) and superfoetation (able to enter additional oestrus cycles and ovulation during pre-implantation pregnancy) (Yamaguchi, Dugdale, and Macdonald 2006). A recent study has found that badgers even adapt the number of oestrus cycles depending on population density by altering oestrogen levels (Sugianto et al., 2021). These adaptable reproductive characteristics have equipped badgers to adjust to different environments and conditions across Europe. Badgers are highly synchronous seasonal breeders, giving birth to a litter of 1 to 5 cubs (in Wytham, average litter size = 1.46± 0.65)(Annavi et al. 2014), although only less than half of all adults reproduce successfully in a given year (see below). In our study population in Wytham Woods, badgers show one distinct post-partum mating season in winter (January – March), although mating is reported across the year in other populations. For example, in Ireland, mating in badgers occurs throughout the whole year, peaks in late summer (Corner et al. 2015). The occurrence of multiple promiscuous matings are likely to make badgers particularly susceptible to STIs.

Interestingly, it has been reported that reproductive failure rates are generally higher in badgers (Page, Ross, and Langton 1994) than in other carnivore species (Creel and Creel 1991; Fournier-Chambrillon et al. 2010). The form and percentage of reproductive failures amongst badgers

differ between populations of different densities. In two intensively studied English high density populations, Carpenter et al. (2005) found only 30% of sexually-mature individuals of each sex reproduce successfully each year in Gloucestershire, using pedigree analysis, which is comparable to the figure estimated by lactation status in our Wytham Woods (Oxfordshire) population that 29% of females breed successfully each year (Macdonald and Newman, 2006). More surprisingly, the pedigree analysis shows only 45% of females and 32% of males of the Wytham Woods population had bred successfully during their lifetime (Annavi et al. 2014). According to the postmortem examination, prenatal mortality is high: in a medium-density Irish population, 34% - 36% of females failed to conceive, whereas, there was less than 1% loss to abortion (Corner et al. 2015; Laura E. Rosen et al. 2019). In high-density populations in England, although 97% of adult females mated, and 80% had blastocysts, only 32% had successful implantation (Page et al. 1994). Furthermore, loss of an implanted foetus before birth was estimated to be 36% (Page et al. 1994). These observations indicate infertility may be widespread in badgers. Thus far, these levels of reproductive failure have been interpreted as a consequence of high intra-sexual competition amongst females or adverse pre-implantation weather conditions (Woodroffe and Macdonald, 1995). The question of whether STIs play a role in the generally low fecundity has, until now, never been explored.

Badger herpesvirus as an STI

Although badgers are susceptible to several pathogens that have sexually transmission potential, such as *Mycobacterium bovis* (causing bovine tuberculosis), the only pathogen that has been isolated from badger genital tract so far is the *Mustelid gammaherpesvirus 1* (MusGHV-1) (Kent et al. 2018). MusGHV-1 belongs to the herpesvirus family (*Herpesviridae*). Herpesvirus has four subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, *Gammaherpesvirinae* and the recently classified *Deltaherpesvirinae* (Pellett 2014). All herpesviruses are large, enveloped viruses with double stranded DNA genome sizes range from 120k to 240k base pairs (McGeoch, Rixon, and Davison 2006), and are featuring in causing lifelong infection by switching between latency and reactivation stage. Herpesvirus is a ubiquitous pathogen in the animal kingdom, it can be found from mollusks to elephants. It is therefore not surprising to find MusGHV-1 is a common pathogen in free-ranging badgers in the UK and Ireland. Previous screening studies reported 95 to 100% prevalence in free-ranging badger populations by virus DNA detection in the blood (King et al. 2004; Sin et al. 2014). It was first discovered in lung tissues sampled from a diseased wild female badger in Cornwall, England, in 1996 (Banks et al. 2002), but the cause of death was not associated with MusGHV-1. Kent and colleagues (2018) subsequently found MusGHV-1 was highly prevalent in genital tracts (55%, 54/98). This genital tract prevalence is much higher than herpesviruses reported in other species: for example, in a population with a 90% seropositive rate of human Epstein-Barr virus (EBV), and viral DNA prevalence of 22-90% in saliva samples; viral DNA prevalence drops to 8-28% in cervical fluids and semen samples (Hjalgrim, Friborg, and Melbye 2007). In horses, screening of *equine herpesvirus 5* (EHV-5) reveals 14.7% from

uterine flushing; 2.6% from genital swabs; while with horse EHV-2 infections, 1.6% from uterine flushing; 2.3% from genital swabs (Marenzoni et al., 2014; Lee and Lee, 2019). In reindeer, screening of CvHV-2 shows 86% prevalence from eye swabs, 64% from nose swabs, but only 24 % from vagina swabs. The relatively high virus shedding rate in the genital tracts of badgers indicates that MusGHV-1 can be highly sexually transmissible, and that this virus could negatively affect host fertility.

After primary infection, herpesviruses - in contrast to other viruses - establish latency in neuron cells or leukocytes, depending on virus species, and can be reactivated repeatedly throughout the host's lifetime (Grinde 2013a). In an immune-competent individual, a variety of stimuli can cause reactivation, usually involving host factors that affect immune balance, such as stress, aging, trauma, or primary infection with other pathogens (Baumworcel et al. 2019; Coskun et al. 2010; Dai, Barrett, et al. 2020; Reese 2016; Seeber et al. 2018; Sehrawat, Kumar, and Rouse 2018; Stowe et al. 2007; Stowe, Pierson, and Barrett 2001). During reactivation, which can be mildly symptomatic or asymptomatic, the virus starts replication, and infectious viral particles (aka virions) will be released from host cells, which can lead to lytic infection. Shedding of virions usually happens in mucous epithelia, facilitating transmission to naïve hosts (Grinde 2013a; Wilson and Mohr 2012).

No herpesvirus has yet been confirmed to be transmitted exclusively via the sexual route, although herpesvirus infections localized in genital organs are commonly reported. In fact, herpesvirus reactivation in maternal organs has proven to be directly and/ or indirectly associated with reproductive disorders and neonatal diseases (Daniel Givens and Marley 2008; Lehtinen et al. 2003; McGee et al. 2017; Smith 1997). These include HSV-2 in humans (Marshall et al. 2001), equine herpesvirus 3 (EHV-3) in horses (Seki et al. 2004), canine herpesvirus 1 (CHV-1) in dogs (Ronsse et al. 2004), bovine herpesvirus 4 (BHV-4) in cattle (Dubuisson et al. 1989; Yang et al. 2017), murine herpesvirus 4 (MHV-4) in mice (François et al. 2013), Otarine herpesvirus-1 (OtHV-1) in sea lions (Deming et al. 2021; Gulland et al. 2020) and bottlenose dolphins (Van Elk et al. 2009). However, detection of herpesviruses in reproductive organs is not always associated with disease (Patel et al. 2019; Yang, Pascottini, et al. 2019). Whether herpesvirus infection in reproductive tracts can be a sole factor causing reproductive failure has therefore been questioned and explored. For example, BHV-4 is common in cattle but does not cause disease on its own. However, reactivation of BHV-4 *in utero* can lead to secondary bacterial infection and result in uterine disease by interrupting immunity and uterine repair mechanisms (Wathes et al. 2019). Recent reviews highlighted how herpesviruses are involved in host immune regulation or management (Rezaee et al. 2006; Sehrawat et al. 2018). In immuno-competent individuals, herpesvirus and host immunity remain in balance. However, factors affecting host immunity such as aging, stress responses, or co-infection with other pathogens

can break this harmony and sometimes cause reproductive disorders (van Maanen et al. 2000; Szenci et al. 2016) or diseases including cancers (Gulland et al. 2020; Münz 2021).

Thesis aims and structure

The aim of my thesis is to provide an original approach to STI research in wildlife by investigating MusGHV-1 herpesvirus ecology and impacts on host reproductive health in a primitively social carnivore species, the European badger.

I collected samples and animal metadata from two sources:
1) a wild population in Wytham Woods (a 6 km^2 deciduous woodland in Oxfordshire, UK; 51°46026N, 1°19019W) from 3 trapping seasons (May, September, and November) in 2018 to study MusGHV-1 epidemiology in a population with well-documented demographic traits; and
2) wild populations from across the Republic of Ireland at two sampling periods in April 2019 and February 2020, to study the correlation between MusGHV-1 presence and reproductive performance via postmortem examination and sampling.

We captured badgers from the Wytham population in mesh traps baited with peanuts and placed near the entrances of setts (Macdonald et al. 2009). We then transported the captured badgers to a central handling facility and sedated them by intramuscular injection of ketamine hydrochloride (details see McLaren et al., 2005). All badgers were tattooed with a unique number during their first capture (usually as a cub) in the left inguinal region for individual identification. We recorded individual information such as sex, age, weight, body length, body condition (categorized as 1 as poor to 5 as very good condition based on thickness of subcutaneous body fat), female reproduction status (determined by teat size to predict lactation status) and trapping location for social group affiliation of each badger. We then collect body-site (oral, nasal, rectal and genital) swabs and whole blood from each individual. Badgers from the Irish populations were either recovered from road-traffic accidents or culled as part of bovine tuberculosis management by the Department of Agriculture, Food and the Marine (DAFM). These badgers were captured by stopped body restraint snares and humanely dispatched by trained and supervised contractors using a 0.22 calibre bullet (O'Keeffe 2006). The carcasses were submitted to the Irish Equine Centre (Naas, Ireland) for postmortem examination by a veterinary pathologist. Individual information such as sex, capture site, weight, subcutaneous fat depth (measured in mm using a ruler), and pregnancy status (determined through criteria published by Rosen et al. (2019) were recorded. In females, the whole reproductive system, including the genital tract, uterus, and ovaries, were removed from the body with assistance from a veterinary pathologist. I then took genital tract swabs and examined genital discharge, signs of infection for each badger. I also recorded placental scars, number and status (normal, aborted, or absorbed) of fetuses. In males, I collected the testes and measured

their weight and size. I also isolated sperm from the epididymis to evaluate the impact of MusGHV-1 infection on sperm abundance in males (Figure 1).

I divided my thesis into four parts: The first part includes a background review of STIs titled ***Chapter 1: Sexually Transmittable Infections: A knowledge gap in Musteloid reproduction?*** In this chapter, I reviewed the STIs reported thus far in musteloids, a superfamily in the order Carnivora, and explored why STIs are likely under-reported in this mostly promiscuous group.

In the second part, I focus on the impact of badger STI on host reproductive health and epidemiology. I explored the association of MusGHV-1 reactivation and reproductive performance by postmortem examination in 144 Irish badgers in ***Chapter 2: Effects of Mustelid gammaherpesvirus 1 (MusGHV-1) reactivation in European badger (Meles meles) genital tracts on reproductive fitness.*** Then I investigated demographic, somatic and seasonal risk factors of MusGHV-1 reactivation, using data from 251 samples collected from the Wytham badger population in ***Chapter 3: Patterns of genital tract Mustelid Gammaherpesvirus 1 (MusGHV-1) reactivation are linked to stressors in European badgers (Meles meles)*.**

In the third part, I explored potential interactions of MusGHV-1 infection with the prevalence of a zoonotic bacterial pathogen in the Wytham population, as detailed in ***Chapter 4: Stress-related herpesvirus reactivation in badgers can result in Clostridium proliferation*.** Furthermore, I explored differences of two MusGHV-1 variants found in Wytham Woods, where I analyse their differential distribution on population-level according to host demographic traits (ecology), and viral load (virulence) as reported in ***Chapter 5: Infection with a newly discovered novel gammaherpesvirus variant in European badgers, Meles meles, is associated with higher viral load in blood*.**

In the last section of the thesis, I expanded the investigation of environmental stressors and herpesvirus infection to prenatal life. I used a sliding window analysis of weather conditions during *in utero* life and genital herpesvirus reactivation status in 2018 of badgers born between 2004 and 2017 which form the basis for ***Chapter 6: Adverse weather during in utero development is linked to higher rates of later-life herpesvirus reactivation in adult European badgers, Meles meles.***

The results of my PhD research will not only help to establish a wildlife host-pathogen model to study STI dynamics but will also further elucidate the role and importance of herpesviruses as STIs and their epidemiology in wildlife. Largely overlooked in the past, the effects of STIs on wildlife population dynamics are likely to increase with anthropogenic stressors due to expanding human wildlife interactions and may well ultimately lead to the extinction of vulnerable wildlife populations.

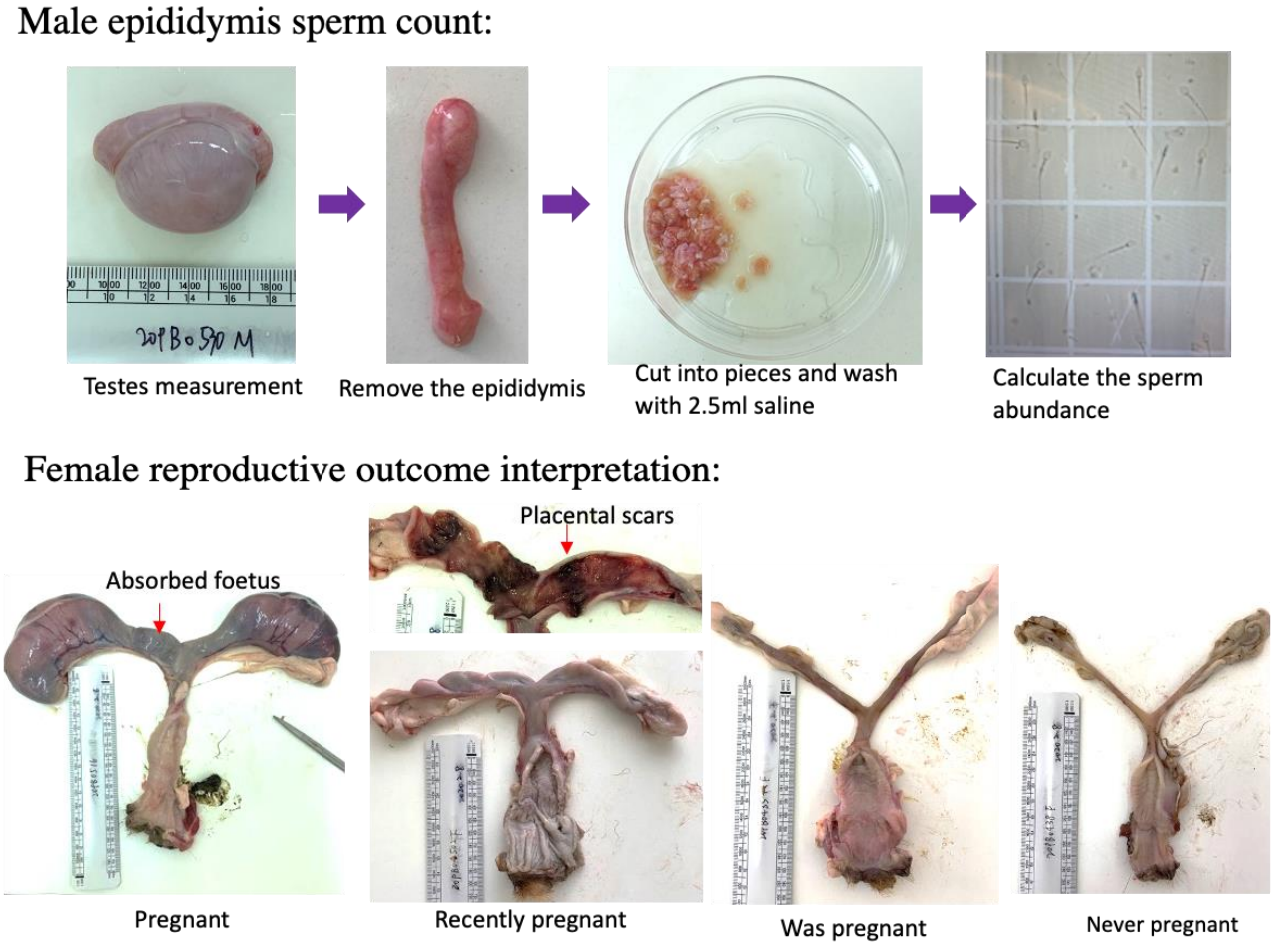

Figure 1: The process of isolating sperm from epididymis and observation of badger sperm under a microscope (for detailed procedures see Chapter 2) and interpretation of female reproductive status

Chapter 1: Sexually Transmittable Infections: A knowledge gap in Musteloid reproduction?

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Abstract

Sexually transmittable infections (STIs) are posited as one of the major drivers of population dynamics, particularly in species with promiscuous mating systems. STIs are theoretically and empirically more likely to drive host extinction than other infections through sterility when the host population size is small or declining. However, due to mostly asymptomatic infection and limited pathogenicity, few studies research STIs in the wild. Particularly few STIs have been reported in the Musteloidea, posing the question if musteloids are less susceptible than other mammals or if STIs are under-reported in this group. Using STI-related keywords in conjunction with musteloid species names in google scholar, we review literature published before December 2020 on musteloid STIs, their transmission routes, signs of disease and impacts on reproductive fitness. Our search returned 24 publications reporting 26 cases of 16 pathogens infecting musteloids that can be sexually transmittable; nine cases in the wild, and 17 from captivity, and involve only 11% (10/90) of musteloid species. Among studies (n=19) that have investigated STI-related reproductive failure/ diseases in females and mostly in captivity (n= 17/19), only one reported no difference of reproductive success between infected and non-infected individuals, the rest are reported with reduced litter size, abortion, stillbirth, neonatal death, uterus rupture or pyometra. Promiscuous species appear susceptible to more STIs are monogamous species. Eight endangered musteloid species have been reported susceptible for STIs that have been proven to be pathogenic in other species. We conclude that musteloids are not less susceptible than other mammalian taxa to STIs, but that STIs are largely understudied in this group. The high proportion of reported cases associated with reproductive diseases and failure and disproportional investigation effort in wild populations highlight the need for further research, especially in species of conservation concern.

Introduction

Sexually transmittable infections (STIs; or sexually transmitted diseases, STDs, to specify those with symptomatic stages) are caused by a very heterogeneous assemblage of pathogen, comprising protozoans, fungi, parasites, bacteria and viruses, as well as cancerous cell lines (e.g. review in mammals and invertebrates: Lockhaert et al., 1996), that share the over-arching characteristic of being transmittable through contact of reproductive organs. Transmission can occur either horizontally between reproductive partners during sexual intercourse or vertically between mother and offspring during pregnancy or parturition. STIs typically have negative effects on host fertility (Lockhart et al. 1996), and, despite long infection periods within hosts that can result in chronic infection, they are generally of low pathogenicity and rarely cause mortality (Lockhart et al. 1996).

In humans, the Word Health Organization (WHO) has identified STIs as a prioritized global health problem (World Health Organisation 2016), with ca. 1 million newly diagnosed cases per day in 2016. Although the majority of the >30 recognized sexually transmittable pathogens in humans are asymptomatic (World Health Organization 2018), several can cause infertility, reproductive failure, congenital infection, disease and/ or death of neonates (Aral 2001), increase the risk of co-infection with other pathogens (e.g., genital herpes and HIV: Zhu et al., 2009), cause cervical cancer (e.g., human papilloma virus, HPV: Castellsagué, 2008), or even increase host mortality rates (e.g., HIV: Thio et al., 2002). Furthermore, increasing evidence shows that some pathogens that can cause severe or even fatal diseases (e.g., tuberculosis: Namavar Jahromi et al., 2001, *toxoplasma gondii*: Flegr et al., 2014 and zika virus: Mead et al., 2018) are typically not classified as STIs, although they can sometimes be transmitted through sexual contact or during pregnancy/ birth.

The theoretical importance of STIs in the evolution of mating systems, mate choice, and even sexual reproduction itself has long been recognised (Kokko et al. 2002; Thrall, Antonovics, and Dobson 2000). Nevertheless, according to previous modelling results, STIs that cause mild sterility are capable to drive the host population towards extinction (Ryder et al. 2007). Disease-induced extinction events were also thought to be more likely in STIs than other infections (Best et al. 2012; De Castro and Bolker 2005; McCallum 2012). Despite their potential to drive particularly K-selected species to near extinction (e.g. the 64% decline of a Koala (*Phascolarctos cinereus*) population in the past decade has been attributed to an 87% infection rate with *Chlamydia pecorum*: Polkinghorne et al., 2013), few studies on wildlife population dynamics test for STIs or even discuss their potential as population drivers. In part this may be due to the fact that, traditionally, STIs have been thought of as a small subset of infectious diseases, rather than

as an important group of pathogens that infect a wide variety of hosts from plants and invertebrates to mammals.

Interestingly, in carnivores, the number of reported STIs is comparatively low (Lockhart et al. 1996), and particularly few STIs have been reported in the *Musteloidea*: Lockhart and colleagues (Lockhart et al. 1996) reported a total of six STIs in the order *Carnivora*, five in the *Canidae* (*Brucella canis*, *Haemophilus hemoglobinophilus*, *Nosema cuniculi,* Canine venereal tumour and Canine herpesvirus) and one in the *Felidae* (*Chlamydia psittaci*), but did not present any reports at all in musteloids. Similarly, a recent review of musteloid diseases (Newman and Byrne 2018) also lists only a single STI, the Aleutian mink disease virus (AMDV). Nevertheless, most musteloids are promiscuous (Macdonald & Newman, 2018), where promiscuity has been shown to be linked to higher intra-host STI diversity, transmission rates and prevalence than monogamy or stable harem mating systems (Nunn et al., 2014; Poiani, 2000; Smith & Mueller, 2015; Thrall et al., 2000). Thus, reports of STIs in the Musteloidea are likely under-represented in the literature. Here, we review the current literature on STIs in this superfamily, and expand our review to also include studies that confirm infections of such pathogens in musteloids that have been shown to be STIs in other taxa. We discuss our findings in light of host susceptibility, transmission routes, social behaviours, reproductive outcomes and potential impact to endangered species.

Materials and Methods

Employing the publication search engine Google Scholar, we performed an extensive literature search using one or several of the following keywords simultaneously: 'infection', 'disease', 'STD', 'genital', 'vagina(l)', 'secretion' 'penis', 'semen', 'sexually transmitted' in combination with 'mustelid' or 'musteloid', and/ or the common or latin name of individual musteloid species (following the phylogeny in Macdonald et al., 2017) to include all studies published before 31st December 2020. In addition, we also searched all musteloid species for ubiquitous STI pathogens known to occur in other mammals.

An infectious pathogen was considered a "confirmed musteloid STI pathogen" when presence in tissue or secretions of reproductive organs has been confirmed, or horizontal/ vertical transmission has been proven in at least one species in this group. These STIs were then searched for in all other musteloids, and recorded for each species as a "potential STI pathogen", where the respective pathogen has been reported, but genital sampling and/ or evidence of vertical/ horizontal transmission is still lacking. For each report, we recorded the musteloid species, the method(s) used for pathogen isolation and identification, pathogen prevalence, pathogen association with reproductive failure, if vertical/ horizontal transmission is likely (i.e.,

pathogen presence in genital tract or foetus confirmed) or confirmed or not, if the study was based on captive (farmed, research or zoo animals) or wild (free-ranging) animals, and if the reported infection was experimentally inoculated or naturally obtained.

Results and discussion

STIs reported in musteloids

Our search returned 24 publications comprising 26 reports and confirming a total of 16 STI pathogens amongst 10 species of *Musteloidea* (Table 1). Detailed description of each pathogen and case can be found in Appendix I. Among those, eight studies (two in captivity, six in the wild) confirmed pathogen presence in the genital tract/ genital mucosa, suggesting a potential for horizontal transmission; five report pathogen presence in foetuses (three in captivity, two in the wild), suggesting vertical transmission during pregnancy, and eight reports (eight in captivity) confirmed infection/ increased death in neonates or new-borns, and thus conclude congenital infection indicating probable vertical transmission during pregnancy or birth, while one additional report from the wild describes a suspected STI-related syndrome in neonates. Six reports isolation of pathogens from uterus or milk, and further evidence is needed to determine the possible extent of vertical transmission. The majority of studies (17/24) recorded clinical signs or manifestations of the respective disease, listing vaginitis and substantial mucosal epithelial ulceration as well as inflammation of the uterus, three studies reported asymptomatic infection, and two studies reported both symptomatic and asymptomatic infection. One study did not discuss symptomology, and one found only the aborted foetus with bacteria infeciton (*Plesiomonas shigelloides*: Weber & Roberts, 1989) but was not able to find the mother. Associated reproductive failure/ disease, including reduced litter size, abortion, stillbirth, neonatal death, uterus rupture or pyometra, were reported in 18/26 cases (16 in captivity and two in the wild) and in females only. One reports no impact on female reproductive output but links vertical transmission to increased mortality in sero-positive offspring (Aleutian mink disease virus, AMDV: Farid et al., 2018), while the remaining seven studies did not investigate pathogen impact on reproductive fitness.

The 16 musteloid STI pathogens belong to three virus families: *Herpesviridae* (1), *Parvoviridae* (2), *Paramyxoviridae* (1); 7 bacteria families: *Campylobacterales* (1), *Clostridiaceae* (1), *Brucellaceae* (1), *Enterobacteriaceae* (4), *Streptococcaceae* (2), *Staphylococcaceae* (1), *Enterococcaceae* (1); and one family of protozoan: *Sarcocystidae* (1). Furthermore, we found 3 additional pathogen genus - *Circovirus*, *Mycobacterium spp.*, and *Leptospira spp.* – that have been confirmed to be sexually transmittable in other species and are known to occur amongst musteloids, but have - thus far - not been reported in musteloid reproductive tracts, foetuses or neonates. The 19 STIs pathogens can be allocated to 17 genera (Table 2).

Six of the 16 STI pathogens (i.e., 37.5%), we found in this review (canine distemper virus, CDV, circovirus, *Clostridium limosum*, *Enterococcus faecalis*, *Escherichia coli*, *Plesiomonas shigelloides*), were not listed as STIs in Lockhart and colleagues' review published in 1996 (Lockhart et al. 1996), partly because these pathogens were confirmed as potential STIs only after their review was completed, but partly also because the authors concluded that sexual transmission of certain pathogens (e.g., CDV and *E. coli*) was so rare compared to other routes that they did not include them in their definition of STDs. Nevertheless, among the 16 confirmed musteloid STI pathogens, CDV has been reported in far more species (n=29) than any other STI (*T. gondii*: 22, amdoparvovirus: 14, *Brucella* sp.: 13, *Salmonella sp.*: 15, *Clostridium sp.*:12, *Stephylococcus sp.*: 12, *Leptospira* sp.: 11, *Streptococcus* sp.: 11, *Mycobacterium* sp.: 11, *E. coli*: 9, herpesvirus: 8, *Proteus* sp.: 6, *Campylobacter* sp.: 4, *Enterococcus* sp.: 4, *Plesiomonas* sp.: 4, circovirus: 3; Table 2 & Figure 1). CDV is highly contagious and is associated with high morbidity and mortality, resulting in intensive epidemiological surveys and disease research in captive and free-ranging wildlife in recent years (Martinez-Gutierrez and Ruiz-Saenz 2016).

Musteloid STIs are frequently associated with reproductive impairment but investigation effort is biased

Although there is evidence from humans or livestock and wildlife species for diminished reproductive success for all the 16 STI pathogens identified by this review, only nine of them have thus far been confirmed to result in reproductive impairment in infected (female) musteloids: parvovirus (AMDV), *T. gondii*; 3 opportunistic bacteria: *S. intermedius, E. faecalis* and *P. shigelloides*; and 4 pathogenic bacteria: *B. abortus, S. enterica, C. jejuni and C. limosum*. In addition to the bias towards testing easily accessible species, and detecting the most conspicuous signs of disease, musteloid reproductive physiology might contribute to even symptomatic STIs escaping diagnosis. All mustelids are induced ovulators (Amstislavsky and Ternovskaya 2000), and females of several species develop pyometra if they are not mated (e.g. ferrets: Orcutt, 2003; mink: Yang et al., 1995; Siberian polecat: Johnson et al., 1999), making it hard to distinguish this condition from STI-induced uterine inflammation (Johnson et al., 1999), one of the prevalent symptoms of many STIs. In addition, mink and badgers, amongst others, undergo delayed implantation (Amstislavsky and Ternovskaya 2000), where further embryonic development is dependent on female body condition (badgers: Macdonald et al., 2015; mink: Tauson & Aldén, 2009). As the length of the latent period between infection and manifestation of clinical symptoms shows considerable variation among STI pathogens (Lockhart et al. 1996), and some STIs, especially those of viral origin, are characterized by lifelong latency (e.g. herpesviruses: Grinde, 2013), distinguishing between reproductive failure due to these natural causes and STI-related sterility is often difficult.

Furthermore, like other diseases, STI infections (with the notable exception of HIV that attacks the immune system directly: Nowak & McMichael, 1995) typically cause increased immune-activity (Lombardo 1998), and thus, as a secondary effect, lower reproductive fitness, and manifest also in fatigue and lowered libido, while showing few or no clinical signs (e.g. *E. coli* in *Neovison vison*: Vulfson et al., 2003). As the majority of the *Musteloidae* are nocturnal (Lode 1995), and spend most of their time sleeping (e.g. ferret: 18-20 hours: Fisher, 2006), increased resting periods would often go unnoticed, even in captivity. The percentage of asymptomatic cases may therefore be particularly difficult to determine for musteloid STIs.

Sexual transmission of musteloid STIs

Since none of the studies identified by this review were conducted to examine pathogen transmission routes *per se*, little can be inferred on the relative importance of vertical vs horizontal transmission of the reported musteloid STIs. However, among the 26 STI cases documented in this review, 19 report indirect evidence of vertical transmission (i.e., either infection of foetuses/ neonates, or pathogen presence in reproductive organs such as uterus or placenta, or uterine fluids), while one reports no vertical transmission of herpesvirus by screening the foetus from mothers that were tested positive in wild badgers (Tsai et al., 2020). Only nine report indirect evidence of possible transmission through sexual intercourse (i.e., pathogen DNA detected or cultured successfully from genital tract swabs or fluids). These include MusGHV-1 in European badgers, *E. coli* in farmed ferrets, CDV in free-ranging fisher, combined infection of *E. coli* and *Proteus mirabilia* in a rehabilitated American river otter, *Enterococcus faecalis* in a captive steppe polecat, *Staphylococcus intermedius* in farmed mink, *Streptococcus phocae* in stranded Southern sea otters and *Streptococcus canis* in a raccoon. All of these pathogens except for herpesvirus and CDV, belong to opportunistic bacteria commonly isolated from the genitals of healthy dogs and cats(Graham and Taylor 2012), although in musteloids they can become pathogenic and can cause minor to severe clinical signs or even mortality if the host suffers from primary viral infection, stress, trauma, invasion of certain strains (e.g., extraintestinal pathogenic *E. coli*, or ExPEC) or other predisposing factors (Graham and Taylor 2012). Thus, further identification of pathogen strains as well as comprehensive evaluation of all predisposing factors is important in future research to determine if these bacteria are also part of the normal genital microbiome of healthy musteloids or if they might cause disease in this host family.

Indeed, other than the 16 STI pathogens we found in this review (Table 1), we included also three pathogens (added in table 2) that have been reported to infect musteloids albeit without evidence of genital infection or sexual transmittability, because they have been proven to be sexually transmittable in other mammals (Table 2): mink circovirus, where the porcine circovirus is present in pig semen (Larochelle et al. 2000), *Leptospira spp.* that is commonly isolated from livestock genitals (Director et al. 2014; Hamond et al. 2013; Heinemann et al. 1999;

Kim et al. 2006; Lilenbaum et al. 2008) and can also be transmitted vertically (Adler 2015), and *Mycobacterium spp*, where *M. tuberculosis* and *M. bovis* are both important STIs in humans (Jacob, Nguyen, and Ray 2008; Neonakis, Spandidos, and Petinaki 2011), the latter being proven to be vertically transmitted in cattle (Phillips et al. 2003), and *M. avium* subspecies being transmitted within herds through bull semen (Khamesipour, Doosti, and Mazrouei Sebdani 2014).

Wide host species range but biased by study intensity

To-date, STIs have been recorded in only 10 of the 90 species (=11%) of the *Musteloidea*: Four *Mustelinae* (American mink, *Neovison vison:* 12 reports; ferret, *Mustela putorius furo*: four reports; steppe polecat, *Mustela eversmanii*: one report; fisher, *Martes pennant*: one report), three *Lutrinae* (Southern sea otter, *Enhydra lutris*: one report; American river otter, *Lontra canadensis*: one report; Eurasian otter, *Lutra lutra*: one report), one member of the *Melinae* (European badger, *Meles meles*: one report), one member of the *Procyonidae* (raccoon, *Procyon lotor*: one report), and one member of the *Mephitidae* (striped skunk, *Mephitis mephitis*: one report). Thus far, American mink and ferrets are the only two species that have been confirmed susceptible to more than one STI pathogen (mink = 10; ferrets = 4, from different studies).

The low proportion of species in the *Musteloidae* (Table 1) with STIs reported is surprising despite wide host range. There are two possible explanations for this phenomenon: either many musteloid species are genuinely less susceptible to STIs or – despite average prevalence - STIs remain disproportionately often undetected, and thus undiagnosed, among other musteloid species.

Are musteloids less susceptible to STIs than other mammal taxa?

While in ordinary infectious diseases the risk of infection is predominantly dependent on the number of infective individuals in the population, and can – if a threshold is passed – become epidemic, STIs generally remain endemic, independent of population density, because risk of infection, and thus spread of disease, depends mostly on the frequency of intimate contact between healthy and infected animals, and therefore on the social structure and (mating) behaviour of the respective species (Kokko et al. 2002). Consequently, STIs have long been proposed as evolutionary drivers of reproductive behaviour and mating structure (Kokko et al. 2002), where individuals can limit the risk of infection through behavioural adaptations (Nunn, 2003) that serve to limit sexual contact between conspecifics. Even human monogamy has been suggested to be an evolutionary consequence of venereal disease transmission (Immerman 1986). Most musteloids, however, are promiscuous or polygynous (Estes, 1989; Fergusen & Larivière, 2005; Johnson et al., 2000), with the few exceptions of monogamy in red pandas (Wei et al. 2005), some freshwater otters (Asian small-clawed otter: Scheifele et al., 2015, and smooth-coated otters: Chanin, 1985), and polygyny in most sea otters (Estes et al., 2009) and polecats (Lodé 2001), and are thus likely to be rather more susceptible to STIs than other taxa. Indeed,

our literature review suggests that even within the musteloids species with promiscuous mating systems tend to have more reported STI pathogens (e.g., European badgers: 15 STIs; American mink: 16 STIs) than equally intensively studied monogamous species (e.g., Oriental small-clawed otters: 5 STIs; red panda: 7 STIs; Figure 2).

This increased STI risk associated with promiscuity has been shown in primates and carnivores to be reflected also on an immunological level in the white blood cell count (WBC), where species with promiscuous mating systems have higher WBC and neutrophil counts than monogamous species (Nunn et al., 2000; Nunn, 2002; Nunn et al., 2003), indicating stronger innate immunity to cope with prolonged and chronic STIs. Interestingly, amongst the musteloids, species with a promiscuous mating system indeed tend to have a higher percentage of neutrophils (67.52%, min=44.77%, max=83.94%, n=6) than species with polygynous (50.55%, min=41.67%, max=51.96%, n=4) or monogamous systems (56.85%, min=41.78, max=66.3%, n=3; Table 3).

Nevertheless, musteloid reproduction is generally confined to a maximum of one or two litters per year, and, in most species, contact between conspecifics is comparatively rare and mostly confined to the mating context (Fergusen and Larivière 2005). Even the few social musteloid species generally exhibit some form of reproductive suppression, largely limiting mating behaviour to either the dominant male in harem-forming species (e.g. wolverine: Copeland et al., 2017) or to the dominant pair in group-living species (e.g. Asian small-clawed otters, *Aonyx cinereus* (Hussain, Gupta, and de SILVA 2011); giant otters, *Pteronura brasiliensis:* Groenendijk et al., 2015). Both mating strategies thus dramatically reduce rates of sexual contact between conspecifics, potentially helping to limit STI prevalence and diversity. Exceptions are the European badger and American mink, that can have superfecundation and superfoetation, where promiscuous mating in additional oestrus cycles during delayed implantation typically results in multiple paternity (Dugdale et al. 2007; Macdonald and Harrington 2003). Interestingly, this increased sexual contact is reflected in the results of our literature review where badgers are susceptible to 15 pathogens and mink to all 16 STI pathogens reported in the Musteloidea (although these figures may be affected by reporting bias, see below). Nevertheless, even European badgers appear to limit the number of mating through density-dependent oestrous patterns, where females in high density populations undergo a maximum of two cycles annually compared to monthly cycles throughout the year in low density populations (Carpenter et al. 2005; Sugianto, Heistermann, et al. 2021).

Are STIs in musteloids diagnosed less often than in other mammals?

Only seven studies describe prevalence rates of the reported pathogens with appropriately large sample sizes to draw conclusions, whereas 19 reports failed to analyse prevalence. Prevalence was high in the majority of cases: *Staphylococcus intermedius* in farmed American mink isolated from the vagina (n= 37): 65%; isolated from milk (n= 37): 54%; in females with affected litters

(n= 21) prevalence in the vagina was 100% and in milk 91%, compared to females with healthy litters (n=16) prevalence in the vagina: 18% and milk: 6%; SKAV in blood of striped skunks (*Mephitis mephitis*) (n=216): 64.8%; MusGHV-1 in vaginal swabs of European badgers (n= 98): 55%; AMDV in spleens of farmed American mink (n= 12 to 7861): 0-94%; *Brucella abortus* cultured from uterus and foetus of farmed American mink (n= 12): 58.3%; and *T. gondii* antibody in blood of farmed American mink (n= 961): 13.84%.

Not surprisingly, of the only 3 musteloid species (American mink, striped skunks and European badgers) from seven studies with confirmed STI-prevalence, the vast majority are either often kept in captivity (American mink are bred in large numbers in fur farms: Frank, 2001), or are of particular (conservation or public health) research interest (e.g., European badgers are extensively studied in the UK due to their role as a wildlife host of bovine tuberculosis (Carter et al. 2007); skunks are studied in the US and Canada as wide-spread carriers of pathogens infecting other, vulnerable wildlife: Glueckert et al., 2019; fisher populations suffer unusually high mortality due to disease outbreaks: Keller et al., 2012). All other reports are opportunistic discoveries from casualties or clinical cases. In contrast to some other carnivore families, many musteloid species have not been studied in the wild, or research has been limited to broad ecological surveys, which are typically based on field signs, aerial surveys or camera trapping (e.g. sea otters: Doroff et al., 2005, Eurasian otters: Manuela Oliveira et al., 2010, fishers, martens, raccoons and weasels: Gomper et al., 2006), rather than on live-trapping, limiting the potential for epidemiological research.

STIs as largely ignored threats to endangered Musteloids

The field of wildlife epidemiology is rapidly expanding and the number of studies published annually has been increasing over the past decades (Figure 3), where the importance of STI research, especially in the context of species conservation, is increasingly recognised (Herrera and Nunn 2019). General studies on wildlife population dynamics, however, often overlook the importance of STIs, and targeted epidemiological studies in wild mammals are still rare. Only 9/ 24 (37.5%) of the studies we identified as STI cases in this review were conducted in the wild. Among those, only 2 had systematically investigated STI's effect on reproductive health (Kent et al. 2018; Tsai et al. 2020 or Chapter 2).

STIs commonly result in reduced reproductive fitness and fecundity in otherwise asymptomatic domestic animals and livestock (Cortizo et al. 2014), so this may also be the case in free-ranging wild species, resulting in a sudden or incremental population decline (Robbins et al. 2019), making the host population susceptible to extinction (Smith et al., 2009). For example, European mink have been documented with significantly lower embryonic litter sizes than free-ranging polecats and American mink, where STIs transmitted from invasive American mink have been suggested as one of the potential causes (Fournier-Chambrillon et al., 2010). The impacts of STI

pathogens on the host can worsen if they interact with those of chronic stress or other complex compromises to immune-competency. For example, chlamydia infection in koalas is not always associated with patent clinical signs but may nonetheless progress to fatal urogenital and ocular disease as a result of complications such as chronic stress and/or coinfection with an immune-suppressive pathogen, such as the koala retrovirus (KoRV) (Robbins et al. 2019). A recent systematic investigation on sexually transmittable herpesvirus infections in badgers also reports patterns of virus shedding in genital tract to be linked to physical stress (Tsai et al. 2021 or Chapter 3). Thus, although the absence of any clinical symptoms in many musteloids that test positive for STI-pathogens could indicate that their immune systems are typically capable of suppressing the infection, the impact of STIs on wild populations in the form of infertility, abortion, or neonatal death (Hurst et al., 1995; Korenromp et al., 2019; Mshelia et al., 2010; Smith, 1997; Thrall et al., 2000) could be substantial. Nevertheless, due to their particular reproductive physiology (incl. suppressed reproduction due to environmental stress and fitness trade-offs, delayed implantation and superfecundation), and their predominantly nocturnal (76%) and solitary (72% of musteloid species) lifestyles, STIs are very difficult to study in free-ranging musteloids, and are thus mostly reported in wildlife casualties like road kills, unusual mortality events (e.g. stranded sea otter: Imai et al., 2009), or are picked up during routine checks in wildlife rehabilitation programmes (Hoover et al. 1985). Reproductive failure in captivity, especially in fur farms, is typically the main reason that STIs are diagnosed in musteloids, explaining the disproportionately high number of reported STIs in farmed American mink and ferret (17/26).

Of the 90 musteloid species, 19 are currently of conservation concern (Macdonald et al., 2018), several of which (e.g., the vulnerable Oriental small-clawed otter, the near-threatened Eurasian otter, the endangered Southern sea otter (Imai et al. 2009), black-footed ferret and red panda, the critically endangered European mink, and the American river otter (Hoover et al. 1985), which has been eliminated from most of its historic range since 1900s due to hunting and water pollution) are susceptible to STIs (Table 1 & Table 2). Of particular conservation concern in this context is that musteloids often contract STIs with high pathogenicity such as CDV (Deem et al. 2000; Trebbien et al. 2014) and AMDV (Reichert and Kostro 2014) that not only affect reproductive fitness, but also lower life expectancy in the adult breeding population.

Aside from the obvious concerns caused by STI-related impacts on fertility (Smith et al., 2009), cross-species infection through non-sexual routes (e.g., contaminated feed: *Clostridium limosum* outbreak in a mink farm through feeding contaminated chicken: Hammer et al., 2017; *Salmonella* outbreak through feeding contaminated cattle offal: Dietz et al., 2006; sniffing/ ingesting contaminated faeces, urine or saliva: *M. bovis* transmission from European badgers to cattle: Phillips et al., 2003; AMDV from mink to other carnivores: Canuti et al., 2015, CDV from domestic dogs to fishers: Brown et al., 2006) is recognised also in STIs. Thus, pathogens may occasionally,

when conditions permit, spread amongst other sympatric musteloid species. Nevertheless, while ca. 20% of musteloids are of conservation concern, others are very wide-spread and some are classed as "pest species" in at least part of their range (e.g., raccoons and skunks: Clark, 1994, American mink: Madonald & Harrington, 2003, weasel: Witmer et al., 2007) or increasing in density and geographical range (e.g. European badger: Bateman & Fleming, 2012), bringing them into ever closer contact with people. For example, American mink are an aggressively invasive species across Europe and South America, and STI infection from invasive American mink to the endangered European mink through mating has been documented in captivity (Fournier-Chambrillon et al., 2010). Furthermore, mink are the primary carrier of AMDV that, in Nova Scotia, has been transmitted from farmed mink to wild musteloids, such as American mink, short-tailed weasels, raccoons, North American river otters, striped skunks, but also to other carnivores (e.g., bobcats, dogs and cats) (Farid 2013), whereas CDV outbreaks in fur farms are suspected to have originated from wildlife reservoirs (Trebbien et al., 2014). Similarly, European badgers are the primary wildlife host for bovine tuberculosis in the UK, and are implicated in cross-species transmission of this economically important disease to cattle (Phillips et al. 2003), where badger densities are increasing and their range is expanding due to global warming (Silva et al., 2017). Other musteloids, such as the short-tailed weasels (Witmer et al. 2007) and American mink (Macdonald and Harrington 2003), have near world-wide distribution (partly due to deliberate or accidental introduction), and thus even localised infection with pathogens could pose considerable risks to livestock and other (endangered) wildlife species throughout their extensive range.

Conclusion

Our results show that, currently, musteloid STI infections are typically detected either post-mortem or in patently diseased animals, rather than recorded during systematic wildlife population surveys. Due to their low pathogenicity and possibility of chronic or latent infection, however, many STIs are thus likely to remain undetected, particularly in wildlife. Nevertheless, STI-associated reproductive failure is evident and can have significant impacts on population productivity (and thus viability), and should be considered in conservation management, particularly of endangered species, while cross-species transmission risks should be taken into account in animal husbandry (e.g., fur farms) and disease outbreaks in non-endangered wildlife.

Systematic trapping and sampling surveys, in combination with targeted STI pathogen screening of genital swabs/ discharge samples using culture and targeted polymerase chain reaction (PCR), or general high-throughput sequencing of genital microbiomes and viromes would be an efficient and cost-effective way to detect STIs in free-ranging wildlife. Long-term monitoring programmes and conservation management plans therefore need to consider STIs as a possible

cause for fluctuations in population productivity and inter-individual differences in reproductive success. Further studies are needed to clarify STI prevalence, pathogenicity, transmission routes and impact to reproductive health in wild musteloids.

Table 1: List of sexually transmitted infections reported in musteloid species

Pathogen type	Pathogen species/ strain	Host species	Captivity/ Wild	Experimental / natural	Affected individual number/ prevalence	Diagnosis methods	Isolation sites	Horizontal sexual transmission *	Vertical transmission **	Reproduction failure	Symptomic	References
Viruses	MusGHV-1 (mustelid gammaherpesvirus 1)	European badger (*Meles meles*)	Wild	Natural	55% (54/98)/ 61.8% (89/144)	PCR	Genital swab	Likely	Unlikely	Yes, lower successful pregnancy rate	Asymptomatic	(Kent et al. 2018; Tsai et al. 2020)
	AMDV (Aleutian mink disease virus)	American mink (*Neovison vison*)	Captivity	Natural	0-34.2% (n=12 to 7861)	CIEP	Spleen		Likely	Yes, kit mortality	Varied	(Farid et al. 2018)
	AMDV	American mink	Captivity	Experimental	94.4% (34/36)	PCR	Placenta, uterus, mammary glands, foetus		Yes	Yes, reduced reproduction capacity	Symptomatic	(Broll and Alexandersen 1996)
	SKAV (skunk amdoparvovirus)	Striped skunks (*Mephitis mephitis*)	Wild	Natural	64.8% (140/216)	PCR	Blood or serum		Likely		Varied	(Glueckert et al. 2019)
	CDV (Canine distemper virus)	Ferret (*Mustela furo*)	Wild	Natural	n=1	PCR	lung, foetus		Yes		Symptomatic	(Keller et al. 2012)
	CDV	Fisher (*Pekania pennanti*)	Wild	Natural	n=4	Histology, PCR, IHC	lung, urothelium, epididymis, uterus, vagina, skin	Likely			Symptomatic	(Trebbien et al. 2014)
Bacteria	*Campylobacter jejuni*	American mink	Captivity	Natural	n=2	Culture	liver of fetus, placenta, uterus, spleen		Yes	Yes, abortion	Symptomatic	(Hunter et al. 1983)
	Campylobacter jejuni	American mink, ferret	Captivity	Experimental	n=4 (mink), n=7 (ferret)	Culture	Placenta, uterine fluids, foetus, faeces		Yes	Yes, abortion and early death of embryo	Symptomatic	(Bell and Manning 1990)
	Brucela abortus	American mink	Captivity	Natural	58.3% (7/12)	Culture	Not specified		Likely	Yes, abortion, small litters, neonatal death	Symptomatic	(Prichard et al. 1971)
	Clostridium limosum	American mink	Captivity	Natural	Not specified	Culture, histology	uterus, liver, spleen, kidneys, lungs, heart, peritoneal fluids		Likely	Yes, stillbirth in uterus, uterine rupture and mortality of pregnant females	Symptomatic	(Biström et al. 2016)
	Clostridium limosum	American mink	Captivity	Natural	n=16	Culture, PCR	uterus, liver		Likely	Yes, abortion and stillbirth	Symptomatic	(Hammer et al. 2017)
	Escherichia coli	Ferret	Captivity	Natural	Yes	PCR	milk, vagina, feces, urine, brain, blood, mammary gland, uterus	Likely		Yes, mastitis	Symptomatic	(Marini et al. 2004)
	Escherichia coli, Proteus mirabilia	American mink	Captivity	Natural	n=1	Culture	uterus		Likely	Yes, uterus rupture	Symptomatic	(Yang et al. 1995)
	Escherichia coli, Proteus mirabilia	American river otter (*Lontra canadensis*)	Wild	Natural	n=2	Culture	vaginal discharge	Likely			Symptomatic	(Hoover et al. 1985)

	Enterococcus faecalis	Steppe polecat (*Steppe polecat*)	Captivity	Natural	n=1	Culture	uterus contents	Likely		Yes, pyometra	Symptomatic	(Johnson et al., 1999)
	Plesiomonas shigelloides	Eurasian otter (*Lutra lutra*)	Wild	Natural	n=1	Culture	Placenta, foetus		Yes	Yes, abortion	NA	(Weber and Roberts 1989)
	Salmonella enterica	American mink	Captivity	Natural	n=52	Culture	uterus, liver, spleen, intestines, lung		Likely	Yes, abortion, neonatal death, stillbirth, perinatal mortality of pregnant mink	Symptomatic	(Dietz et al. 2006)
	Staphylococcus intermedius	American mink	Captivity	Natural	n=21	Histology, culture	Vagina, milk	Likely	Likely	Yes, neonatal death	Symptomatic	(Hunter and Prescott 1991)
	Streptococcus phocae	Southern sea otter (*Enhydra lutris nereis*)	Wild	Natural	Not specified	Culture, PCR	Orogenital mucosa, lesion	Likely			Not specified	(Imai et al. 2009)
	Streptococcus canis	Raccoon (*Procyon lotor*)	Wild	Natural	n=1	Culture	Vagina mucosa	Likely			Symptomatic	(Rentería-Solís 2017)
Protozoa	*Toxoplasma gondii*	American mink	Captivity	Natural	13.84% (133/961)	Antibody, IF	liver, brain		Likely	Yes, low reproduction performance	Asymptomatic	(Śmielewska-Łoś and Turniak 2004)
	Toxoplasma gondii	American mink	Captivity	Natural	26% (1,976/7,800)	Microscope, IHC	Lung, brain, heart, liver, kidney		Likely	Yes, abortion, neonatal death	Symptomatic	(Frank 2001)
	Toxoplasma gondii	Ferret	Captivity	Natural	n=7	Microscope	lung		Likely	Yes, neonatal death	Asymptomatic	(Thornton 1990; Thornton and Cook 1986)

* Likely = pathogen isolated in genital tract or semen

**: Yes= pathogen isolated from foetus; likely = pathogen isolated from neonates, youngs, maternal reproductive organs (uterus, placenta or mammalian gland) or milk

Table 2: Musteloid species susceptible to STIs (see Appendix II for references)

H: likely horizontal sexual transmission (pathogens isolated from genital tract/ semen) ; V: likely vertical transmission (pathogens isolated from neonates, foetus or placenta) ; R: reported associated reproductive failure/ impaired reproductive fitness; S: reported to be susceptible

	Morbillivirus (CDV)	Herpesvirus	Amdoparvovirus	Circovirus	*Brucela sp.*	*Leptospira sp.*	*Staphylococcus sp.*	*Clostridium sp.*	*Salmonella sp.*	*Mycobacterium sp.*	*Streptococcus sp.*	*Escherichia coli*	*Proteus sp.*	*Campylobacter sp.*	*Enterococcus sp.*	*Plesiomonas sp.*	*Toxoplasma gondii*
Mustelidae																	
American mink	S[1]	S[2]	V/R/S[3–7]	S[8,9]	V/R/S[10,11]	S[12]	H/V/R/S[13–15]	V/R/S[16,17]	V/R/S[18]	S[19]	S[20,21]	R/S[14,21–23]	R/S[14]	V/R/S[24–26]	S[21]	S[27]	V/R/S[28–31]
European mink	S[32]		S[33,34]		S[10]	S[12]											S[35]
European badger	S[32,36]	H/S[37]	S[38]	S[39]	S[40]	S[41]	S[42]	S[36]	S[43–45]	S[46]	S[36,42]	S[36,42,47]		S[48]	S[49]		S[29,42,50]
Asian badger					S[10]												
American badger	S[51,52]				S[10]												S[53]
Chinese ferret-badger	S[32]																S[54]
Ferret	V/S[1,55]	S[55]	S[55]				S[56]	S[56]	S[57]	S[19,58,59]	S[60]	H/S[61]	S[56]	V/R/S[25]			V/R/S[29,50,56,62,63]
Black-footed ferret	S[64]							S[65]				S[65]	S[65]		S[65]		S[66]
Polecat	S[32]		S[33]			S[41]	S[15]		S[43]	S[67]							S[29,42]
Siberian polecat															R/S[68]		
Sea otter	S[32]	S[69]		S[70]	S[10,71]	S[72,73]	S[73]	S[74]	S[75,76]		H/S[73,76–78]	S[76]		S[74,76]		S[74]	S[72–74]
Eurasian otter	S[52]		S[34]		S[10,11,79,80]		S[81]	S[82]	S[83,84]	S[85]	S[81]	S[81]	S[83]			V/R/S[86]	S[50]
American river otter	S[32]		S[87]			S[88]	S[89]	S[89]	S[90]		S[89]	H/S[89,90]	H/S[89,90]				S[28,88]
Asian small-clawed otter	S[91]	S[92]						S[93]		S[94]			S[95]				
Spotted-necked otter																	S[96]
Stone marten	S[32]		S[33]			S[12,41]		S[42]	S[43]			S[97]					S[50]
Pine marten	S[32]		S[33]			S[12,41]	S[42]		S[43]		S[42]	S[97]					S[42,50]

American marten	S[98]	S[99]	S[98,100]			S[98]											S[98]
Fisher	H/S[101,102]	S[103]															S[104]
Stoat	S[32]		S[87]		S[10]	S[41]			S[42]	S[58,67]							S[29]
Least weasel	S[32]					S[41]				S[58]							
Japanese marten	S[32]																
Siberian weasel	S[32]																
Greater grison	S[32]																S[105]
Lesser grison	S[32]																
Wolverine	S[32]				S[10]												
Ailurudae																	
Red panda	S[32]		S[106]				S[15]	S[107]		S[108]	S[109]						S[96,110]
Mephitidae																	
Skunk	S[52]		V/S[87,111–113]		S[10]		S[15,114]		S[115]	S[116]	S[114]				S[114]		S[117]
Western spotted skunk					S[10]												
Hooded skunk							S[15]		S[118]								
Libyan striped skunk									S[108]								
Procyonidae																	
Raccoon	S[32,119]	S[120]	S[87]		S[10,121,122]		S[15]	S[123,124]	S[125]	S[116,126]	H/S[127]					S[128]	S[117,129,130]
Cozumel raccoon	S[32]																
Crab-eating raccoon											S[131]						
Kinkajou	S[52]																
Coati	S[32]				S[10]			S[132]									S[133]
White-nosed coati									S[134]								S[105]

Table 3: White blood cell counts of some musteloid species

Species common name	Species scientific name	Mating system	Total WBC count (10^3/µl)	Lymphocyte (10^3/µl)	Monocyte (10^3/µl)	Neutrophil (10^3/µl)	Neutrophil %	References
Raccoon	*Procyon lotor*	Polygynous or promiscuous	12.14	1.55	0.3	10.19	83.94%	(Rendón-Franco et al. 2019)
Mink	*Neovison vison*	Promiscuous	4.11	1.73	0.37	1.84	44.77%	(Bozena et al. 2013)
Badger	*Meles meles*	Promiscuous	5.1	1.49	0.26	3.29	64.51%	(Mahmood et al. 1988)
Eurasian otter	*Lutra lutra*	Promiscuous	6.44	1.66	0.2	3.99	61.96%	(Lewis et al. 1998)
American river otter	*Lontra canadensis*	Promiscuous	11.3	1.25	0.45	8.88	78.58%	(Tocidlowski et al. 2000)
American marten	*Martes americana*	Promiscuous	4.94	0.68	0.21	3.93	79.55%	(Spriggs et al. 2018)
White-nosed coati	*Nasua narica*	Polygynous	9.45	3.71	0.4	4.39	46.46%	(Rendón-Franco et al. 2019)
Sea otter	*Enhydra lutris*	Polygynous	8.66	2.79	0.33	4.86	56.12%	(Williams and Pulley 1983)
Island spotted skunk	*Spilogale gracilis amphialus*	Polygynous	8.54	2.85	0.2	4.95	57.96%	(Crooks et al. 2003)
Ferret	*Mustela furo*	Polygynous	7.2	3.4	0.2	3	41.67%	(Hein et al. 2012)
Giant otter (Captive)	*Pteronura brasiliensis*	Monogamous	5.4	1.25	0.21	3.58	66.30%	(Colares and Best 1991)
Red panda (Captive)	*Ailurus fulgens*	Monogamous	7.85	3.74	0.41	3.28	41.78%	(Burrell et al. 2018)
Small-clawed otter	*Aonyx cinereus*	Monogamous	6.9	1.33	0.21	4.31	62.46%	Species 360 database

Figure 1: Number of musteloid species reported to be susceptible to STI pathogens collated through a literature review conducted in 2020.

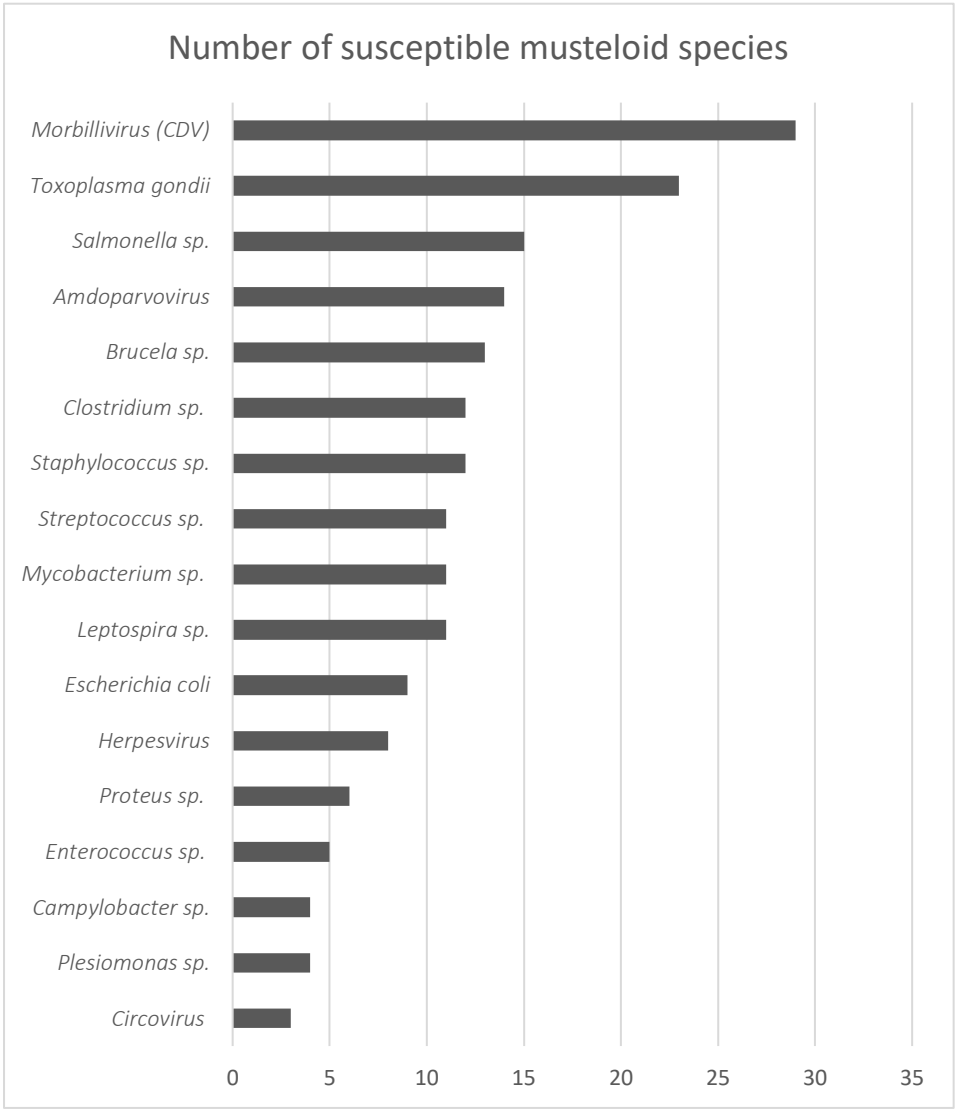


Figure 2: Number of STIs reported in musteloid species collated through a literature review conducted in 2020.

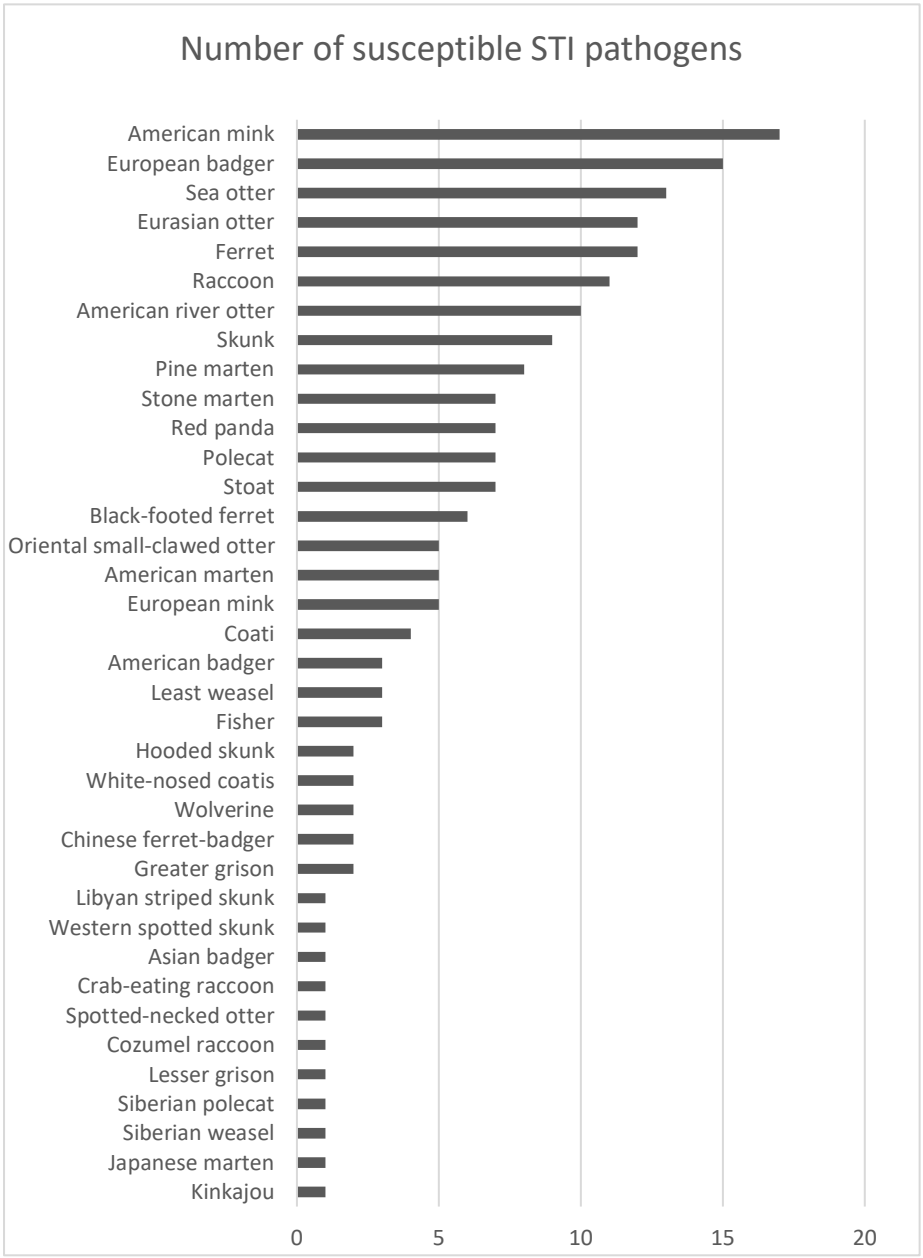
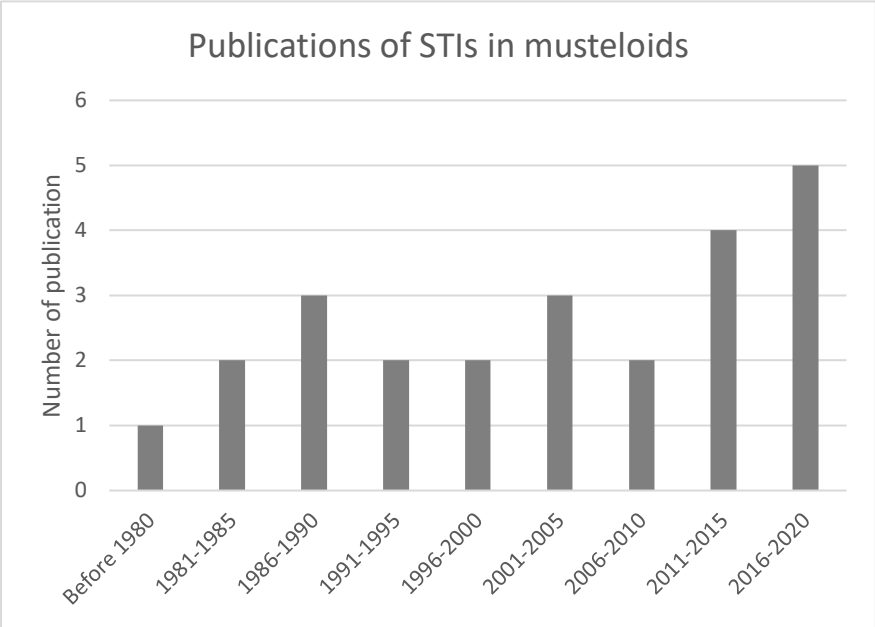


Figure 3: Publications reported STIs in musteloids (n=21) since 1980 to 2020 collated through a literature review conducted in 2020.



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To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	Sexually Transmittable Infections: A knowledge gap in Musteloid reproduction?
Publication Status	□Published □ Accepted for Publication □Submitted for Publicatio ■Unpublished and unsubmitted work written in a manuscript style
Publication Details	N/A

Student Confirmation

Student Name:	Ming-shan Tsai		
Contribution to the Paper	Performed literature reviews, analysis, interpretation of data and manuscript writing.		
Signature		Date	11th November 2021

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor David Macdonald		
Supervisor comments The candidate took the intellectual lead, and contributed very substantially to this research, which was primarily dependent upon her role.		
Signature	Date	12th November 2021

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 2: Effects of *Mustelid Gammaherpesvirus 1* (MusGHV-1) Reactivation in Badger (*Meles meles*) genital tracts on Reproductive Fitness

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Abstract

Reactivation of latent Gammaherpesvirus in the genital tract can lead to reproductive failure in domestic animals. Nevertheless, this pathophysiology has not received formal study in wild mammals. High prevalence of *Mustelid gammaherpesvirus 1* (MusGHV-1) DNA detected in the genital tracts of European badgers (*Meles meles*) implies that this common pathogen may be a sexual transmitted infection. Here we used PCR to test MusGHV-1 DNA prevalence in genital swabs collected from 144 wild badgers in Ireland (71 males, 73 females) to investigate impacts on male fertility indicators (sperm abundance and testes weight) and female fecundity (current reproductive output). MusGHV-1 reactivation had a negative effect on female reproduction, but not on male fertility; however, males had a higher risk of MusGHV-1 reactivation than females, especially during the late-winter mating season, and genital MusGHV-1 reactivation differed between age classes, where 3–5 year old adults had significantly lower reactivation rates than younger or older ones. Negative results in foetal tissues from MusGHV-1 positive mothers indicated that cross-placental transmission was unlikely. This study has broader implications for how wide-spread gammaherpesvirus infections could affect reproductive performance in wild Carnivora species.

Introduction

The herpesviridae comprise four subfamilies, the alphaherpesvirinae, betaherpesvirinae, gammaherpesvirinae (Davison 2010), and the newly assigned deltaherpesvirinae (Pellett 2014), which can all maintain lifelong infection, and exhibit prolonged latency and repeated reactivation. Infection typically causes no, or only mild subpatent, disease (Azab et al. 2018), although serious symptoms may be observed in immuno-compromised individuals (Barton, Mandal, and Speck 2011). In most cases, reactivation and virus shedding are not associated with symptomatic disease (Grinde 2013b), but even asymptomatic reactivation results in host infectiousness (Grinde 2013b).

In general, the extent of clinical symptoms during the lytic stage of herpesvirus infection depends on both, innate and acquired host immunity (Chew, Taylor, and Mossman 2009). Across species, stimuli associated with reactivation include physical or mental stress (Glaser et al. 1994; Stowe et al. 2001), trauma (Wilson and Mohr 2012), hypoxia (Davis et al. 2001), coinfection with other pathogens (Odumade, Hogquist, and Balfour 2011), pregnancy (Haeri, Baker, and Boggess 2010), hormonal changes (François et al. 2013), and aging (Stowe et al. 2007).

Primary herpesvirus infection, or reactivation from latency, during pregnancy can be serious, causing reproductive failure, including infertility, embryonic reabsorption, foetal abortion, preterm birth, stillbirth, poor neonatal condition, or neonatal death (Avgil and Ornoy 2006; Smith 1997). The mechanisms involved may be direct or indirect (Smith 1997). Direct herpesvirus-induced abortions are usually associated with viremia by primary infection or reactivation during pregnancy, leading to heavy viral loads in uterine vessels and cross-placental transmission to the foetus(es), resulting in reabsorption, abortion, or neonatal death. This has been demonstrated in alphaherpesviruses, such as *Canine herpesvirus 1* (CHV-1) in dogs (Evermann, Ledbetter, and Maes 2011), Bovine herpesvirus 1 (BHV-1) in cattle (Crook et al. 2012), *Equine herpesvirus 1* (EHV-1) and occasionally *Equine herpesvirus 4* (EHV-4) in horses (Patel and Heldens 2005; Smith 1997), and *Cervid herpesvirus 2* (CHV-2) in reindeer (das Neves et al. 2009). In contrast, indirect abortigenic sequelae associated with herpesvirus reactivation usually involve secondary bacterial infection. This may occur because viral infection can decrease foetal protection from bacteria ascending the vaginal tract or peritoneal cavity (Racicot et al. 2013), or they may change the maternal immune response to commensal bacteria and cause preterm birth (Mor, Aldo, and Alvero 2017). This has been demonstrated for Gammaherpesvirinae, such as the *Murid herpesvirus 4* (MHV-4) in mice (Cardenas et al. 2010), *Bovine herpesvirus 4* (BHV-4) in cattle (Chastant-Maillard 2015), and has been suggested for *Equine herpesvirus 2* and *5* (EHV-2 and EHV-5) in horses (Marenzoni et al. 2014).

Despite these potential impacts on reproduction, herpesvirus reactivation and associated diseases have rarely been investigated in wildlife. In the Carnivora, reported herpesvirus infections belong mostly to the gammaherpesvirinae, and have been documented from casualties (Gagnon et al. 2011; Tseng et al. 2013), epidemiological surveys (Hendrikse et al. 2019; Lozano et al. 2015; Subudhi et al. 2018), endangered species management (Black et al. 2019; Cabello et al. 2013; Makundi et al. 2018), or incidentally from studies that were focused on other pathogens (Cabello et al. 2013). Here we report for the first time the results of a systematic screening study of wild European badgers (*Meles meles*; henceforth 'badger') from populations across the Republic of Ireland, investigating how *Mustelid gammaherpesvirus 1* (MusGHV-1) reactivation in genital tracts affects host reproductive performance.

Badgers are an informative wildlife model to study the effects of sexually transmittable infections (STIs), because they have a highly promiscuous, polygynandrous mating system characterized by repeated mounting behaviour (Dugdale et al. 2007), likely putting them at particular risk of contracting STIs (Kokko et al. 2002). Their social system is highly flexible and depends largely on resource availability, ranging from solitary or pair-living to living in social groups comprising up to 29 adults (Johnson, Jetz, and Macdonald 2002). They are seasonal breeders, and produce one litter of one to a maximum of five cubs annually between mid-January to end of February after six weeks of gestation (Corner et al. 2015). In Ireland and the UK, 93% of litters comprise less than three viable cubs (Annavi et al. 2014), with an average litter size of 2.33–2.5 (min = 1, max = 4, Ireland: (Corner et al. 2015; Laura E Rosen et al. 2019)). The primary mating season occurs during the postpartum oestrus (UK/Ireland: from mid-January to end of April) with possible additional conceptive matings occurring through the summer months into October (Yamaguchi et al. 2006). Badger reproduction is characterized by delayed implantation (i.e., the fertilized ova develop to the blastocyst stadium, but then remain in diapause for up to 11 months until implantation in mid-late December) and superfoetation (i.e., females can continue to ovulate and conceive during delayed implantation) (Yamaguchi et al. 2006). Nevertheless, in areas with high population density, badger fecundity is low (e.g., Southern England (Page et al. 1994)): although conception rate can reach 90% (Page et al. 1994), Roper (Roper 2010) estimated that on average only 14% of blastocysts result in viable cubs, of which a further 60% die pre-emergence (Roper 2010). In contrast, in lower density populations, female badgers have lower conception rate (66%), but their abortion rate is also lower (estimated in Ireland as <1% in January from post-mortem studies: (Laura E Rosen et al. 2019)), corroborating previous evidence (Corner et al. 2015; Rosen et al. 2019) that prepartum reproductive success may be largely affected by population density. Previous studies have linked this high reproductive failure rate in part to intra-sexual reproductive suppression (Carpenter et al. 2005), somatic vs. reproductive fitness trade-offs (Bright Ross et al. 2020), infanticide in Southern

England (Roper 2010) (but not in Ireland (Rosen et al. 2019)), and high neonatal cub mortality due to coccidiosis (Newman, Macdonald, and Anwar 2001); but no single suggested cause can fully explain the observed phenomenon (Roper 2010). Surprisingly, the potential for this reduced fecundity, infertility, or abortigenic disease to be linked to STIs (Apari et al. 2014), has been largely ignored in badgers (but see (Kent et al. 2018)).

The *Mustelid gammaherpesvirus 1* (MusGHV-1), a member of the genus Percavirus, was first isolated from lung tissue of a wild badger in 1996 without obvious association to disease (Banks et al. 2002). It is highly prevalent amongst wild badgers in the UK (Sin et al. 2014) and Ireland (King et al. 2004), where MusGHV-1 DNA isolation from blood shows near 100% infection rate at some localities. Nevertheless, unlike alphaherpesviruses that reside in neuron cells during latency, gammaherpesviruses reside in B-lymphocytes (EBV, MHV-4, EHV-5) (Barton et al. 2011; Van Cleemput et al. 2019; Young, Yap, and Murray 2016) or monocytes (BHV-4) (Yang, Xie, et al. 2019), and therefore it is not possible to differentiate latency from reactivation when isolating viral DNA from whole blood samples. Confirming sexual transmission in a free-ranging population without using manipulative experiments is extremely challenging, but indirect evidence permits inference of horizontal and/or vertical transmission, for example, by isolating the pathogen DNA using PCR from swabs of genital tracts, placenta tissues, or foetal tissues. A population-wide screening survey revealed a reactivation rate of 55% in genital tract mucosae of badgers in a high density population in the UK (Kent et al. 2018), indicating a high likelihood of sexual transmission. This same study also linked reactivation of genital MusGHV-1 to reduced future reproductive success in female badgers (Kent et al. 2018), but did not investigate the factors contributing to herpesvirus reactivation. Here, we use badger samples collected post-mortem from across the Republic of Ireland under a national culling programme for the management of bovine tuberculosis to investigate:

1. Patterns of MusGHV-1 reactivation in badger genital tracts by comparing prevalence among females culled at the start of the primary mating season with those culled at the end (where many females are pregnant at the beginning, but all will have given birth by the end);
2. Differences in MusGHV-1 reactivation between males and females, and between different age groups;
3. Effects of MusGHV-1 reactivation on female reproductive success, male testes weight, and sperm abundance;
4. The potential for vertical transmission of MusGHV-1 to the foetus via the placenta during gestation, or via the maternal vaginal tract during parturition; and
5. The potential for horizontal transmission through semen.

We discuss our results in the framework of potential impacts of MusGHV-1 infection on badger population dynamics and reproductive health and in the broader context of carnivore conservation.

Materials and Methods

Source of Post-Mortem Badgers

Samples were collected at the Irish Equine Centre (IEC) between 23rd and 26th April 2019 (lactation/end of primary mating season) and 3rd–7th February 2020 (end of gestation/onset of the primary postpartum mating season) post-mortem from 144 badgers (71 males and 73 females) from across the Republic of Ireland culled under licence by the National Parks and Wildlife Service, with powers conferred by the Wildlife Acts 1976 to 2010 in the context of a national removal scheme to control bovine tuberculosis led by the Department of Agriculture, Food and the Marine (DAFM) (O'Keeffe 2006) (see Table 2 for details). Carcasses were preserved in a cold room at 0–2 °C as soon as possible after death and were examined between 1 and 10 days after death (average = 4.58 ± 1.95 days, median = 4 days). For each badger, culling site, sex, weight, and approximate age group (estimated from tooth wear: (Hancox 1988) and categorized as cubs: <1 year old; yearling: <2 years old; young adult: 2–3 years old; middle-aged adult: 3–5 years old; old adult: >5 years old) were recorded. All badgers were examined for genital discharge and lesions in the reproductive system.

Assessment of Reproductive Fitness

In females, appearance and interior of the uterus were examined and reproductive status was classified as follows: pregnant (carrying developing foetus(es)), recently pregnant (enlarged and thickened uterus with dark pigmented placental scars and mammary development/lactation), recently pregnant but aborted (enlarged and thickened uterus with no or pale placental scars, or no mammary development), not pregnant (determined by opaque round uterine appearance, absence of placental scars and small nonelongated teats; or thin translucent uterus with no evidence of uterine thickening, placental scars, or mammary development) (Laura E Rosen et al. 2019). Uterine flushing with 0.9% saline solution and following examination of the flush exudate under a dissecting microscope at 4× magnification were used to determine recent, successful mating (i.e., presence and number of blastocysts in uterus). Litter size was determined through direct counts of foetuses if the female was pregnant, or placental scar counts if it had already given birth (Fournier-Chambrillon et al. 2010). Lactational status was determined peripartum in winter by gross and histological examination of the mammary gland tissues, and postpartum in spring by mammary development and presence of milk (Laura E Rosen et al. 2019).

In males, one testis was collected using sterile scissors, and after removing all attached fat, muscles and blood vessels, was weighed in g using a precision balance (Sugianto et al. 2018). The epididymis was then detached and placed on a sterile petri dish, before being macerated and mixed with 2.5 mL 1× PBS to release the semen. The diluted semen was transferred to a sterile 2 mL microcentrifuge tube and stored at 4 °C before processing. Relative abundance of sperm was determined using a haemocytometer (1:10 diluted by 1× PBS) under a microscope at 40× magnification (Scott-Wilson 1982).

Assessing Potential for Vertical and Horizontal Transmission of MusGHV-1

To investigate if MusGHV-1 can be transmitted vertically prebirth (transplacentally), we collected maternal placentas and foetal brains, lungs, spleens, and livers from eight pregnant females (four females that tested MusGHV-1 positive in genital swabs and four females that tested negative in genital swabs—i.e., still with potential for latent infection or reactivation in other body-sites). To investigate if horizontal transmission through cell-free MusGHV-1 DNA in semen is plausible, we selected diluted semen samples from 10 males (five genital swab MusGHV-1 positive and five genital swab-negative control samples to elucidate if potential cell-free viral DNA originates from sperm or from epithelial cells in the genital tract), and then collected seminal plasma from the supernatant after centrifugation at 1500 rpm for 10 min. All swabs, tissue, and semen samples were frozen immediately and stored at −20 °C before being shipped frozen and packaged in dry ice to the University of Oxford for DNA extraction and PCR.

Histopathological Examination

Mammary gland tissues from all females with recent pregnancy but uncertain lactation status were collected to confirm lactation by histological examination. Representative lower genital tract tissue samples with lesions were collected from five females and two males (according to availability) and fixed in 10% neutral buffered formalin. These formalin-fixed tissues were trimmed, dehydrated through a graded ethanol series, and then infiltrated with paraffin. These embedded tissues were then sliced at 4 μm and stained with haematoxylin and eosin (H&E) for microscopic examination.

MusGHV-1 DNA Detection in Genital Tracts, Semen, and Tissues

To determine MusGHV-1 reactivation in badger genital tracts (i.e., presence of cell-free MusGHV DNA), lower genital tracts were swabbed with sterile cotton tops with wooden shafts (Dynarex, New York, USA) to secure virions without damaging the epithelium. Any swabs contaminated with blood (from culling/prior dissection) were centrifuged at 1500 rpm for 10 min to eliminate circulating white blood cells that could contain latent MusGHV-1. Samples were then placed in empty 2 mL sterile microcentrifuge tubes and stored at −20 °C.

A ca. 25 mg subsample of each tissue was taken, and each swab was reconstituted with 400 µL sterile doubly distilled water, vortexed gently at room temperature for 10 min, before decanting a 200 µL aliquot from the supernatant. DNA was processed using DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. Purified DNA was then eluted in 100 µL of the buffer provided.

Purified DNA was screened for MusGHV-1 using a specific primer pair (forward primer: 5′-CCA AGC AGT GCA TAG GAG GT-3′; reverse primer: 5′-TGG ACT TCT CCA ACA TGC GTC GCC CTT C-3′) modified from King et al. (2004), targeting 771 base pairs of a partial DNA polymerase gene. For each reaction, a total of 20 µL PCR solution was mixed with 10 µL HotStartTaq Master Mix (Qiagen) containing 1 unit of HotStartTaq DNA Polymerase, 12 µM of $MgCl_2$, and 1.6 µM of each dNTP), 0.5 µM of forward and reverse primer, 2 µL Coralload gel loading dye, and 5 µL DNA template. Amplification conditions were kept at 95 °C for 5 min to activate DNA polymerase, followed by 45 cycles of denaturation at 95 °C for 45 s, primer annealing at 60 °C for 45 s, and chain elongation at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Finally, the PCR products were loaded in 2% agarose gel to check amplification results under UV light. Representative PCR products with positive results were variantd by Sanger sequencing (Zoology Sequencing Facility, University of Oxford), and one representative sequence was submitted to the NCBI Genbank under accession number MT332102. Trimmed sequences were compared to previously published sequences on GenBank by the online NCBI Blast service.

Statistical Analysis

Statistical analyses were performed using *R* Studio software (version 1.21335). Prevalence of MusGHV-1 was calculated as the percentage of PCR positive cases over the total number of samples examined, and 95% upper and lower confidence intervals were calculated using the Wilson method (Wilson 1927). Univariate analysis using Chi-square tests with Yates continuity correction and Fisher's exact test (if any column of the contingency table was less than 5) were used to investigate if age, sex, or age groups were risk factors for genital MusGHV-1 reactivation in badgers, and whether genital MusGHV-1 reactivation affects female reproductive fitness. To investigate whether there was an interaction between MusGHV-1 reactivation and badger weight, herpes-positive and negative captures of each sex, in each culling period, were compared separately with *t*-tests to standardize for sexual and seasonal weight differences (Laura E Rosen et al. 2019). To investigate effects of genital MusGHV-1 on testes weight and relative sperm concentration, herpes-positive and negative males were compared using *t*-tests. Logistic regression multivariable analysis and odds ratio were used to estimate the size effect of different risk factors on MusGHV-1 reactivation, and the area under receiver operating characteristic curve (AUROC) was calculated for diagnostic ability of the final model (Zweig and Campbell 1993). Due to data availability, age group analysis was only applied to samples collected in

winter, and yearlings were excluded (due to very low sample sizes). We used a different (n = 251) dataset from a UK population, where we show that age has strong correlation to MusGHV-1 prevalence (quadratic term, adjusted R^2 = 0.602, p < 0.001) (Tsai et al. 2021 or Chapter 3). We use the adjusted R^2 as effect size, significance level = 0.05 to calculate the minimum size required to reach higher statistical power (set as 0.8) (Statistics Kingdom 2017), and determined that a minimal sample size as eight has sufficient statistical power to reach a valid statistical result.

Results

Risk Factors of MusGHV-1 Reactivation in Badgers

Overall, prevalence of MusGHV-1 in genital swabs was 61.8% (95%CI: 53.7–69.3%, 89/144 swabs). The trimmed 23 sequences (691 bps) were 100% identical to each other and to the previously reported MusGHV-1 sequence (AF275657) isolated from lung tissue cultures of a female badger in South England in 1996 (Banks et al. 2002).

In winter (i.e., for which we had data on both, sex and age), sex and age were significant risk factors for genital MusGHV-1 reactivation (see results of multivariable logistic regression analysis in Table 1, area under receiver operating characteristic curve (AUROC) = 0.74. If AUROC equals 0.5 means the model has no accuracy of diagnostic; while AUROC equals 1 means the model has perfect accuracy). At the beginning of the mating season, MusGHV-1 reactivation in genital tract was 6.7 times more likely to happen in males (82.5%, 33/40) than in females (47.5%, 19/40), and 4.7 times more likely in young adults (72.4%, 21/29) than in middle-aged adults (50%, 10/20) (prevalence in Table 2 and odds ratio in Table 1). Particularly, middle-aged males had a 6-fold higher prevalence of genital MusGHV-1 than did females in the same age category (75%, 9/12 vs. 12.5%, 1/8, respectively). If yearlings were excluded (due to very low sample size), our data suggested no difference of prevalence between age groups was found in males, but in females, prevalence in middle-aged individuals (12.5%, 1/8) tended to be lower than in young (58.8%, 10/17) or in old (63.6%, 7/11) adults, although this association did not reach significance (Chi-squared = 5.8476, df = 3, p = 0.054).

Univariate follow-up analyses for both seasons, however, showed that males had a significantly higher risk of MusGHV-1 reactivation than females only in winter, but not in spring (winter prevalence in males = 82.5% (33/40), females = 47.5% (19/40), Chi-squared = 9.2857, df = 1, p-value = 0.002; spring prevalence in males = 64.5% (20/31), females = 51.5% (17/33); Chi-squared = 0.6388, df = 1, p-value = 0.424, see Table 2 for full results).

Interactions of Genital MusGHV-1 Reactivation with Body Weight

There was no overall body weight difference between MusGHV-1 positive and negative individuals, with the exception of females in winter. As pregnancy is energetically costly, we further separated females into pregnant and nonpregnant, and evidenced that MusGHV-1 was associated with lighter bodyweight only in pregnant females, but not for those that had already given birth or had not been pregnant; nevertheless, this effect was not statistically significant, perhaps due to the small sample sizes (t = 1.8691, df = 4.8914, p-value = 0.122; t-test) (Figure 1).

Effects of Genital MusGHV-1 Reactivation on Reproductive Fitness

Relative sperm abundance ranged from 0 to 160.38 × 10^5/mL with an average of 43.89 × 10^5 spermatozoa/mL (±40.18 × 10^5) of equally diluted semen collected from 39 males. However, there was no significant effect of genital MusGHV-1 reactivation on relative testicular weight (p = 0.82), but a trend—albeit not significant (p = 0.085)—for positive males to have higher sperm abundance (Figure 2).

In the winter dataset (2020), 71.8% (28/39) of females implanted successfully while 61.5% (24/39) reproduced successfully (i.e., still pregnant or recently pregnant with fresh placental scars at time of culling) with an average litter size of 2.46 ± 0.72 (min = 1, max = 4). In the spring dataset (2019), 51.6% females (16/31) had signs of recent pregnancy with an average of 2.41 ± 0.9 (min = 1, max = 4) placental scars (representing most recent litter sizes); blastocysts were present in the uterus of 52.2% (12/23) of adult females with an average of 2.08 ± 0.63 blastocysts (min = 1, max = 3, n = 12) per pregnant female. There was no seasonal/annual difference in reproductive fitness between the two sampling periods (Chi-square = 0.3486, df = 1, p-value = 0.555).

Notably, females with genital MusGHV-1 reactivation (see Table 3) had a significantly lower rate of successful pregnancy (41.2%, 14/34) than MusGHV-1 negative females (72.2%, 26/36) (odds ratio = 0.269, 95%CI = 0.1–0.73, Chi-square = 5.672, df = 1, p-value = 0.017). This indicates that MusGHV-1 reactivation had a negative effect on female reproductive fitness, although when separated by season this trend was only significant in winter but not in spring (Table 4). No significant association was found between MusGHV-1 genital reactivation and the presence of blastocysts in spring (Table 5).

Potential for Horizontal and Vertical Transmission

One (20%) seminal plasma sample from genital MusGHV-1 negative males tested positive for MusGHV-1, but interestingly none of the five samples from genital MusGHV-1 positive males contained cell-free MusGHV-1 DNA. From 18 well-preserved pregnant females, we dissected a total of 43 foetuses (partial dissection of 33 and full dissection of 10 foetuses), which all appeared to be healthy and normally developed, irrespective of the mother's herpes infection

status. Three out of 10 placentas tested were positive for MusGHV-1 DNA, (one from a genital MusGHV-1 negative mother; two from mothers with partial foetal loss—one tested positive for genital MusGHV-1, one negative); however, no MusGHV-1 DNA was detected in any foetal tissues.

Abnormalities in Female Reproductive Tracts Associated with Genital MusGHV

In 25% (7/28) of females (three young and four old adults) that were pregnant or showed signs of recent pregnancy, we recorded possible abortions (enlarged uterus but no foetuses/placental scar(s) present, but with signs of reabsorption, such as dark, amorphous mass of dead cells in the enlarged uterus (Page et al. 1994)) of entire 14.3% (4/28) or partial (10.7%; 3/28) litters. All four females (three were > 5 years old, one was < 3 years old) with complete litter loss showed no sign of lactation and tested positive for genital MusGHV-1 reactivation. Furthermore, one of the four pregnant MusGHV-1 positive females had reddened cyst-like gross lesions surrounding the cervix; one had submucosal haemorrhage around the cervix, and one had the remains of an absorbed foetus (in addition to two healthy ones) in her uterus; only one showed no abnormalities in her reproductive tract.

We also observed that 48.38% females (30/62, 40 from winter and 22 from spring) and 52.5% males (21/40, all from winter) displayed macroscopic lesions that resembled lymphoid hyperplasia on the epithelium of the lower genital tracts or around the cervix in females, and the mucosal epithelium of the outer urethra in males. These gross lesions took the form of vesicle-like (Figure 3A), congestive and oedemic (Figure 3B), or pigmented dotted scars (Figure 3C), but were not always associated with genital MusGHV-1 reactivation (Table 5). We only tested one lesion tissue (Figure 3B) for MusGHV-1 and the result was positive, but the genital swab of this specific animal tested negative.

Histopathological examination confirmed that all seven samples from animals with gross lesions investigated (females: one cervix and four inner genital tract tissue samples from females; males: two inner genital tract tissue samples) showed inflammation, with five out of the six genital tract tissues exhibiting epithelial hyperplasia (Table 6). Among these, only one male mucosal tissue collected from the base of the penis had intra-nuclear inclusion bodies in the epithelium, likely indicating viral infection (Figure 4).

Genital discharge was present in 74.2% (23/31) of females during spring, and 7.5% (3/40) of females and none of the males (0/40) in winter, but was not associated with MusGHV-1 reactivation. A cervical mucus plug (CMP) was observed in 44% (8/18) of the pregnant females, none of which tested positive for genital MusGHV-1 reactivation (compared to 40% genital MusGHV-1 prevalence in the remaining 10 pregnant females without a CMP).

Discussion

Genital MusGHV-1 reactivation rates in our Irish dataset are comparable to those previously reported in an English badger population (Kent et al. 2018). Nevertheless, the overall badger MusGHV-1 reactivation rates of 61.8% found in this study and of 50.6% (excluding cubs) in Kent et al. (Kent et al. 2018) at a near 100% prevalence in blood (Sin et al. 2014) are higher than gammaherpesvirus reactivation rates reported in other species: human Epstein-Barr virus (EBV) (22–90% seropositive rate) has been reported with viral DNA isolation from 8–28% from cervical fluids and semen samples (Hjalgrim et al. 2007); horse EHV-5 DNA has been found in 14.7% of uterine flushing samples and 2.6% of genital swabs; horse EHV-2 (79.7% seroprevalence (Craig, Barrandeguy, and Fernández 2005)): 1.6% from uterine flushing; 2.3% from genital swabs (Lee and Lee 2019; Marenzoni et al. 2014); reindeer CvHV-2 DNA has been found in 24% of vaginal swabs (unspecified seroprevalence) (das Neves et al. 2009); cattle BHV-4 (96.9% seroprevalence) DNA in 48.5% of uterine swabs and 51.5% of vaginal swabs (Yang, Pascottini, et al. 2019). The exceptionally high prevalence of MusGHV-1 DNA in badger genital tracts further strengthens that sexual transmission may be an important route in gammaherpesvirus transmission comparable to human KSHV (Kaposi's sarcoma-associated herpesvirus) (Dittmer and Damania 2013) and murine MHV-4 (François et al. 2013).

Nevertheless, MusGHV-1 reactivation rates fluctuated over a badger's lifetime: middle-aged (3–5 years old) individuals had lower reactivation rates than young (2–3 years old) and over 5 year old animals, and all MusGHV-1 positive pregnant female badgers belonged to the old age group. This mirrors results from humans and some domestic animals where primary infection occurs predominantly during infancy (Kangro et al. 1994), with initial reactivation in mucosae near the entry portal (Longnecker, Kieff, and Cohen 2013) and repeated reactivation throughout life (Young et al. 2016), which increases with aging due to declining immune-competency (Stowe et al. 2007).

Overall, we found no difference in the body weights of genital MusGHV-1 positive and negative individuals suggesting that MusGHV-1 reactivation does not affect—nor was triggered by—host body condition, mirroring results from in England (Kent et al. 2018). We did, however, identify a nonsignificant trend in pregnant females, where genital MusGHV-1 positive individuals had lighter body weights than those that tested negative (Figure 1), indicating that during pregnancy, reactivation may lead to decreased body condition (Bandyopadhyay et al. 2010), or—conversely—that reduced body condition during pregnancy may promote MusGHV-1 reactivation (Cavignac and Esclatine 2010).

Sex-Specific Seasonal Patterns in Genital MusGHV-1 Reactivation

Our study shows that male badgers are significantly more at risk of genital MusGHV-1 reactivation than females, particularly at the beginning of postpartum mating season (end of January and early February). Latent sexually transmittable pathogens likely evolved to be reactivated during their host's mating season to maximize host-to-host transmission (Lockhart et al. 1996); a pattern well-established for cyprinid herpesvirus CyHV-3 in wild common carp (Uchii et al. 2014). The main mating season in badgers coincides with the postpartum oestrus (in the UK and Ireland typically in the middle of February (Corner et al. 2015; Sugianto, Heistermann, et al. 2021), but males start their "rutting behaviour" several weeks earlier from the middle of January (Charlton et al. 2020), coinciding with a rise in their testosterone levels (Sugianto et al. 2018). Testosterone, however, acts as an immune-suppressant (Trumble et al. 2016) and may thus be partly responsible for the corresponding peak in male MusGHV-1 prevalence rates, mirroring results in domestic cats where males also have a higher risk than females to be infected with the feline gammaherpesvirus FcaGHV-1 (Makundi et al. 2018; Troyer et al. 2014), and entire males are at higher risk of infection with FcaGHV-1 than neutered males (McLuckie et al. 2016). Female badger MusGHV-1 reactivation rates in the genital tract, in contrast, are higher in spring at the end of the main mating season than at its start in January. This could be due to the physiological stress linked to late-term pregnancy, birthing, lactation (Woodroffe and Macdonald 1995), and postpartum mating (Macdonald et al. 2004), but may also arise due to (re-)infection through mating with males shedding MusGHV-1 in their genital tract (e.g., equid alphaherpesvirus 3 reported in seropositive mares re-shed after mating with virus-shedding stallions (Barrandeguy et al. 2012)), where our results show that over 80% MusGHV-1 prevalence in genital tracts of male badgers, and 10% semen contained viral DNA.

Effects of Genital MusGHV-1 Reactivation on Reproductive Fitness

Although reproductive failure (Page et al. 1994) and premature cessation of lactation (Laura E Rosen et al. 2019) are often reported in badgers, specific studies investigating potential links to reproductive tract diseases are lacking. MusGHV-1 reactivation did not decrease male sperm production; in fact, there was a nonsignificant trend for MusGHV-1 positive males to have a higher sperm count than MusGHV-1 negative ones (Figure 2A), although we found no difference in testicular weight connected to genital herpes (Figure 2B). This suggests that, in males, genital MusGHV-1 reactivation does not cause sterility, although effects on sperm morphology, motility, and viability remain unknown (see also humans: (Kaspersen and Höllsberg 2013)).

In females, in contrast, genital MusGHV-1 reactivation has a negative effect on reproductive fitness, manifesting in failure to carry pregnancy to term as seen in in other species (Smith 1997). The abortion rate (entire litter: 14.3% and partial 10.7%) confirmed in this study is considerably higher than the <1% previously reported in Irish badgers (Corner et al. 2015; Laura E Rosen et

al. 2019). This is likely due to the concentrated sampling immediately before the main birthing period in this study, which appears to be more likely to observe abortions through gross examination, but comparable to rates reported from England (complete litter loss: 20–33% (Roper 2010), partial loss: 10–14.3% (Roper 2010)). In winter, genital MusGHV-1 prevalence rates were significantly higher in females that did not reproduce than in those carrying healthy foetuses or having carried their pregnancy to term. All four females with signs of spontaneous abortion of their full litters, as well as one out of the three females with late partial abortion tested MusGHV-1 positive. This further substantiates the negative association of genital MusGHV-1 reactivation with badger female reproductive fitness. In addition, 4/5 of these were old, potentially indicating that aging in combination with increasing stress when approaching parturition (Carr et al. 1981) may favour latent MusGHV-1 reactivation, thus increasing the likelihood of abortion (Egyed et al. 2011).

In domestic animals, gammaherpesvirus-associated reproductive failure has frequently been shown to be associated with bacterial coinfection (Cardenas et al. 2010; Chastant-Maillard 2015; Mor et al. 2017; Racicot et al. 2013). During pregnancy, cervical mucous plugs (CMPs) likely form a physical and immunological barrier to prevent microbes ascending into the uterus from the lower reproductive tract (e.g., humans: (Becher et al. 2009); mares: (Loux et al. 2017)). While we did not investigate potential differences in the genital microbiome community structure, all pregnant females with a CMP tested MusGHV-1 negative, compared to a 40% prevalence of genital MusGHV-1 reactivation in females without a CMP, suggesting that reproductive success may be affected by complex relationships between MusGHV-1 reactivation, bacterial microbiome dynamics, and host immunity (Racicot and Mor 2017).

Genital Lesions

Both sexes commonly had gross lesions in their genital tracts resembling the lymphoid hyperplasia and hyperaemia of the vaginal mucosa characteristic of female dogs infected with CHV-1 (Decaro, Martella, and Buonavoglia 2008), and similar to the progress of equine coital exanthema caused to horse genitalia by EHV-3 infection (Blanchard, Kenney, and Timoney 1992; Bryans 1980). In the case of badgers, however, we did not find a significant correlation between the presence of gross genital lesions and MusGHV-1 reactivation, nor with female reproductive success, although intranuclear inclusion bodies—an indicator of herpesviral lytic infection (Josey and Zaki 1973)—were found during histopathological examination in epithelial cells of the genital mucosa of one male with epithelial hyperplasia. This may be due to lytic infection and virion-shedding not necessarily being synchronized with the formation of lesions, as reported from experimental CvHV-2 reactivation in reindeer (das Neves et al. 2009).

Vertical Transmission of MusGHV-1

Our findings suggest that vertical transmission of MusGHV-1 through the placenta is possible but unlikely. This corroborates other studies on gammaherpesvirus transmission (e.g., MHV-4 transmission in mice (François et al. 2013); EBV in humans (Avgil and Ornoy 2006)), but contradicts a study of BHV-4 transmission in cattle, where intrauterine infection was found in 4/7 stillbirth foetuses (Egyed et al. 2011). Nevertheless, primary infection in foetuses may likely happen during parturition, or through close postnatal contact with the mother, explaining results from a previous study that found 75% 3-month old prepubescent cubs presenting positive for genital MusGHV-1 (Kent et al. 2018).

The Significance of Gammaherpesviruses as Drivers of Wildlife Population Dynamics

Gammaherpesviruses are of increasing concern in humans (Dittmer and Damania 2013; Young et al. 2016) and animals (livestock: (Smith 1997), pets: (Beatty et al. 2019), wild (Banks et al. 2002; Cabello et al. 2013; Lozano et al. 2015; Troyer et al. 2014), and captive wildlife populations: (Gagnon et al. 2011)). EBV is prevalent in 90% of the human population, and is associated with diseases such as infectious mononucleosis, childhood leukaemia from maternal infection and, more rarely, lymphoma (Hachkuga 1992; Lehtinen et al. 2003; Young et al. 2016). Bovine herpesvirus 4 is associated with abortion in cattle (Smith 1997), and the widespread equid herpesvirus 2 and 5 are associated with rare diseases such as fatal equine multinodular pulmonary fibrosis (Van Cleemput et al. 2019), and there is increasing evidence of their connection to other reproductive disorders (Marenzoni et al. 2014). Most recently, infections by a novel gammaherpesvirus *Leporid gammaherpesvirus 5* (LeHV-5) were identified in wild Iberian hares (*Lepus granatensis*) with genital and skin lesions, and will likely intensify the recent population decline (Abade dos Santos et al. 2020).

Although gammaherpesvirus associated diseases have rarely been reported in wild animals, this observation might be due to the difficulty of diagnosis in the field, and to asymptomatic infection, especially for abortigenic diseases. However, reports of novel herpesviruses in wildlife have strengthened the assumption of ubiquity across the animal kingdom. Broad surveys of occurrence and in-depth research on associated diseases in wildlife are warranted to enhance understanding of gammaherpesvirus effects on epidemiology, pathogenicity, evolution, and disease ecology. As theory predicts (De Castro and Bolker 2005; Ryder et al. 2007) and empirical studies evidence (McCallum 2008; McCallum et al. 2018), STI-induced sterility can constitute an important driver of population dynamics, and superficially benign infections without clinical manifestation may in fact drive already endangered wildlife populations to extinction.

Table 1. Final model of multivariable logistic regression to identify risk factors for genital *Mustelid gammaherpesvirus 1* (MusGHV-1) reactivation (function = glm(MusGHV ~ Sex + Age, family = binomial), degrees of freedom = 68, AIC = 83.66) of winter samples with yearlings (*n* = 2) being excluded. Area under ROC = 0.74 in the final model.

Group	Estimate	Standard Error	z Value	Adjusted OR	95% CI of OR	*p* Value
(Intercept)	−1.1697	0.6466	−1.809	0.31	0.09–1.1	0.07
Sex (vs. Female)						
Male	1.9023	0.6442	2.953	6.7	1.9–23.67	0.003
Age group (vs. middle-aged adults)						
Young adults	1.5527	0.7243	2.144	4.72	1.14–19.53	0.032
Old adults	1.3507	0.7671	1.761	3.86	0.86–17.36	0.078

Table 2. Univariate analysis of risk factors affecting MusGHV-1 DNA prevalence in badger genital tracts.

	Positive *n*	Total *n*	Prevalence	95% CI	Chi-Square Test
Season					
Winter	52	80	65%	54.1–74.5%	$X^2 = 0.503$, df = 1, $p = 0.478$
Spring	37	64	57.80%	45.6–69.1%	
Sex					
Male	53	71	74.60%	63.4–83.3%	$X^2 = 8.741$, df = 1, $p = 0.003$
Female	36	73	49.30%	38.2–60.5%	
Age group [a,b]					
Yearling	2	2	100%	34.2–100%	$X^2 = 2.559$, df = 1, $p = 0.11$ (Young:Middle-age)
Young	21	29	72.40%	54.3–85.3%	$X^2 = 0.034$, df = 1, $p = 0.854$ (Young:Old)
Middle-age	10	20	50%	29.9–70.1%	$X^2 = 1.667$, df = 1, $p = 0.197$ (Middle aged:Old)
Old	14	20	70%	48.1–85.4%	
Season:Sex					
Winter:Male	33	40	82.50%	68.1–91.3%	$X^2 = 9.2857$, df = 1, $p = 0.002$
Winter:Female	19	40	47.50%	32.9–62.5%	
Spring:Male	20	31	64.50%	46.9–78.9%	$X^2 = 0.6388$, df = 1, $p = 0.424$
Spring:Female	17	33	51.50%	35.2–67.5%	
Sex:Age group [a,b]					
Male:Yearling	1	1	100%	20.7–100%	$p = 0.59$ (Young:Middle aged)
Male:Young	11	12	91.60%	64.6–98.5%	$p = 0.553$ (Young:Old)
Male:Middle-age	9	12	75%	46.8–91.1%	$p = 1$ (Middle-age:Old)
Male:Old	7	9	77.80%	45.3–93.7%	
Female:Yearling	1	1	100%	20.7–100%	$p = 0.042$ (Young:Middle-age)
Female:Young	10	17	58.80%	36–78.4%	$p = 1$ (Young:Old)
Female:Middle-age	1	8	12.50%	2.2–47.1%	$p = 0.059$ (Middle-age:Old)
Female:Old	7	11	63.60%	35.4–84.8%	

a: Yearlings are not included in the statistic calculation due to small sample size. b: Only winter samples as age group data was not obtained in spring samples.

Table 3. Female reproductive status and genital MusGHV-1 prevalence in European badgers separated by seasons and age groups (only in winter).

Reproductive Status	Spring				Winter				Young		Middle-age		Old	
	(+)	Total	%	95% CI	(+)	Total	%	95% CI	(+)	(-)	(+)	(-)	(+)	(-)
Not pregnant	11	17	64.7%	41.3–82.7%	8	12	66.7%	39.1–86.2%	6	1	1	3	-	-
Pregnant [a]	-	-	-	-	4	18	22.2%	9.0–45.2%	0	5	0	3	4	3
Recently pregnant	7	16	43.8%	23.1–66.8%	3	6	50.0%	18.8–81.2%	3	1	0	1	0	1
Recently pregnant, aborted	-	-	-	-	4	4	100.0%	51.0–100%	1	0	-	-	3	0

a: Three of the pregnant females showed signs of partial litter loss.

Table 4. Risk factor analysis of female reproductive fitness.

	Pregnant *n* [a]	Total *n* [b]	Rate	95% CI	Chi-Square Test
Season					
Winter	24	39	61.50%	45.9–75.1%	$X^2 = 0.3486$, df = 1, $p = 0.555$
Spring	16	31	51.60%	34.8–68%	
Genital MusGHV-1					
Positive	14	34	41.20%	26.4–57.8%	$X^2 = 5.672$, df = 1, $\mathbf{p = 0.017}$
Negative	26	36	72.20%	56–84.2%	
Season:Genital MusGHV-1					
Winter:Positive	7	18	38.90%	20.3–61.4%	$X^2 = 4.0032$, df = 1, $\mathbf{p = 0.045}$
Winter:Negative	17	21	81%	60–92.3%	
Spring:Positive	7	16	43.80%	23.1–66.8%	$X^2 = 0.2972$, df = 1, $p = 0.586$
Spring:Negative	9	15	60%	35.7–80.2%	

a: This includes females that were pregnant or successfully gave birth (i.e., had fresh placental scars). b: This excludes two prepubescent yearlings from spring and one from winter.

Table 5: Risk factor analysis for presence of genital discharge, presence of genital lesions and presence of blastocyst using logistic regression analysis (formula = MusGHV ~ Variable, family = binomial)

	Estimate	Std.Error	z value	p-value
Model 1 (df=110)				
(Intercept)	0.6061	0.227	2.671	0.00757
Genital_discharge Yes	-0.6061	0.4532	-1.338	0.18104
Model 2 (df=101)				
(Intercept)	0.5213	0.2896	1.8	0.0719
Genital_lesion Yes	-0.3246	0.4038	-0.804	0.4215
Model 3 (df=22)				
(Intercept)	-0.1823	0.6055	-0.301	0.763
Blastocyst Yes	-0.1542	0.8423	-0.183	0.855

Table 6: List of badger samples for histology (collected by Ming-shan Tsai at Feburary 2020; histopathological examination by Dr. Ursula Fogarty)

19PB3066 Female, was pregnant, vaginal tract
Gross description: Vaginal tract of a sexually mature resting *Meles meles*. The reproductive tract was opened with an incision along the central midline. The upper vaginal tract was pale and has several vaginal folds. There were multiple red vesicles found on the surface of the lower vaginal tract. Histopathological description: Histopathological examination showed epithelial hyperplasia with a subepithelial chronic inflammatory infiltrate of macrophages, lymphocytes, plasma cells and a few eosinophils.
20PB0384 Female, Recently pregnant, vaginal tract
Gross description: Vaginal tract of a sexually mature and recently pregnant *Meles meles*. The reproductive tract was opened with an incision along the central midline. The vaginal tract has a hyper-enlarged skin in the upper and lower vaginal tract with grey papules on the vaginal folds. Histopathological description: Histopathological examination showed epithelial hyperplasia. There was a low grade diffuse subepithelial chronic inflammatory infiltrate primarily lymphocytes with occasional perivascular foci.
20PB0398 Female, Pregnant (2 fetus), vaginal tract
Gross description: Vaginal tract of a sexually mature pregnant *Meles meles*. The reproductive tract was opened with an incision along the central midline. The vaginal tract was enlarged with no vaginal folds present and has several visible pink papules found on the surface of the upper vaginal tract. Histopathological description: (Two tissues examined) Histopathological examination showed tissue oedema with diffuse and focal subepithelial inflammatory cells primarily macrophages, plasma cells, lymphocytes and eosinophils. The eosinophils were especially prominent in the deeper layers. In

the second sample there was epithelial hyperplasia, a folded surface epithelium with subepithelial inflammatory foci of macrophages, lymphocytes, plasma cells and eosinophils.

20PB0444 Female, Not pregnant, cervix

Gross description: Cervix of a sexually immature *Meles meles*. The reproductive tract was opened with an incision along the central midline. There were several visible red vesicles on and surround the cervix.

Histopathological description: Histopathological examination showed a diffuse low grade chronic inflammatory infiltrate.

20PB0527 Female, vaginal tract

Gross description: Vaginal tract of a *Meles meles*. The reproductive tract was opened with an incision along the central midline. The vaginal tract has blanketed flat red dots found on the cervix and on the surface of upper genital tract.

Histopathological description: Histopathological examination showed a diffuse subepithelial chronic inflammatory infiltrate. Occasional subepithelial focal accumulations of cells were primarily macrophages, lymphocytes, plasma cells with an occasional neutrophil.

20PB0350 Male penis

Gross description: Penis of a sexually mature *Meles meles*. The penis was revealed by opening the male genital tract. The entry of the genital tract (in the bottom of the penis) has several transparent papules on the mucosa surface.

Histopathological description: Histopathological examination showed epithelial hyperplasia with keratinisation of superficial cells. There were subepithelial foci of chronic inflammatory cells, primarily lymphocytes.

20PB0496 Male penis

Gross description: Penis of a sexually mature *Meles meles*. The penis was revealed by opening the male genital tract. The entry of the genital tract (in the bottom of the penis) has several transparent papules on the mucosa surface.

Histopathological description: Histopathological examination showed epithelial hyperplasia with intranuclear inclusions and there was a subepithelial inflammatory infiltrate of macrophages, lymphocytes and plasma cells.

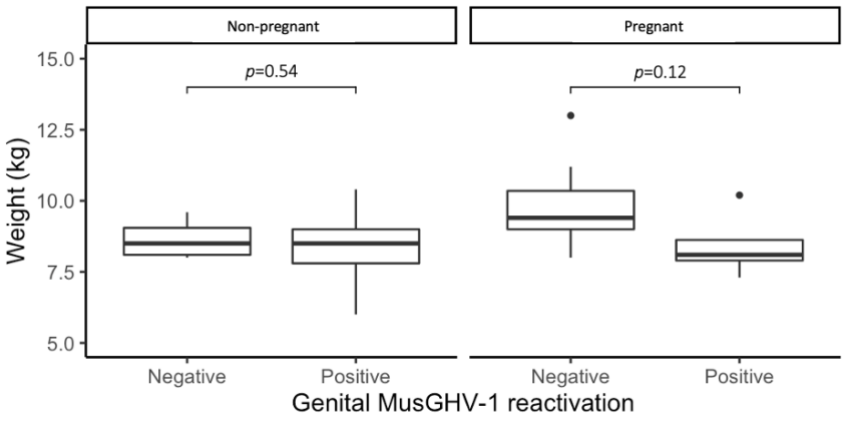

Figure 1. Weight comparison using *t*-test between genital MusGHV-1 positive (n=4) and negative females (n=14) in pregnant females, and genital MusGHV-1 positive (n=32) and negative females (n=23) in nonpregnant females.

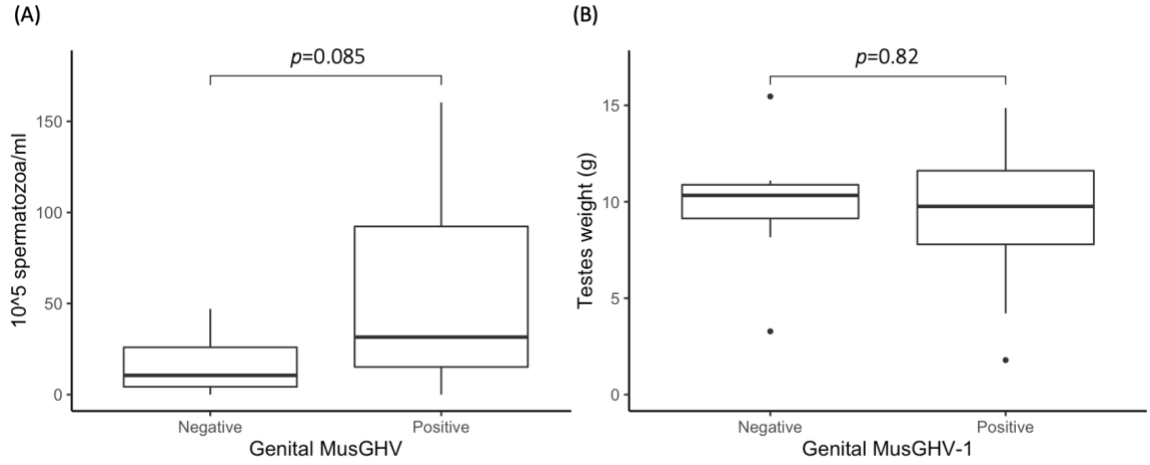

Figure 2. Comparison of sperm concentration (A) and testes weight (B) between genital MusGHV-1 positive (n=33) and negative males (n=7). The black dots are referred to outliers.

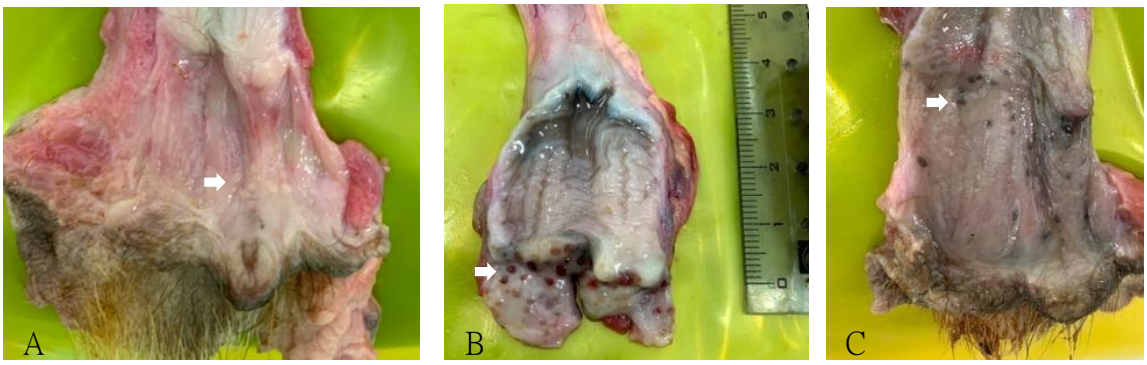

Figure 3. Lesions in the lower genital tract (possibly lymphoid hyperplasia); differences in appearance may reflect different stages of infection (A–C).

Figure 4. Image of gross lesion (A) found on preputial mucosa located at the base of the penis (arrows) and histology (B) showing intranuclear inclusion bodies (circles) in the apical side of epithelium.

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	Effects of *Mustelid Gammaherpesvirus 1* (MusGHV-1) Reactivation in Badger (*Meles meles*) genital tracts on Reproductive Fitness
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Student Confirmation

Student Name:	Ming-shan Tsai		
Contribution to the Paper	Performed field works, sample collection, lab experiments, data analysis, interpretation of data and manuscript writing.		
Signature	Ming-shan Tsai	Date	11th November 2021

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor David Macdonald		
Supervisor comments The candidate took the intellectual lead, and contributed very substantially to this research, which was primarily dependent upon her role.		
Signature	Date	12th November 2021

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 3: Patterns of genital tract *Mustelid gammaherpesvirus 1* (MusGHV-1) reactivation are linked to stressors in European badgers (*Meles meles*)

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Abstract

Gammaherpesvirus reactivation can promote diseases or impair reproduction. Understanding reactivation patterns and associated risks of different stressors is therefore important. Nevertheless, outside the laboratory or captive environment, studies on the effects of stress on gammaherpesvirus reactivation in animals are lacking. Here we used *Mustelid gammaherpesvirus 1* (MusGHV-1) infection in European badgers (*Meles meles*) as a host-pathogen wildlife model to study the effects of a variety of demographic, physiological and environmental stressors on virus shedding in the genital tract. We collected 251 genital swabs from 150 free-ranging individuals across 3 seasons and screened for MusGHV-1 DNA using PCR. We explored possible links between MusGHV-1 DNA presence and 7 variables reflecting stressors, using logistic regression analysis. The results reveal different sets of risk factors between juveniles and adults, likely reflecting primary infection and reactivation. In adults, virus shedding was more likely in badgers in poorer body condition and younger than 5 years or older than 7; while in juveniles, virus shedding is more likely in females and individuals in better body condition. However, living in social groups with more cubs was a risk factor for all badgers. We discuss possible explanations for these risk factors and their links to stress in badgers.

Introduction

Alongside the *Alphaherpesvirinae, Betaherpesvirina* and *Deltaherpesvirinae*, the Gammaherpesvirus is a group of double-stranded DNA viruses belong to the family Herpesviridae (Pellett 2014; Roizman and Baines 1991) and can be classified into 4 genuses, the *Macavirus*, *Rhadinovirus*, *Lymphocryptovirus* and *Percavirus*. After primary infection, most known gammaherpesviruses establish a latent stage in the B lymphocytes or macrophages, and can then be reactivated repeatedly throughout life. Numerous factors are known to trigger reactivation, including stress (Seeber et al. 2018; Stowe et al. 2001), trauma (e.g. surgery:(Grinde 2013b)) or primary co-infection with other pathogens (Donofrio et al. 2005). Reactivation is a process of viral lytic infection, which involves virus replication within the host cell, eventually causing the cells to burst and release infectious virions. Reactivation of gammaherpesviruses occurs in plasma cells and epithelial cells of mucosa that function as portals for external contact (e.g., mouth, nose, eyes and genital tract), thus facilitating transmission (Shannon-Lowe et al. 2009). Reactivation is typically asymptomatic or induces only mild disease, but depending on strain pathogenicity (Goodman et al. 2007), can also contribute to the development of other severe diseases such as cancer (Barton et al. 2011) and is associated with a higher risk of contracting co-infection with additional pathogens with high virulence (e.g. herpesvirus DNA detection is a risk factor for the presence of *Chlamydia pecorum* DNA in koalas (Stalder et al. 2015)) or pathogens causing immunodeficiency (e.g. Human herpesvirus 8 and HIV (Zhang et al. 2020)). Although not as extensively studied as the human Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), gammaherpesvirus reactivation has long been linked to sometimes severe diseases in domestic animals (Chastant-Maillard 2015; Marenzoni et al. 2014; McLuckie et al. 2018), and increasingly also to illness in wildlife species (Abade dos Santos et al. 2020; Gagnon et al. 2011; Nicolas de Francisco et al. 2020; Tseng et al. 2012).

European badgers, *Meles meles* (henceforth 'badgers'), are seasonally breeding mustelids that are commonly infected with the *Mustelid gammaherpesvirus 1* (MusGHV-1, genus *Percavirus*), for which prevalence of viral DNA in blood samples can reach up to 100% in the UK and in Ireland across all age groups, where primary infection likely occurs in early life (King et al. 2004; Sin et al. 2014). Furthermore, MusGHV-1 DNA has also been detected in 55% - 82.5% badger genital swab samples (Kent et al. 2018; Tsai et al. 2020, or Chapter 2), suggesting local reactivation in genital tract is common, and MusGHV-1 is a sexually transmittable pathogen. Sexually transmitted infections (STIs) are often found to be associated with infertility in animals (Lockhart et al. 1996; McLeod and Day 2019), and in badgers, previous research has linked otherwise asymptomatic MusGHV-1 shedding in genital tracts to poorer female reproductive performance (Tsai et al. 2020, or Chapter 2). Considering the potentially considerable impact of

STI-induced sterility on population dynamics (Ryder et al. 2007), understanding the patterns of MusGHV-1 shedding in genital tracts is important in identifying the risk factors. Two previous studies on genital tract MusGHV-1 screening have indicated that cubs, old individuals and adult males during the mating season are at particular risk of genital MusGHV-1 shedding (Kent et al. 2018; Tsai et al. 2020 or Chapter 2). Nevertheless, the impact of social, physiological and seasonal effects, which may contribute to immune dysregulation due to stress, on MusGHV-1 reactivation has not been investigated.

Since the local extinction of brown bears (*Ursus arctos*) and wolves (*Canis lupus*) (Wilson 2004), badgers do not have natural predators in the UK, and their survival is mainly dependent on weather conditions (Macdonald et al. 2010; Nouvellet et al. 2013), which are closely linked to food availability. Faecal corticoid levels indicate that badgers experience seasonal variation in stress levels (George et al. 2014a; Virgós et al. 2004). There are several potential underlying causes: Seasonal stress may be linked to seasonally-varying food availability (George et al. 2014a; Virgós et al. 2004) that can result in mortality (Macdonald et al. 2010; Nouvellet et al. 2013). Higher cortisol levels are also associated with poor body condition and coinfection with *Mycobacterium bovis*, the pathogen causing bovine tuberculosis (Cheeseman et al. 1985). Therefore metrics of body condition, especially reduced body-condition as a consequence of recent lactation (George et al. 2014a; Woodroffe and Macdonald 1995), can indicate that individuals experience physiological stress (George et al. 2014a). Sociologically, higher social group density is associated with female reproductive suppression (Creel and Creel 1991; Woodroffe and MacDonald 1995), reduced body condition and fecundity (Macdonald et al. 2002), and increased bite wounding among male badgers (Macdonald et al. 2004). Furthermore, aging reduces tolerance to stress (Hunter and McEwen 2013), specifically altering the balance between innate and acquired immunity in badgers (van Lieshout et al. 2020), and increasing their risk of herpesvirus reactivation (Tsai et al. 2020, or chapter 2), as also observed in other carnivora species (Dall'Ara et al. 2019; Troyer et al. 2014), sometimes resulting in chronic or continuous herpesvirus reactivation (Sebastiano et al. 2016).

Here we conducted a population-wide molecular screening using genital swabs taken from a free-ranging badger population in the south of England across three seasons (spring: May, summer: September, autumn: November). We investigated whether environmental factors (i.e., season, social group size and structure), host demographic parameters (i.e., sex, age and lactation), and host body condition are risk factors of genital MusGHV-1 shedding. Furthermore, we investigated if risk factors differ between juveniles and adults, given that virus shedding in juveniles is more likely associated with primary infection, whereas in adults it is likely due to reactivation from latency.

Materials and Methods

Samples were collected from live-trapped badgers in Wytham Woods, Oxfordshire, UK (51◦46'26"N, 1◦19'19"W; caught in May, September and November 2018 following the methodology described in Macdonald et al. (Macdonald et al. 2009); for details see Table 1). For each capture, we recorded sex, sett (i.e., communal den used by a social group) of capture, body condition score (BCS, categorized as 1= very thin to 5= very fat), and lactational status (determined by teat measurements of females in spring (Dugdale, Davison, et al. 2011)). Because each badger in Wytham is given an individual tattoo at first capture (usually as a cub (Macdonald et al. 2015)), exact age (in years) was known for most (243 of 251) animals in the dataset. For the remaining 8 badgers first caught as adults, age was inferred by toothwear according to the method described by Bright Ross et al. (2020). We defined four age classes based on sex-steroid levels (Sugianto et al. 2020) and age: i) juveniles < 2 years old (cubs and yearlings were combined to increase sample sizes, as there was no difference between MusGHV-1 prevalence in cubs and yearlings: Fisher's exact test: p-value=0.7449); ii) young adults: 2≤ x <5 years old; iii) old adults: 5≤ x <8 years old; iv) very old adults: ≥ 8 years old. The number of cubs and adults resident in each sett was estimated using minimum number alive (MNA) estimates (Bright Ross et al. 2020; Macdonald et al. 2009).

Sterile cotton tops with wooden shafts were used to swab the genital tracts of all females (cubs and adults) and all males (except for very small male cubs in spring for animal welfare reasons), and stored in 2 ml sterile microcentrifuge tubes. All samples were frozen and stored at -20°C immediately after sampling. Badgers were released at their site of capture on the same day, after full recovery from anaesthesia.

Each swab was reconstituted with 400µl sterile double distilled water and vortexed gently at room temperature for 10 minutes. A 200µl aliquot was taken from the reconstituted swab fluids, and viral DNA was extracted and purified using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen) following manufacturer's instructions. Purified DNA was then eluted in 100µl of the provided buffer.

The purified DNA was screened using a MusGHV-1-specific primer pair designed by King et al. (2004), targeting 281 base pairs of the partial DNApol gene. For each reaction, a total of 20 µl PCR solution was mixed with 10µl HotStartTaq Master Mix (Qiagen, containing 1 unit of HotStartTaq DNA Polymerase, 12µM of $MgCl_2$ and 1.6µM of each dNTP), 0.5 µM of each primer, and 2µl CoralLoad gel loading dye and 5 µl DNA template. Amplification conditions were kept at 95°C for 5 mins to activate DNA polymerase, followed by 45 cycles of denaturation at 95°C for

45 seconds, primer annealing at 60°C for 45 seconds, and chain elongation at 72°C for one minute, followed by a final extension at 72°C for 10 minutes. Finally, the PCR products were loaded in 2% agarose gel to check the amplification results under UV light. Samples with positive results were then amplified again with substituted front primer (5' CCA AGC AGT GCA TAG GAG GT 3') to generate longer sequences (771 base pairs) to check if there are genetic variations among the sequences. PCR products were then purified and sent for genotyping using Sanger sequencing to confirm the identity of produced amplicon. Sequences returned were then aligned by Clustal W method (Thompson, Higgins, and Gibson 1994) and analyzed for variation using MEGA X (10.1.7) (Kumar et al. 2018). Representative sequences were selected and deposited on GenBank under accession numbers MT332100 and MT332101.

Statistical analyses were performed with the R and R Studio softwares (version 1.21335) (R-Development-Core-Team 2019; RStudio Team 2020) . Detection rate of genital MusGHV-1 DNA was calculated by dividing the number of PCR positive cases by the total number of tested cases, and 5% upper and lower confidence intervals were calculated using the Wilson method (Wilson 1927). Logistic regression (glmer function, R package lme4) with badger identity (tattoo) number as a random effect was used to measure univariate effects of MusGHV-1 reactivation in genital tracts with season, sex, age class, BCS, number of residents per social group (total, adult and cub MNA), and percentage of cubs per social group (calculated by cub MNA divided by total MNA) as fixed effect variables. We categorized the percentage of cubs per social group into low and high using 30% as the dividing point according to distribution of the data (Figure 1). Effects of lactation were analyzed using Fisher's exact tests due to low sample sizes. Because juveniles (especially cubs) are generally thinner than adults, and thus have a different body condition distribution (Macdonald et al. 2002), in the univariate analysis we analyzed the association of MusGHV-1 detection and individual BCS for juveniles and adults separately. The effect size of each variable is presented using odds ratios. Variables with p-values < 0.1 were retained in the multivariable analysis. We constructed 2 multivariable models based on data from juveniles and adults as we expected patterns to be different between these 2 groups. Manual backwards selection was used to select the final model with best fit (determined by lowest Akaike information criterion, or AIC, value). Model residual diagnostics were conducted using the R package DHARMa (version 0.3.3.0). Model fit was established using area-under-receiver-operating characteristics (AUC) with an acceptance threshold of 0.7 (Mandrekar 2010). Collinearity of variables was checked using variance inflation factor (VIF), where variables with a VIF value of more than 5 were excluded from the model. We defined variables in the final models with p-values of less than 0.1 as risk factors for genital tract MusGHV-1 shedding.

Results

Univariate analysis

The overall detection rate of genital tract MusGHV-1 DNA was 35.5% (89/251, 95% CI: 30% - 41.6%), and was generally higher in summer (43.8%, 35/80) than in spring (34.4%, 33/96) and autumn (28%, 21/75) (Table 2). There was strong evidence for an effect of age on genital MusGHV-1 shedding where genital tract MusGHV-1 DNA detection rate in juveniles (45.2%, 33/73) and very old badgers (47.7%, 21/44) was higher than in middle-aged adults (i.e., young (30.9%, 30/97) and old (13.5%, 5/37) individuals; Table 2). However, no effect of sex was observed (logistic regression analysis, p=0.679). Adults with lower BCS had a higher probability of genital MusGHV-1 DNA detection rate (p-value=0.012, Table 2). There was no evidence for the number of adults, nor the total number of badgers resident in each social group affecting MusGHV-1 DNA detection rates, except for the number of cubs per social group (Table 2): The prevalence of MusGHV-1 in the genital tract was significantly higher in badgers living in social groups comprising >30% cubs (logistic regression analysis, p-value = 0.004). Finally, there was no evidence for recent lactation affecting the MusGHV-1 DNA detection rate in sexually mature females (Fisher's exact test, p-value=1; Table 2).

Multivariable analysis

We generated multivariable models of MusGHV-1 detection for juveniles (n=72) and adults (n=171). The most parsimonious models for juveniles (Table 3; Figure 2) and adults (Table 4; Figure 3) had both diagnostically acceptable (Mandrekar 2010) AUC area of 0.727 and 0.774, respectively. In juveniles, multivariable analyses showed that being a female, being in better body condition and living in a social group with a high proportion of cubs (over 30%) were risk factors of genital MusGHV-1 shedding (Table 3). In contrast, in adults, young (2-5 years old) or very old (≥8 years) badgers in poorer body condition and living in social groups with a high proportion of cubs are at particular risk of shedding MusGHV-1 in their genital tract (Table 4).

Genetic diversity of MusGHV-1 in the Wytham badger population

We sequenced 5 MusGHV-1 positive PCR products of partial DNA polymerase gene. All sequences were trimmed to 694 base pairs and confirmed to be MusGHV-1 according to the NCBI BLAST results, returning 98.7% (n=3) and 100% (n=2) nucleotide identity to the published MusGHV-1 sequence isolated from a badger in Cornwall, England (Accession number: AF275657).

Discussion

In this study, we investigated patterns of MusGHV-1 shedding in badger genital tracts, and identified associated risk factors comprising age, sex, season and social group composition. We confirmed that juveniles and adults are subject to different sets of risk factors, putatively reflecting primary infection and reactivation from latency, respectively.

In terms of age class effects, the high genital reactivation rate detected in cubs and yearlings suggests that badgers contract MusGHV-1 early in life, before reaching sexual maturity. Although MusGHV-1 reactivates repeatedly throughout life, reactivation tends to be less frequent in young and old adults, although rates increase in very old individuals. This matches patterns in humans where most people become infected with herpes during their childhood/ adolescence (e.g. 100% and 70% seroprevalence of EBV before age 14 in Hong Kong and the United Kingdom (Kangro et al. 1994)), then typically experience viral latency during their prime, but can suffer from increasingly longer and more frequent herpesvirus reactivation that can cause mild disease (e.g., shingles (Donahue 1995) in old age due to lowered immune response (Gouin, Hantsoo, and Kiecolt-Glaser 2008; Stowe et al. 2007)).

Since vertical transmission of MusGHV-1 through the placenta is unlikely (Tsai et al. 2020, or Chapter 2), and the potential for infection from the vaginal tract during parturition is equally low due to low genital MusGHV-1 detection rate in pregnant females (Tsai et al. 2020, or Chapter 2), we hypothesize that cubs contract MusGHV-1 through close contact with virus-shedding conspecifics (Tsai et al. 2020, or Chapter 2). Thereafter genital virus shedding in cubs may arise after primary acute infection through non-sexual routes and subsequent latency, as observed in the murine model where *Murine herpesvirus 4* (MuHV-4, also a gammaherpesvirus), inoculation in the nasal cavity results in acute infection in the respiratory tract and lungs and establishes latency in the spleen, but then reactivates in the vaginal tract 17-21 days after inoculation (François et al. 2013). MuHV-4 nasal cavity inoculation, however, does not result in reactivation in male genital tracts, and transmission is only possible from females to males. After sexual intercourse with virus-shedding female mice, the virus replicates in male penis for 3 weeks. Interestingly, also in badgers, female juveniles are at higher risk of MusGHV-1 reactivation in the genital tract than males. Furthermore, juveniles in better body condition, regardless of sex, exhibit higher virus detection rate. Indeed, juvenile males in better body condition enter puberty earlier than thinner males (11 months compared to 22 – 28 months: (Sugianto, Newman, et al. 2019)). Once juveniles enter puberty they will experience an increased risk of contracting MusGHV-1 through sexual contact and/or that their latent infection is reactivated through mating resulting in viral shedding in the genital tract.

Faecal corticosteroid measurements from badgers in Ireland (George et al. 2014a) evidence higher stress levels in summer likely associated with dry environmental conditions that result in lower earthworm availability (i.e., the badgers' main food type (Macdonald et al. 2015)); similarly, in our own study population, summer drought is an established mortality factor due to starvation/malnutrition (Macdonald et al. 2010; Nouvellet et al. 2013). This corresponds to our finding that, in all adults, seasonal MusGHV-1 reactivation rates were highest in summer, but females tended to have higher viral reactivation levels than males in spring – possibly due to reproductive stresses, while the reverse was true in autumn (Table 2) (George et al. 2014a). Badgers captured in summer generally have lower BCS than in other seasons, this is reflected in our multivariable model (Table 4) that when season and BCS are both included in the model, the effect of seasons found in univariate model (Table 2) has been eliminated, while BCS remains strong negative effect on MusGHV-1 DNA presence in genital tract. This implies that BCS is the main driver to cause the seasonal effect of MusGHV-1 DNA detection rate in genital tract. However, we did not detect any collinearity between seasons and BCS, and removing season from the model decreased model fit (i.e., an increase in the AIC value), suggesting season contributed to the response value through a different explanatory variable that was not included in our study.

Chronic stress has proven a significant risk factor of herpesvirus reactivation, causing immune system dysregulation, where corticosteroids inhibit the pro-inflammatory cytokine responses, allowing viruses to (re-)activate and undergo lytic proliferation unchecked (Morey et al. 2015). This link between elevated corticosteroid levels and herpesvirus reactivation has been proven experimentally (horses: (Barrandeguy et al. 2008); captive reindeer: (das Neves et al. 2009) and through observation (e.g. humans: (Glaser et al. 1994; Stowe et al. 2001); captive Grévy's zebras (*Equus grevyi*): (Seeber et al. 2018), but has not been investigated in free-living wildlife populations. Herpesvirus reactivation triggered by stress has been widely confirmed naturally and experimentally by corticosteroid injection in humans and domestic animals (Barrandeguy et al. 2008; Seeber et al. 2018). Linking stress and viral reactivation in wildlife, however, is particularly challenging due to the difficulties of monitoring individual stress levels in the field, and typically this relationship can only be confirmed experimentally by taking subjects into captivity (das Neves et al. 2009). Using indicators that have been linked to stress hormone levels in previous studies can thus provide an informative way to study the relationship between stress and herpesvirus reactivation in free-ranging wildlife.

Our finding of no sex-related effect on herpesvirus reactivation in adults contradicts our recent survey of Irish badger populations (Tsai et al. 2020, or Chapter 2), where the rate (over 82.5%) of genital MusGHV-1 DNA presence in adult males was significantly higher than in females

(47.5%), during the peak (postpartum) mating season (i.e. from mid-January to mid-February). This implies not only a link to mating activity, but also a mechanism enhancing sexual transmission where sex-biases in viral shedding in badger genital tracts are most pronounced during the mating season. This corroborates our finding in the same study where males with more spermatozoa have a higher detection rate of genital MusGHV-1 DNA (Tsai et al. 2020, or Chapter 2); linking higher sexual activity to higher STI prevalence as predicted by theory (Kokko et al. 2002) and reported also in many other studies (Munday et al. 1983; Poiani 2000; Ryder et al. 2013; White et al. 2011). We were unable to test effects relating to badger mating behaviour explicitly because we did not trap individuals during late pregnancy and neonatal cub care to avoid stressing mothers or depriving cubs of maternal care. Nevertheless, reactivation rate in autumn, when food sources are most abundant, and badgers undergo a period of reproductive quiescence (Sugianto, Dehnhard, et al. 2021), and thus experience less implicit stress, was significantly lower compared only to summer, but not to spring. This suggests that other factor(s) (e.g. sex hormone cycles (François et al. 2013), oxidative stress (Costantini et al. 2018), and/or genital microbiome (Urbaniak et al. 2020) and linked coinfections (Nordengrahn et al. 1996) might also be affecting reactivation rates, beyond the scope of our current study.

Our results also show that social group structure can affect the risk of genital herpesvirus shedding, particularly the higher proportion of cubs within a residential group. This may be because badger cubs generally carry higher pathogen burdens than adults (particularly lice *Trichodectes melis* and coccidia *Eimeria melis*) (Albery et al. 2020; Newman et al. 2001), and thus increase per capita immunity burden among all badgers resident in the respective sett.

Conclusion

Our study demonstrates - for the first time in the wild - the link between host stressors and herpesvirus shedding, which is likely reflecting both primary infection and reactivation from latency according to host age. Amplified stress levels induced by human disturbance as well as food insecurity and more frequent catastrophic weather events arising from human induced rapid environmental change could therefore not only increase the risk of disease development and promote pathogen transmission within a population, but also negatively impact host reproductive fitness through localised herpesvirus reactivation in the reproductive tract (Tsai et al. 2020, or Chapter 2). Careful monitoring of endemic latent virus infection as well as surveillance for possible newly emerging strains should therefore be included when planning *in situ* and *ex situ* conservation programmes for endangered species (Kelly et al. 2017).

Table 1: Summary of sampling effort

Age group	Age	Spring		Summer		Autumn		Total
		Female	Male	Female	Male	Female	Male	
Cub	0	15	15	10	4	9	9	62
Yearling	1	1	3	2	2		3	11
Young	2	7	7	7	9	5	6	41
	3	4	8	3	5	2	8	30
	4	2	5	4	5	2	8	26
Old	5	2	1	6	1	3	1	14
	6	3	1	3	1	3		11
	7		2		2		2	6
	NA	1	1	1	1		2	6
Very old	8	4	4	4	3	1	3	19
	9	2	1	2	1	1	1	8
	10	3	1	3		4		11
	11							0
	12							0
	13	1	1		1	1		4
	NA		1				1	2
	Total	45	51	45	35	31	44	251

Table 2: Overview of MusGHV-1 DNA detection rate in badger genital tract and univariate logistic regression analysis. Formula: MusGHV ~ Variable + (1|Tattoo); number of observations: 251; groups by tattoo number: 150

Variable	Positive	Total	%	95% CI	Odds ratio (OR)	OR 95% CI	P-value
Sex							
Male	45	130	34.62%	27% - 43.1%			
Female	44	121	36.36%	28.3% - 45.2%	0.93	0.55 - 1.56	0.774
Season							
Spring	33	96	34.38%	25.6% - 44.3%	1.36	0.7 - 2.65	0.368
Summer	35	80	43.75%	33.4% - 54.7%	2.02	1.02 - 4.03	**0.044**
Autumn	21	75	28.00%	19.1% - 39%			
Age group							
Juvenile (<2 years old)	33	73	45.21%	34.3% - 56.6%	5.28	1.85 - 15.08	**0.001**
Young (2 - 4 years old)	30	97	30.93%	22.6% - 40.7%	2.87	1.02 - 8.08	**0.046**
Old (5 - 7 years old)	5	37	13.51%	5.9% - 27.8%			
Very old (>7 years old)	21	44	47.73%	33.8% - 62%	5.9	1.92 - 17.78	**0.002**
Body Condition[a]							
Body condition score (1-5)		182					**0.012**
Social group size							
Total		251					0.653
Adult		251					0.405
Cub		251					**0.035**
Cub percentage per sett							
Low (<30%)	58	191	30.37%	24.3% - 37.2%			
High (>30%)	31	60	51.67%	39.3% - 63.8%	2.35	1.03 - 4.24	**0.004**
Lactational status[bc]							
Not Lactated	3	10	30.00%	10.8% - 60.3%			
Lactated	6	18	33.33%	16.3% - 56.3%	0.9	0.16 - 4.92	0.856

a: Only adults were included in this analysis; b: Only females captured in spring were included in this analysis; c: Fisher exact test

Table 3: Final general mixed effect model of multivariable logistic regression analysis for juveniles. Formula: MusGHV ~ Sex + Body condition + (1|Tattoo); number of observations: 72; groups by tattoo number: 48

Group	Estimate	Standard error	z value	Adjusted OR	95% CI	p value
(Intercept)	-1.204	0.813	-1.482			
Sex						
Female						
Male	-0.985	0.528	-1.863	0.37	0.13 - 1.05	0.062
Body Condition						
BCS	0.785	0.306	2.565	2.19	1.2 - 3.99	**0.01**
Cub percentage						
Low (<30%)	-0.977	0.539	-1.812	0.38	0.13 - 1.08	0.07
High (>30%)						

Table 4: Final general mixed effect model of multivariable logistic regression analysis for adults. Formula: MusGHV ~ Sex + Season + AgeGroup + Cub percentage + Sex*Cub percentage + (1|Tattoo); Number of observations: 171; Groups by tattoo number: 99

Group	Estimate	Standard error	z value	Adjusted OR	95% CI	p value
(Intercept)	0.059	1.075	0.055			
Season						
Spring	-0.289	0.584	-0.495	0.74	0.24 - 2.35	0.620
Summer	0.684	0.520	1.318	1.98	0.72 - 5.48	0.188
Autumn						
Age Group						
Young	1.767	0.673	2.627	5.85	1.57 - 21.87	0.009
Old						
Very old	1.75	0.695	2.522	5.77	1.48 - 22.55	0.012
Body condition						
BCS	-0.60	0.238	-2.513	0.54	0.34 - 0.88	0.012
Cub percentage						
Low (<30%)	-1.15	0.544	-2.115	0.31	0.11 - 0.92	0.034
High (>30%)						

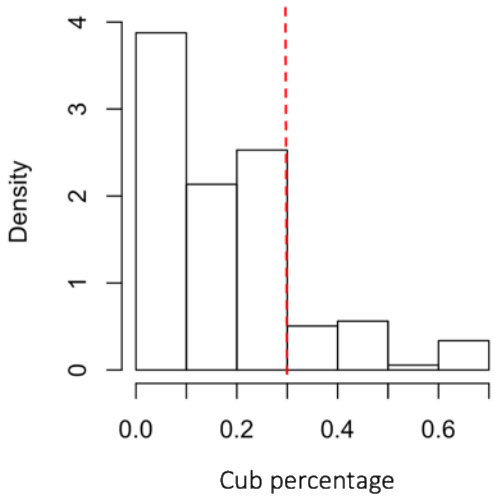

Figure 1: Data distribution of cub percentage (n=251) according to group density

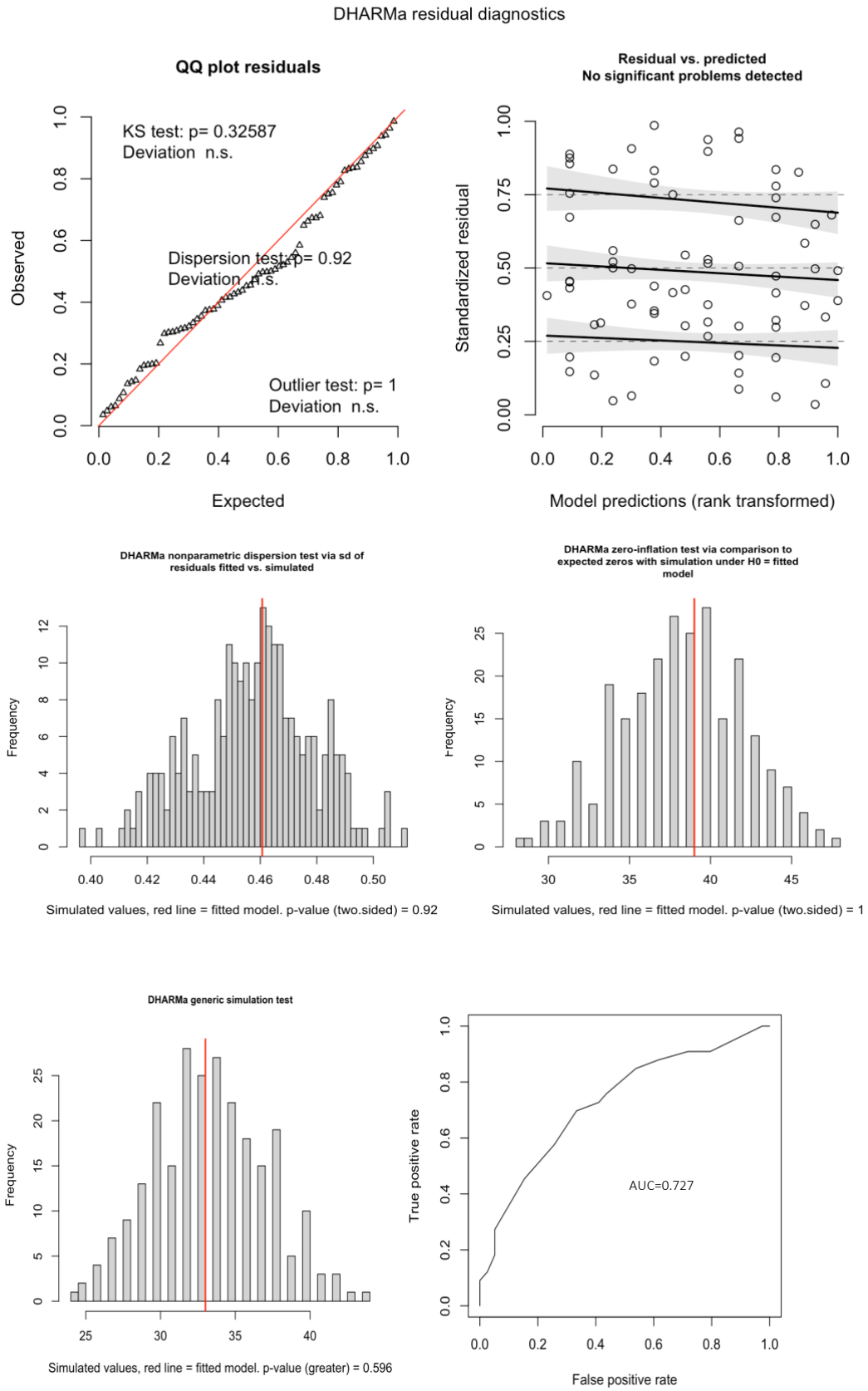

Figure 2: Residual diagnostic plot of the final multivariable logistic regression model of juvenile MusGHV-1 shedding in genital tract;

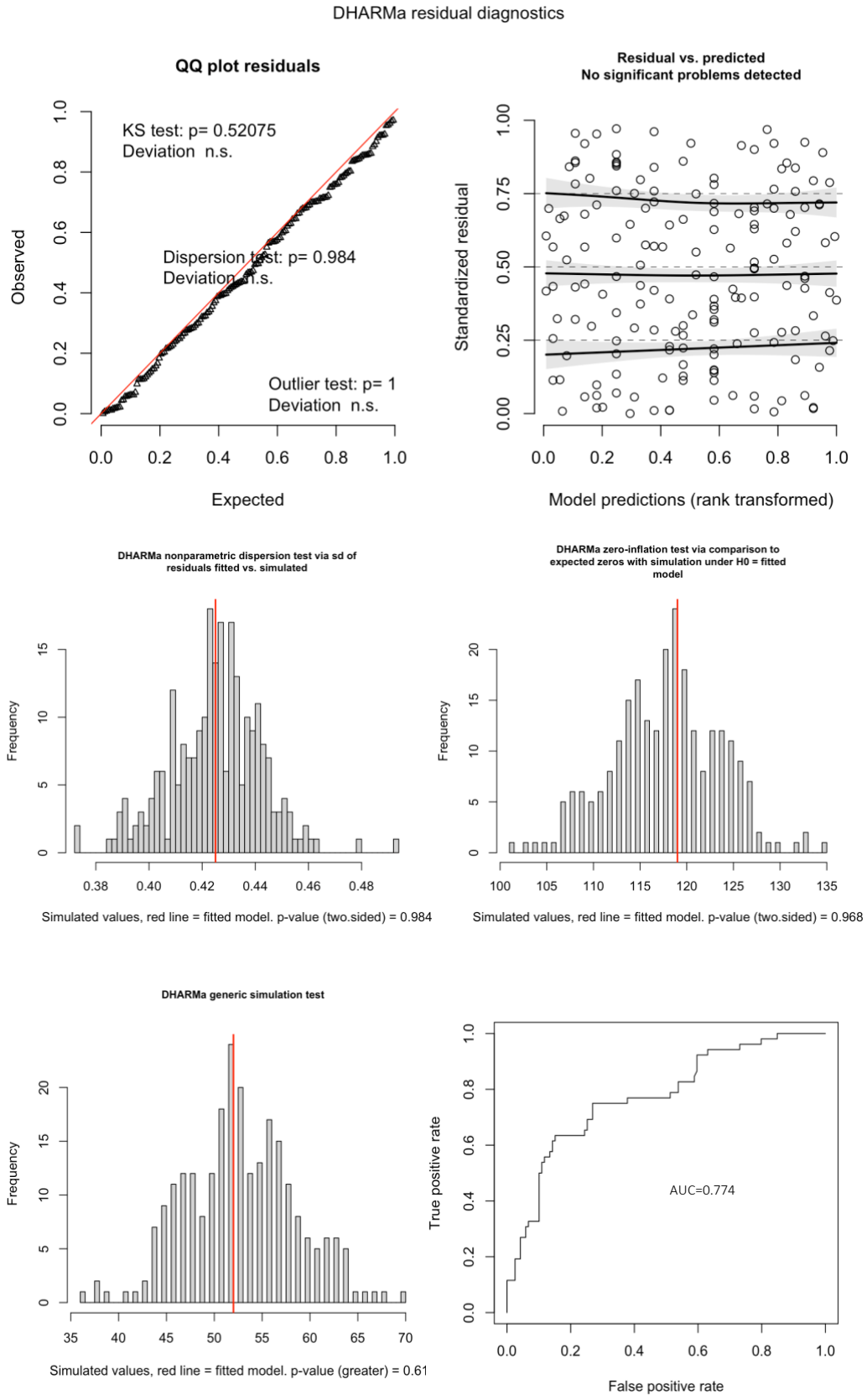

Figure 3: Residual diagnostic plot of the final multivariable logistic regression model of adult MusGHV-1 shedding in genital tract

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	Patterns of genital tract *Mustelid gammaherpesvirus 1* (MusGHV-1) reactivation are linked to stressors in European badgers (*Meles meles*)
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Student Confirmation

Student Name:	Ming-shan Tsai		
Contribution to the Paper	Performed field works, sample collection, lab experiments, data analysis, interpretation of data and manuscript writing.		
Signature		Date	11th November 2021

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor David Macdonald		
Supervisor comments The candidate took the intellectual lead, and contributed very substantially to this research, which was primarily dependent upon her role.		
Signature	Date	12th November 2021

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 4: Stress-related herpesvirus reactivation in badgers can result in Clostridium proliferation

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Abstract

Clostridium perfringens is an important food-borne zoonotic pathogen, and a member of the commensal gut microbiome of many mammals. Predisposing factors such as coinfection with other pathogens or diet change can, however, cause overgrowth and subsequent disease development. Here we investigated the occurrence of *C. perfringens* in a free-ranging badger population with up to 100% prevalence of herpesvirus infection. Herpesvirus reactivation is known to be associated with increased susceptibility to bacterial infections. PCR screening of rectal swabs from 69 free-ranging badgers revealed 15.9% (11/69, 95%CI=9.1% - 26.3%) prevalence of detectable *C. perfringens* (Type A) DNA in the digestive tracts of assymptomatic animals. The results of Fisher's exact test revealed *C. perfringens* detection was not biased by age, sex and seasons. However, badgers with genital tract gammaherpesvirus (MusGHV-1) reactivation (p=0.007) and infection with a specific MusGHV-1 variant (p=0.019) were more prone to *C. perfringens* proliferation, indicating coinfection biased dynamics of intestinal *C. perfringens*. An inclusion pattern analysis further indicated that, causally, MusGHV-1 reactivation potentiated *C. perfringens* detection. Whether specific MusGHV-1 variantinfection or reactivation plays a role in *C. perfringens* overgrowth or disease development in badgers will require further investigation. Nevertheless, a postmortem examination of a single badger that died of fatal disease, likely associated with *C. perfringens*, revealed MusGHV-1 detection in the small intestine.

Introduction

Most vertebrates test positive for one or several herpesvirus species (Shrawat et al. 2018) because the host immune system is unable to eradicate the virus from the body after primary infection. Instead, herpesviruses undergo a period of latency inside host cells but can reactivate when the host immune system is weakened (e.g., due to stress: (Baumworcel et al. 2019; Seeber et al. 2018) or coinfection with other pathogens: (Dai, Barrett, et al. 2020)): For example, the two human gammaherpesvirus species, the Epstein-Barr virus (EBV, causing mononucleosis) and the Kaposi's sarcoma-associated herpesvirus (KSHV), establish latency in lymphocytes after primary infection (Barton et al. 2011; Johnson and Tarakanova 2020; Liang et al. 2009; Young et al. 2016) and shed infectious virions into the oral cavity or genital tract when reactivated. The Equine gammaherpesviruses 2 and 5 (EHV-2 and EHV-5) are associated with abortion in mares, although the causal relationship remains unclear (Galosi et al. 2005; Marenzoni et al. 2013). In wild carnivores, gammaherpesviruses also produce pervasive, lifelong infection (e.g., felids: (Lozano et al. 2015; Tateno et al. 2017), musteloids: (King et al. 2004; Nicolas de Francisco et al. 2020), and ursids: (Black et al. 2019)). Repetitive reactivation, or active virus replication and shedding, happens asymptomatically but can occasionally cause recrudescent symptoms. These include pruritic (mucocutaneous) lesions or ulcers in the skin or genitalia, as well as neoplasms (Abade dos Santos et al. 2020; Gagnon et al. 2011; Nicolas de Francisco et al. 2020; Tsai et al. 2020, or Chapter 2; Tseng et al. 2013). Although infections generally remain sub-patent, clinically severe disease can occur when host immunity is compromised due to senescence, stress responses, coinfections or neoplastic disease conditions (Sehrawat et al. 2018). One such co-morbidity factor is the proliferation of *Clostridium perfringens*, as reported in Asian elephant calves (*Elephas maximus*) diagnosed with fatal Elephant endotheliotropic herpesvirus (EEHV) infection (Boonsri et al. 2018), and dairy cows (Frazier et al. 2002) and other captive artiodactyls (Flach et al. 2002) with Bovine Herpesvirus-4 (BHV-4) infection.

C. perfringens is a spore-forming gram-positive bacterium and a common member of mammals, birds, and reptiles' commensal gut microbiome (Lyhs et al., 2013; Meng et al., 2017; Milton et al., 2017; Ramos et al., 2019; Razmyar, Kalidari, Tolooe, Rad, & Movassaghi, 2014). However, in some cases, it can result in pathogenic zoonotic infections (Van Immerseel et al. 2004; Silva and Lobato 2015; Weese and Staempfli 2000). Eating foods, particularly undercooked meat contaminated with *C. perfringens*, is a common source of food poisoning due to the bacterium's ability to tolerate extreme high and low temperatures (Li and McClane 2006) and aerobic conditions (Briolat and Reysset 2002). If dysbiosis occurs and *C. perfringens* proliferates in the small intestine, watery diarrhea and gastroenteritis can develop into necrotic enteritis. It can also cause emphysematous cholecystitis in the gallbladder and fulminate gas gangrene (also

known as clostridial myonecrosis, caused by α-toxins) in humans and other animals (Kiu and Hall 2018; Miyahara et al. 2013). Symptoms of *C. perfringens* food poisoning may include nausea, vomiting, abdominal pain, and fever. Symptoms usually develop within 8-12 hours but can take up to 24 hours from ingestion. In extremis, *C. perfringens* can prove fatal in domestic animals and wildlife (Silva and Lobato 2015), although in human food poisoning cases it usually self-resolves within 24 hours (Kiu and Hall 2018). The major virulence factor of *C. perfringens* arises from the secretion of various enterotoxins, which are used to classify the strain as Type A, B, C, D, E, F and G; (Kiu and Hall 2018). The key enterotoxin of type A strain, also called the α-toxin, causes hemolysis of erythrocytes (Sakurai, Nagahama, and Oda 2004), cell death, necrosis (Navarro, McClane, and Uzal 2018) and disintegration of tight junctions between epithelial cells in the gut (Morris et al. 2012). Infections will respond to various antibiotic treatments, where cindamycin, metronidazole, rifampin and tetracycline are more efficacious than penicillin.

C. perfringens has frequently been isolated from the feces of healthy wild animal species in captivity or the field. Severe patho-morbidity is rare, although mortality due to necrotic enteritis has been reported in captive wild mammals and birds, and - more rarely - also in the field (Asaoka et al. 2004; Butler et al. 2008; Gartrell et al. 2017; Silva and Lobato 2015). A three-year study by Vierheilig et al. (2013) revealed higher prevalence and abundance of *C. perfringens* in the feces of Carnivora than of ruminant wildlife species; another study by Cox et al. (2005) reported similar findings. These results suggest diet plays an important role in the epidemiology of *C. perfringens* (Silva et al. 2014), and secondly, that carnivores (such as feral dogs, cats, fish-eating avian species, foxes or badgers), omnivores (wild boar or domestic chickens) or scavengers (vultures) (Meng et al. 2017) may be significant sources of environmental *C. perfringens* contamination. It is thus essential to monitor and identify risk factors associated with high prevalence/ prolific shedding of *C. perfringens* in animal feces at the human-livestock-wildlife interface, in order to establish and track transmission routes, as well as contamination levels in food or water sources (Van Immerseel et al. 2004; Kiu and Hall 2018).

The *Mustelid gammaherpesvirus 1* (MusGHV-1) was first reported in 2002 (Banks et al. 2002) and confirmed to have an almost 100% prevalence in wild badger populations (King et al. 2004; Sin et al. 2014). MusGHV-1 infection is typically asymptomatic but results in a high occurrence of viral shedding in the genital tract (Kent et al. 2018). Genital MusGHV-1 reactivation in adults is linked to stressors (Tsai et al. 2021, or Chapter 3) and associated with impaired female reproductive capacity (Tsai et al. 2020, or Chapter 2). Although gammaherpesvirus reactivation generally involves no or only mild disease, it has been identified in other species as a predisposing factor for secondary bacterial infection or cancer development (Nordengrahn et al. 1996).

As part of an ongoing investigation into the causes and consequences of MusGHV-1 reactivation in the European badger, *Meles meles* (hereafter 'badger') (Tsai et al. 2021, or Chapter 3), here we examined demographic traits, seasons and coinfection with different variant of MusGHV-1 to identify risk factors associated with intestinal *C. perfringens* proliferation. We also report the results of a badger necropsy, where *C. perfringens* was abundant in the ileum, in Appendix III.

Materials and methods

To assess the background prevalence of *C. perfringens* in the study population, we tested 69 rectal swabs collected from 50 badgers sampled in 3 seasons (spring, summer and autumn) in 2018 (for detailed trapping and sampling methods, see Tsai et al., 2021, Chapter 3). None of these sampled animals exhibited any clinical sign indicative of *C. perfringens* infection at the time of sampling. DNA from the swabs was extracted and purified based on the method described in Tsai et al (2020) (or Chapter 2) and the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instruction. Purified DNA samples were screened using *C. perfringens* toxin genes specific multiplex PCR (Baums et al. 2004) (Table 1). We also used a MusGHV-1 specific PCR (King et al. 2004; Tsai et al. 2020, or Chapter 2) targeting the DNA polymerase gene to detect MusGHV-1 DNA from the same rectal and genital swab samples. Based on substitutions in the partial MusGHV-1 DNA polymerase gene, two different MusGHV-1 variants circulate in our study population (Tsai et al. 2021, or Chapter 3). We determined the MusGHV-1 variant for each individual with Sanger sequencing results of successfully amplified PCR products from genital swab samples (Tsai et al. 2021 or Chapter 3).

We used Fisher's exact test to identify the association between *C. perfringens* presence in rectal swabs with sampling seasons, demographic traits, including sex and age group (juvenile: <2 years old; young adults: 2≤ x <5 years old; old adults: 5≤ x <8 years old; very old adults: ≥ 8 years old), MusGHV-1 presence in rectal/ genital swabs and MusGHV-1 variants. We further applied an inclusion pattern analysis to explore the causal relationship between detection of MusGHV-1 and *C. perfringens*. We used the output derived from the equation $\frac{(b-c)^2}{(b+c)}$, which applied values taken from the diagonal of the *b* and *c* quadrants of the 2x2 table in Figure 1A. This output was then compared with the critical χ^2 value at 1 degree of freedom with p-values of 0.05 and 0.001. An output higher than these critical χ^2 values indicated a significant imbalanced diagonal quadrant. This would evidence that detection of either pathogen was not equally dependent and that one caused a predisposition to infection with the other; that is, when b is greater than c (or when c is greater than b), then infection of pathogen *x* is highly likely to predispose infection with pathogen *y* (or y is highly likely to predispose for x) (Cavali-Sforza and Bodmer 1972).

Results

From the *C. perfringens* toxin genes specific multiplex PCR results, in apparently asymptomatic animals the prevalence of *C. perfringens* was 15.9% (11/69, 95%CI=9.1% - 26.3%), with all positive instances classified as type A.

We found no overall association between rectal swab MusGHV-1 and *C. perfringens* detection rate (Fisher's exact test: p=1), nor any effect of season, sex, or age (Table 2). However, we identified a strong positive correlation between genital MusGHV-1 detection and intestinal *C. perfringens* detection (Pearson's r value= 0.31, Fisher's exact test p-value=0.019). We also identified an imbalance of observations in the opposite diagonal of the inclusion correlation matrix (Figure 1, provides a 2x2 matrix of MusGHV-1 DNA detected in genital swabs (x) and *C. perfringens* DNA detected in rectal swabs (y), collected concurrently from the same individual, where a=9, b=23, c=2, d=34, Figure 1B). This inclusion pattern analysis gave a χ^2 of 17.64, which was much larger than the critical χ^2 value at $p > 0.05$ (3.84, with1 degree of freedom) or at $p > 0.001$ (10.83, with1 degree of freedom). This indicated that *C. perfringens* detection was a consequence of MusGHV-1 reactivation. Thus, when *C. perfringens* was detected in the gut, MusGHV-1 was almost always reactivated; but when MusGHV-1 was reactivated, *C. perfringens* was not always detectable. Consequently MusGHV-1 reactivation appeared to act as a predisposing factor for *C. perfringens* proliferation in badger intestines.

Upon testing for any correlation between *C. perfringens* detection with either variant, we found that *C. perfringens* occurrence was significantly more likely with the MusGHV-1 novel variant, with a prevalence of 41.2% (7/17), compared to only 9.1% (4/44) in badgers infected with the MusGHV-1 common variant (Fisher's exact test: p= 0.007).

Discussion

Our study demonstrates that *C. perfringens* detection was highly correlated with MusGHV-1 reactivation in the badgers we sampled. Furthermore, co-occurrence with *C. perfringens* was more likely to occur among individuals infected with the MusGHV-1 novel variant, which is recently found to cause higher relative blood viral load (see Chapter 5). This shows that different individuals within the same population can have markedly different pathogen profiles and risks of disease pathogenesis. Although we did not investigate the directionality of causation empirically (whether the bacterium caused viral reactivation or viral reactivation compromised

immunity, allowing bacterial proliferation to detectable levels), the inclusion pattern we found, plus C. perfringes positive cases were more likely in badgers infected with higher virulent MusGHV-1 variant (Figure 1B) suggested that MusGHV-1 reactivation promotes *C. perfringens* overgrowth.

From postmortem results (see Appendix III), we found that *C. perfringens* can proliferate in the badgers' ileum. However, given the duration over which the cadaver had been decomposing, it was impossible to determine whether necro-hemorrhagic enteritis was ante- or postmortem, and thus whether *C. perfringens* infection contributed to the cause of death of this individual, or whether this was solely autolysis leading to putrefaction. Diagnosis of the pathophysiology arising from *C. perfringens* is very challenging because this pathogen is frequently present in the environment and often in the gut microbiome of healthy animals. Consequently, the differential elimination of competing pathogens from the diagnosis, combined with the application of multiple stands of symptomatic and clinical evidence, is required. Notably, however, at the final examination of this same individual in November 2019 (i.e., about 15 weeks before its death), as part of our long-term trapping and monitoring program (see Macdonald et al. 2015), the rectal swab we collected tested negative for *C. perfringens.* This suggests that the infection levels were either too low to be detected via conventional PCR or that the animal subsequently contracted this pathogen, perhaps fatally. However, the MusGHV-1 PCR sequencing product amplified from postmortem gut samples confirmed this individual carried the common variant of MusGHV-1, which suggests that *C. perfringens* disease development may not be associated with the specific reactivated MusGHV-1 strain, despite being one of the risk factors for *C. perfringens* proliferation.

Only one previous report has detected of *C. perfringens* infection in a European badger. The bacterium was isolated from the lung and spleen of a dead individual in Italy (Di Sabatino et al. 2016). The cause of death was diagnosed as canine distemper virus infection, with no discussion of any pathogenic role of *C. perfringens* (Di Sabatino et al. 2016). Pathologic infections with *C. perfringens* have been reported for other members of the Family Mustelidae, such as captive mink (*Mustela (*now *Neogale) vison*) (Macarie et al. 1980) and among captive breeding colonies of the highly endangered black-footed ferrets (*Mustela nigripes*) (Schulman, Montali, and Hauer 1993). Wild Sea otters (*Enhydra lutris nereis*) are also susceptible to *C. perfringens,* where prevalence rates are 7.3 times higher among necropsied sea otters than live-sampled individuals (Miller et al. 2010). In captivity, several Carnivora species have been reported suffering from disease or mortality associated with *C. perfringens* type A. These include a group of cheetahs (*Acinonyx jubatus*) infected with *C. perfringens* enterotoxin (cpe), which recovered after treatment (Citino 1995); 1 African lion (*Panthera leo*) and 1 Amur tiger (*Panthera tigris altaica*) with fatal consequences in a zoo (Zhang, Hou, and Ma 2012); and 2 Amur leopards (*Panthera*

pardus orientalis) although no *C. perfringens* genotyping was done in this case to confirm the diagnosis (Neiffer 2001).

Ours is the first report of any link between MusGHV-1 and *C. perfringens* occurrence, although this co-stressor of immunity is similar to other examples of coinfections with *C. perfringens* and other pathogens that can damage intestinal mucosa. Examples include CPV in dogs (R. O. S. Silva et al. 2017), coccidia in chickens (*Gallus gallus domesticus*) (Collier et al. 2008), nematodes in Hamadryas baboons (*Papio hamadryas*) (Nikolaou et al. 2009) and turkeys (*Meleagris gallopavo*)(Norton et al. 1992). Compromised host immunity generally is a risk factor, as infection with *C*. *perfringens* diminishes mature neutrophils in bone marrow, leading to lowered replenishment of mature neutrophils in the peripheral circulation, resulting in an innate immune deficiency of the host (van Lieshout et al. 2020; Takehara et al. 2016). Indeed, any stressful conditions causing an adrenocortical response (Herman et al. 2016), such as high-density stocking in poultry (Tsiouris et al. 2015) and disruption of gut microbiome composition (Zaytsoff, Uwiera, and Douglas Inglis 2020), pose potential risks. Other causes of *C. perfringens* enteritis include food poisoning (Grass et al., 2013) or the ingestion of feces infected with abundant *C. perfringens* cells; for example, from scavenging carcasses. In addition, a change to a high protein (Zentek et al. 2003) or high carbohydrate (Allison et al. 1975; Butler et al. 2008) diet can also predispose the gut to *C. perfringens* overgrowth.

Several studies report that gammaherpesvirus reactivation is associated with coinfection with other pathogens; for instance, EHV-2 was confirmed experimentally as a predisposing factor for *Rhodococcus equi* pneumonia in foals (Nordengrahn et al. 1996). Furthermore, more than 80% of infertile cows that tested positive for Bovine herpesvirus 4 also tested positive in pathogenic bacterial and/ or fungal culture results, including for *C. perfringens* as one of the primary species (Chastant-Maillard 2015). In humans, a series of studies examining KSHV revealed that the pathogen-associated molecular patterns (PAMPs) produced by *Staphylococcus aureus* can promote virus entry, latency establishment and reactivation in the oral cavity of HIV-positive patients (Dai et al. 2014; Dai, Qiao, et al. 2020). In addition, several recent studies have applied high throughput sequencing to search for microbiome signals to indicate EBV and KSHV reactivation (Gruffaz et al. 2020; Urbaniak et al. 2020). Experimentally, Murid herpesvirus 4 primary infection has been established to sensitize mice to abortion induced by bacterial PAMPs, even at low doses (Cardenas et al. 2011). The above examples thus demonstrate that herpesvirus reactivation/ viral shedding interacts closely with host microbiomes. Our results here imply a similar relationship between MusGHV-1 and *C. perfringens* in badgers, and stress-related MusGHV-1 reactivation may enhance shedding of this zoonotic bacterium into the environment. Generally, further investigation into disease causality and development is warranted to better understand the underlying mechanisms and consequences of infection. This will be informative

for zoonotic disease management using the One Health approach in the interface between humans, domestic animals and wildlife.

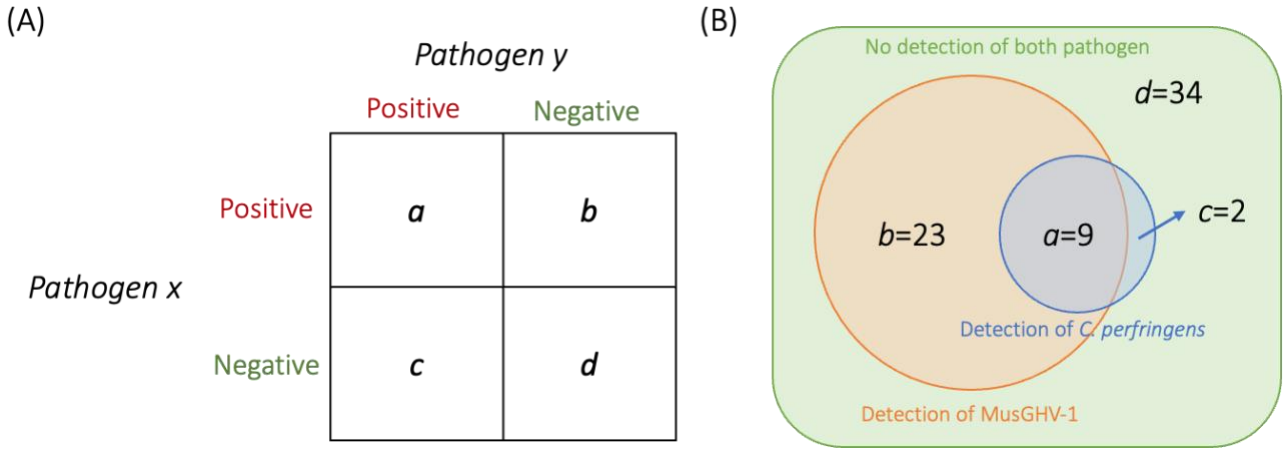

Figure 1: (A) A 2x2 table used for testing unequalness of observations (a= counts for individuals with positive detection of both pathogen x and y; b= counts for individuals with positive detection of pathogen x but negative for pathogen y; c= counts for individuals with negative detection of pathogen x but positive detection of pathogen y; d= counts for individuals with negative detection of both pathogen x and y). (Cavali-Sforza and Bodmer 1972). (B) Illustration of detection events of genital MusGHV-1 and *C. perfringens* of the sampled individuals (n=68), where *C. perfringens* detection in gut is likely to be a consequence event of MusGHV-1 detection in genital tract.

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	Stress-related herpesvirus reactivation in badgers can result in Clostridium proliferation
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Student Confirmation

Student Name:	Ming-shan Tsai		
Contribution to the Paper	Performed field works, sample collection, lab experiments, data analysis, interpretation of data and manuscript writing.		
Signature	Ming-shan Tsai	Date	11th November 2021

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor David Macdonald		
Supervisor comments The candidate took the intellectual lead, and contributed very substantially to this research, which was primarily dependent upon her role.		
Signature	Date	12th November 2021

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 5: Infection with a newly discovered novel gammaherpesvirus variant in European badgers, *Meles meles*, is associated with higher relative viral load in blood

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Abstract

Herpesviridae are a ubiquitous family of pathogens infecting a variety of animals from mollusks to mammals. Although virulence has coevolved over millennia in competition against host immunity resistance, occasionally herpes virus variants emerge with enhanced transmission or virulence capability, which can lead to higher disease burdens in host populations. *Mustelid gammaherpesvirus 1* (MusGHV-1) is the only herpesvirus so far identified in European badgers. No MusGHV-1 associated pathomorbidity has been diagnosed in badgers, however reactivation of MusGHV-1 in genital tract is linked to poorer female reproductive performance. A previous study in a free-ranging badger population in southern England identified both a common and novel variant through analysis of a short sequence from the DNA polymerase (DNApol) gene, which is a highly conserved gene across different herpesvirus species. Here we used samples collected from 66 known badgers to identify genetic and phenotypic differences (relative blood viral load) between these 2 MusGHV-1 variants. We compared genetic variance using a partial sequence of the DNApol gene that comprised a total sequence length of 2,874 nucleotides, among which we identified 15 nucleotide and 5 amino acid differences in the DNA polymerase domain. The relative prevalence was 86.4% (59/66) for the common variant and 16.6% (11/66) for the novel variant, with 6.1% (4/66) coinfected. MusGHV-1 variants were distributed unevenly across the population, where individuals infected with the novel variant were clustered in three (out of 25) contiguous social groups. Mixed effect linear regression analysis revealed that individuals infected with the novel variant had significantly higher MusGHV-1 relative viral load (measured through Ct values) in their blood ($p=0.002$) after adjusting for age (juveniles > adults, $p<0.001$) and season (summer>spring and autumn, $p=0.005$), likely indicating its higher virulence. The evolutionary origin of either variant is unknown but may have arisen from intra-host speciation. Further genome-wide analysis of MusGHV-1 host resistance genes and host phenotypic variations are required to clarify the drivers and sequelae of the new variants in this population.

Introduction

According to Jones et al (2008), 60% of emerging infectious diseases are of animal origin, predominantly (72%) from wildlife. Under the concept of 'One Health' (a framework recognizing the interconnection between human, animal and environmental health), emerging pathogen strain monitoring and pathotype identification are considered important for disease surveillance and prevention, not only in humans and domestic animals, but particularly also in wildlife (Xiao et al. 2021). Due to a lack of fidelity during replication, viruses have a higher mutation rate compared to cellular organisms. This results in higher evolutionary plasticity, which favors viral adaptation to changing environments (i.e., novel hosts, new host immune strategies, different medical treatments, etc.) and the emergence of new strains (Biek et al. 2015; Duffy, Shackelton, and Holmes 2008). The resulting arms race between pathogen virulence and host resistance continually shapes the variants of each through repeated cycles of adaptation and counter-adaptation; a process formalized under the Red Queen hypothesis (RQH) (Van Valen 1977). When applied to host-pathogen coevolution the RQH predicts that, under natural conditions, changes of host allele frequencies within a population cause pathogens to adjust allele frequencies to match with those of the host, and vice versa (Carius, Little, and Ebert 2001; Lively and Dybdahl 2000; Salathé, Kouyos, and Bonhoeffer 2008). This can drive intra-host speciation (i.e., several closely related virus species can evolve independently within the same host species) (Davison 2002; Escalera et al. 2016). Recently, this so-called 'duplication' has been found to be more frequent than expected in the evolution of herpesviruses (was believed to be dominated by co-speciation with their host species), especially among the gammaherpesvirinae (Brito et al. 2021).

European badgers, *Meles meles,* are promiscuous, polygynandrous mustelids (Carpenter et al. 2005). Their social system is highly flexible (Johnson et al. 2000), ranging from solitary or pair-living (Woodroffe and Macdonald 1993) to the formation of extensive family groups comprising upwards of 30 individuals (Macdonald and Newman, 2006). *Mustelid gammaherpesvirus 1* (MusGHV-1) is the only gammaherpesvirus species known to infect badgers (Banks et al. 2002). Like other gammaherpesviruses, after primary infection, MusGHV-1 may establish latency in white blood cells (Barton et al. 2011). Screening badger blood samples has revealed a 98-100% prevalence of MusGHV-1 in wild British and Irish populations (King et al. 2004; Sin et al. 2014). Reactivation from latency causes viral replication and shedding in mucosa in oral, nasal and/ or genital tracts. Depending on season and population (Tsai et al. 2021, or Chapter 3), prevalence of MusGHV-1 genital shedding ranges from 55% to 62% (Kent et al. 2018; Tsai et al. 2020, or Chapter 2). No specific signs or pathomorbidity have been linked to MusGHV-1 reactivation in badgers, although viral shedding is associated with lower female reproductive success and

lesions in the genital tract of both sexes (Tsai et al. 2020, or Chapter 2). In our previous analysis of partial DNA polymerase (DNApol) gene sequences (Tsai et al. 2021, or Chapter 3) we identified two distinct variants through genotyping of MusGHV-1 in a single closed badger population in Wytham Woods, Oxfordshire (for details of study population see Buesching et al., 2010 (Buesching et al. 2011)).

The DNApol gene codes for exonuclease and polymerase proteins that are essential for virus replication and genome proofreading and can therefore be used to determine virulence (Dourmishev et al. 2003). The DNApol gene is thus one of the most conserved genes, and likely to be under strong purifying selection with a low substitution rate. Nevertheless, any mutations found in these domains are typically associated with lower replication fidelity (see review: (Trimpert and Osterrieder 2019)), which in turn can contribute to higher mutation rates. If these mutations are beneficial to virus replication efficiency, they can in turn result in faster evolution rates (Tian et al. 2009). Consequently, herpesvirus strains that show differences in their DNApol genes can have different disease expression and outcomes. For example, although the exact mechanisms are as yet unclear, a single nucleotide difference in the equine herpesvirus 1 (EHV-1) polymerase gene on amino acid position 752 (N to D) is associated with neurological disease outbreaks (Lunn et al. 2009; Nugent et al. 2006; Stasiak et al. 2015), and higher viremia levels in infected horses compared to those infected with the EHV-1 N_{752} strain, suggesting a higher virus replication efficiency (Goodman et al. 2007). Differences in host susceptibility to the two strains has not yet been explored, but simultaneous infections with both strains have been reported (Pusterla et al. 2009; Pusterla, Mapes, and Wilson 2010).

Here we investigated genetic, epidemiological and phenotypic differences between these two previously reported (Tsai et al. 2021, or Chapter 3) MusGHV-1 variants in a natural host population. Specifically, we aimed to:

1) acquire longer sequences of the MusGHV-1 DNA polymerase gene in order to investigate for additional mutations/ genetic differences between the 2 viral variants;
2) establish the relative prevalence of the two MusGHV-1 variants within the population, investigate the rate of coinfection, and whether potential differences in variant prevalence are associated with demographic (i.e., host sex and age) and/or socio-geographic (i.e., badger sett and social group) patterns;
3) analyze potential differences in viral loads in blood samples between individuals infected with each or both MusGHV-1 variants.

Materials and methods

Animal sampling

We captured free-ranging badgers in Wytham Woods, Oxfordshire, United Kingdom (51◦46'26"N, 1◦19'19"W; for details of study site see Savill et al. 2010; for details of badger trapping and handling protocols see Macdonald et al.(2009) and Tsai et al. (2021) (or Chapter 3) in spring (21st May to 2nd June), summer (3rd to 15th September) and autumn (13th to 24th November) 2018. Individual badgers were identified by their serial tattoo numbers, given at first capture (Macdonald and Newman 2002) as part of a long-term badger research programme (Macdonald et al. 2015). After sedation with ketamine hydrochloride (Sugianto, Buesching, et al. 2019), oral, nasal, genital and rectal swab samples were collected using wooden shafted cotton-tips (Dynarex). Blood samples (not exceeding 2ml) were collected by jugular venipuncture in heparin-coated tubes and stored immediately in -20 °C. All protocols and procedures were approved by the Oxford University's Zoology Department's Animal Welfare and Ethical Review body, and were conducted under the Animals (Scientific Procedures) Act, 1986 (PPL: 30/3379).

Genotyping of MusGHV-1 DNA

DNA was extracted and purified from swab samples using DNeasy Blood and Tissue Kits (Qiagen), and from 200µL whole blood samples using Monarch Genomic DNA Purification Kits (New England BioLabs Inc.) following manufacturers' instructions (for full methodological details see Tsai et al (2021) or Chapter 3). MusGHV-1 DNA was amplified from each sample by conventional PCR using primers (pol3-F and pol3-R in Table 1) specifically targeting the MusGHV-1 DNA polymerase gene. After checking amplification results on 1.5% agarose gels following electrophoresis, we sent 66 successfully amplified PCR products to Zoology Sequencing, University of Oxford, for genotyping using Sanger sequencing to confirm the identity of those amplicons produced. Variant allocation was determined according to nine substitutions across 691 nucleotides identified between the two previously reported MusGHV-1 sequences (accession numbers MT332100 and MT332101) in the same study population: at position 363 (C to T), 378 (T to C), 383 (A to G), 480 (T to C), 486 (T to C), 537 (A to G), 540 (G to A), 546 (G to A), 558 (C to A) (Tsai et al. 2021, or Chapter 3). Coinfection was reported if the aforementioned signals for both nucleotides were present in a given sequencing product.

MusGHV-1 DNA polymerase gene mapping and phylogenic analysis

One sample of each variant was chosen for extended DNApol gene genotyping using primer pairs designed specifically for this study (Table 1). The same PCR procedures and conditions were used as described above, and successfully amplified PCR products were also sent to the Zoology Sequencing, University of Oxford for Sanger sequencing. Sequences were then mapped to the

reference gene (DNA polymerase gene of MusGHV-1, Accession number: AF275657), assembled, translated to amino acids and aligned using the ClustalW method (Thompson et al. 1994) applying published GenBank gammaherpesvirus sequences (selected to share either complete or more than 1000 nucleotide basepairs of the DNA polymerase gene), using the software Unipro UGENE (version 37) (Okonechnikov et al. 2012). For each isolate pair a purifying selection test using the Nei-Gojobori method was employed to determine whether the number of nonsynonymous substitutions per nonsynonymous site (dNS) was fewer than the number of synonymous substitutions per synonymous site (dS) (Nei and Gojobori 1986). Phylogenic trees were constructed using the Neighbor-Joining method (Saitou and Nei 1987) with bootstrap tests (1,000 replicates) (Buczkowska et al. 2012) in MEGA X (version 10.1.7) (Kumar et al. 2018).

Geographic distribution of MusGHV-1 strains and social group-specific prevalence

The social group affiliation of each individual was determined using previously established residency rules (Annavi et al. 2014; Sugianto, Newman, et al. 2019), with social group ranges established through baitmarking surveys (Annavi et al. 2014; Macdonald et al. 2009). Social group-specific strain prevalence was calculated by dividing the number of total positive badgers by the total of tested badgers resident in each social group. All maps were drawn using QGIS (version 3.14.15).

Blood viral load quantification

We next selected blood samples from individuals with known MusGHV-1 variant infections. Some individuals (see Table 2) were sampled in more than one trapping season. We analyzed relative MusGHV-1 genome copy numbers in each sample using real-time PCR, performed using StepOnePlus PCR Systems (ABI) to obtain Ct values. We used primers designed by Sin et al (Sin et al. 2014) targeting specifically the MusGHV-1 DNA polymerase gene (forward: 5'-GGAGAGTGCTGACCGATGGA; reverse: 5'-AAAAGCCTGGAATTGGATCAATAA, product length:150 bp). We prepared 20µL of reaction mix containing 0.5mM of each primer, 10µL of Luna Universal qPCR Master Mix, 3µL of RNase-free water and 5 µL of DNA template to be run at 95°C for 60 seconds (initial denaturation), 45 cycles of 95°C (denaturation) for 15 seconds, and 60°C for 30 seconds (extension). ~~Another set of primers targeting badger MHC Class II DRB exon 2 (forward: 5'-CGTCCCCACAGGACATTTC; reverse: 5'-CTCGCCGCTGCACC, 248bp) (Sin, Dugdale, Newman, MacDonald, et al. 2012) was used to quantify the relative white blood cell volume. These results were used to adjust viral load calculations, since gammaherpesviruses in the blood stream are cell-associated, and titres can thus be biased by differences in white blood cells counts between samples.~~

Statistical analyses

All statistical analyses were performed using R studio (version 1.2.1335). Fisher's exact tests were used to test if infections of MusGHV-1 variant differed with sex, age group (adult: ≥2 year-old, and juvenile: <2 year-old) or social group (n=25). Differences of viral loads between variants were analysed using a mixed effect linear regression analysis with R package *lme4*. We used Ct values adjusted for host genome copies as the response variable and variants (common, coinfection and novel), age group and sampling seasons (spring, summer and autumn) as fixed effects. Tattoo ID was included as a random effect, as some individuals contributed more than one sample to the data set.

Results

Substitutions in the DNApol gene of the two MusGHV-1 variants

We sequenced 66 MusGHV-1 PCR products amplified from the partial DNApol gene. All sequences were trimmed to 694 base pairs and were confirmed to be MusGHV-1 according to the NCBI online blasting service, returning between 98.7% to 100% identity with 100% congruence with the published MusGHV-1 sequence isolated from a badger in Cornwall, England (Accession number: AF275657).

From extended genotyping of the DNApol gene we obtained a total sequence length of 2,874 nucleotides for each MusGHV-1 variant. This enabled us to test for additional variations between the two variants. In total, we found 15 nucleotides and five amino acid differences in the DNA polymerase domain, which we classified here as the 'common variant' and the 'novel variant', as the former was almost identical (99.9% nucleotide identity) to the reference sequence, while the novel variant only shared 99.4% similarity. Both MusGHV-1 variants had three nucleotides and two amino acids substitutions in consensus with the reference sequence, all located at the exonuclease domain (details of these substitutions are listed in Table 3 and Figure 5). There was support for strong purifying selection having occurred between the two variants in the Wytham badger population (dNS<dS, p-value=0.004, dS-dNS=2.66).

Among these 66 individuals the common variant was detected in 59 (89.4%), including in genital swabs (n=49), oral swabs (n=5), rectal swabs (n=2) and blood samples (n=5). The novel variant was detected in 11 (16.7%) individuals, including in genital swabs (n=9) and blood samples (n=5). Coinfection was confirmed in 4 individuals (6.1%) in 3 blood samples and 1 rectal swab (Table 5).

Phylogenetic relationships between the two MusGHV-1 variants and other gammaherpesvirus species

All Carnivora-infecting gammaherpesviruses are grouped in a monophyletic clade, including also the MusGHV-1 virus, which likely belongs to the genus *Percavirus* (Figure 1). Phylogenic analysis using the DNApol gene sequence showed that the novel and the common variant share a common ancestor, and the Cornish variant (AF275657) was derived from the common variant more recently, making the novel variant the ancestral variant.

Novel MusGHV-1 variant infections were clustered within just 3 social groups

MusGHV-1 variant frequencies did not differ between males and females (Fisher's exact test, p=0.19) or between age groups (p=0.19), but varied between social groups (p=0.052) (Table 7). The novel variant (and coinfection with both variants) was confined to badgers from three neighboring social groups, while the common strain was evenly distributed across the Woods (Figure 2). To investigate further, we screened 14 DNA samples from blood collected from badgers resident at these three social groups during 2009 and 2010. Two individuals were infected with novel variant, one was infected with both variants and the rest were infected with the common variant. This retrospective scrutiny indicated that the novel variant originated in the CHO social group before spreading to the CH and RC groups (Table 8).

MusGHV-1 novel variant was associated with higher viral load in the blood stream

Ct values from real-time PCR on 40 blood samples collected from 24 individuals at different sampling seasons (Table 2) showed that MusGHV-1 relative viral load was highest (i.e., low Ct value) among badger cubs (age class) then decreased after in yearling (age class 1) (Figure 3). Furthermore, juveniles had higher relative viral loads than adults ($p<0.001$), and samples taken in summer had higher loads than in spring or autumn (p=0.005) (Table 4). Mixed effect linear regression analysis results suggest that badgers infected with the novel variant generally had a higher MusGHV-1 viral load than did those infected with the common variant (p=0.002).

Discussion

Understanding epidemiological traits can aid interpretation of viral load data across wildlife species. Despite the relatively low sample size in this study, we observed clear epidemiological variations in viral loads. Higher relative viral loads in juveniles infected with either MusGHV-1 variant corroborated a previous study using animals from the same population (Sin et al. 2014), where primary herpes infection with a higher viral load typically occurs early in life (Bauer et al. 2005). Seasonally higher viral loads in summer correlated with higher prevalence of genital tract viral DNA reactivation (Tsai et al. 2021, or Chapter 3). Most intriguingly, we found relative blood viral load was also depending on the MusGHV-1 variant detected. This indicates the two MusGHV-1 variant found in Wytham have not only genotypic but also phenotypic difference.

Furthermore, previous findings show the novel variant, which is found with higher relative viral loads in this study, is associated with higher risk of infection with *Clostridium perfringens,* a zoonotic bacterial pathogen that can cause severe enteritis (Tsai et al. 2021 or Chapter 4).

Considering the generally low mutation rate of herpesviruses and their strong host co-speciation traits (McGeoch, Dolan, and Ralph 2000), the coexistence of two closely related gammaherpesvirus variants in the same population is unusual. Strain genetic diversity of herpesviruses found within a single host species is typically very low, even between host populations separated by geographical barriers. For example, the gammaherpesvirus (LcaGHV-1) found in two geographically distinct (from Maine and Newfoundland) Canadian lynx (*Lynx canadensis*) populations remains identical apart from just only two synonymous substitutions across the 3.4 kb nucleotide sequences of its partial gB protein and DNApol gene (Hendrikse et al. 2019). Molecular analysis of the DNApol gene of twelve EHV-2 strains circulating in horse herds in Iceland, isolated for more than 1000 years, still showed 99% to 100% similarity across a region of 483 base pairs to seven EHV-2 strains from other European countries (Thorsteinsdóttir et al. 2013). Furthermore, country-wide screening for MusGHV-1 in Irish badgers in 2019, found that sequences from 23 individuals were 100% identical at 691 base pairs to the reference MusGHV-1 DNApol sequence (Tsai et al. 2020, or Chapter 2); all of which demonstrates the stability of species-specific herpesvirus DNApol genes.

Determining whether either MusGHV-1 variant in the Wytham Woods population originated from introduction or evolution is challenging without genome-wide analysis of mutations and sequencing results from other badger populations across the UK and other nearby countries. Potentially, MusGHV-1 may have co-evolved with badgers from a different population (likely subject to greater isolation by distance than the Cornish and Irish populations, e.g. continental Europe (Marmi et al. 2006)), and then have been introduced into the Wytham population more recently, but at least prior to 2009 (Allen et al. 2020). However, there are no historic records of badgers either colonizing or being introduced into Wytham from other sub-populations, and badger genetic diversity in this population is low (Domingo-Roura et al. 2003); although any such introduced badger could, plausibly, have caused contagion with the novel variant and died without leaving any decedents. Ultimately, therefore, it seems unlikely that the novel variant originated from an ex-situ source.

Phylogenetic analysis revealed that the node from/at the Cornwall variant branched from the ancestral MusGHV-1 root occurred earlier than the node at which either Wytham population branched which separated and evolved independently from an earlier time point. Although the common variant is dominant in the Wytham and the Irish populations, this does not infer that the novel variant emerged (branched) later than the common variant. It is also possible that the

common variant became dominant by selectively outcompeting the novel variant, even if that novel strain actually branched earlier. For instance, if the higher virulence of the novel variant (rising from replicative advantage) caused higher host mortality, prior to some immune adaptation, or triggered a more robust host immune responses to eliminate the pathogen (Alizon et al. 2009), then a less-virulent variant (common) would spread among surviving badgers and become dominant. Some explanation based on balancing, or fluctuating selection between specific variant advantages (Abdul-Rahman, Tranchina, and Gresham 2021; Gibson, Xu, and Lively 2016), or among host immune alleles (Tollenaere et al. 2008), could then explain occasional co-infection, and why infections of the novel variants were limited in only few social groups, due to incomplete over-dominance.

The hypothesis that virulence maybe be a trade-off against transmission is debated (Acevedo et al. 2019; Alizon et al. 2009). Although higher viral load can be an indicator of more efficient replication (Acevedo et al. 2019), invertebrate models show that selection for an intermediate replication level can maximize pathogen fitness (De Roode, Yates, and Altizer 2008). This may prevent the novel variant being completely wiped out from the population. Although the high replication efficiency of the novel variant may result in it being outcompeted by the common variant due to host immune activities, this advantage may aid the novel variant to transmit and seed in the population through virus-shedding individuals. The life-long infection trait of herpesvirus may allow the novel variant to persist in a latency stage in the host at extremely low quantities, possibly beyond the detection limit of PCR most of the time.

An alternative, but not mutually exclusive explanation is that differences in host pathogen resistance determined by immune-related genes, such as MHC genes, may be a causal factor not only for new strain emergence but also for the coexistence of multiple strains within a population. In badgers, Sin *et al.* (2014) found that individuals with certain MHC variants had different blood viral loads of MusGHV-1. Similarly, in horses, EHV-1 induced downregulation of cell surface molecules expressed by MHC I is allele-dependent (Rappocciolo, Birch, and Ellis 2003), and the extent to which MHC I is downregulated is EHV-1 strain-dependent (Ma et al. 2012). In humans, herpesvirus infection has been linked to dementia (Itzhaki 2018), as some human leucocyte antigen (HLA; equivalent to MHC) alleles that protect against dementia have a high binding ability to a variety of human herpesvirus strains, suggesting that this link may be dependent on host variant (Charonis, James, and Georgopoulos 2020). Moreover, the subtypes of human herpesvirus 8 (HHV-8, or Kaposi's sarcoma-associated herpesvirus, KSHV) are clustered by ethnic group, and infection with different subtypes affects the risk of Kaposi's sarcoma occurring (Hulaniuk et al. 2020). However, Wytham's badgers engage extensively in extra-group mating, and thus should a specific MHC variant be more susceptibility to the novel variant reactivation, it should spread quickly across social groups; contrary to our observations

where the novel variant expanded from one to three social groups over nine badger cohorts (from 2009 to 2018). This would necessitate a recessive MHC gene circulating within these social groups only expressed through inbreeding (Annavi et al. 2014). Currently, we lack data to corroborate or refute this hypothesis.

In conclusion, much as the SARS-CoV-2 Delta variant is more virulent and contagious than other variants (Liu and Rocklöv 2021), so too here we illustrate that variant variation in MusGHV-1 results in different epidemiological and spatial patterns of infection in badgers. Only by better understanding viral genetics and pathotypes, and how these interact with co-evolving host immunity, can we accurately inform conservation management and initiatives such as One Health, tasked with disease surveillance and prevention (including the spread of zoonosis).

Table 1: Primers used for MusGHV-1 DNA polymerase gene PCR amplification

	Start position	End position	Primer sequence (5' - 3')	Product length
pol5-F	3	22	GGCAGGGAATTTTTATAACC	699
pol5-R	681	701	CCACCCAAAAGTAGAAAATCC	
pol6-F	345	364	CCCCTCTGGAACTGTGCTAA	637
pol6-R	962	981	AGGGTCACATGTCCCCAAAT	
pol7-F	863	846	ATGTCTGGGGGAAAATGG	486
pol7-R	1329	1348	GACCTCCTATGCACTGCTTG	
pol10-F	1185	1204	TGAAGTTCACACACCCCAGA	361
pol10-R	1524	1545	TCCATCGGTCAGCACTCTC	
pol3-F	1328	1347	CCAAGCAGTGCATAGGAGGT	771
pol3-R	2072	2098	TGGACTTCTCCAACATGCGTCGCCCTT	
pol11-F	1684	1703	CCGATCTTGGTGGTTGATTT	627
pol11-R	2291	2310	CTTAATTGGCTCCTCGAACA	
pol12-F	2015	2034	CAGGTGTGTCCTCGGGTATT	561
pol12-R	2556	2575	TCACTTTGAAAAGTGGAAGTGG	
pol9-F	2393	2412	TGATGAAGGGAGTGGATCTC	597
pol9-R	2936	2989	TCACAGCTTTGTCTGCACTG	

Table 2: Blood samples used for relative viral load analysis

Tattoo number	Sex	Age	Spring	Summer	Autumn
1012	Male	13		v	
1045x	Female	13	v		v
1232	Female	10	v		v
1234x	Female	10		v	v
1283	Male	9	v	v	
1330	Male	8		v	
1379	Male	>8*	v		v
1435	Female	6		v	v
1478	Female	5		v	
1487	Male	7	v		v
1520	Female	4	v	v	
1622	Male	3	v	v	
1694	Female	2		v	v
1699	Male	1		v	v
1735	Male	0	v		
1746	Male	0		v	
1749	Female	0	v		
1750	Female	0		v	v
1751	Female	0	v	v	v
1753	Female	0	v		
1754	Male	0	v		
1755	Male	0	v	v	v
1778	Male	0		v	
1785	Female	0			v

* Age not determined as this individual was firstly captured as an adult, it was estimated as older than 8 years old according to toothwear (Hancox 1988).

Table 3: Amino acid substitutions (nonsynonymous differences) between the two variants in the MusGHV-1 DNA polymerase gene

Position	Reference (AF275657d)	Common variant	Novel variant	Conserved protein domain family[a]	In conserved region[b]	In conserved region within host order[c]
253	P	L	L	3'-5' exonuclease Exo I	Yes	Yes
274	N	Y	Y	3'-5' exonuclease Exo I	Yes	Yes
591	K	K	R	Polymerase	No	No
649	N	N	K	Polymerase	No	Yes
819	E	E	G	Polymerase	No	No
872	T	T	N	Polymerase	No	No
889	S	S	L	Polymerase	No	Yes

a: Data from the NCBI Conserved Domain Database (CDD) (Lu et al. 2020)

b: Determined by whether the substitution is located in previously identified conserved motif of viral DNA polymerase genes (Knopf 1998)

c: Determined by whether the substitution is located in a position with a higher conservation degree within herpesvirus species confirmed in the order Carnivora (Figure 4)

d: GenBank accession number

Table 4: Results of Linear mixed model fit by REML. t-tests using Satterthwaite's method (formula= Ct ~ Variant + Age Group + Season + (1 | Tattoo)). Number of observations: 39, groups (by tattoo): 23

Variable	Estimate	Std. Error	df	t-value	Pr(>|t|)
(Intercept)	36.1298	0.5053	29.4718	71.495	
MusGHV-1 variant					
Common	(reference)				
Novel	-2.0821	0.588	20.6962	-3.541	**0.002**
Coinfection	-0.3549	0.9188	32.9006	-0.386	0.702
Age group					
Adult	(reference)				
Juvenile	-3.1531	0.5822	19.7504	-5.416	**<0.001**
Season					
Spring	(reference)				
Summer	-1.5584	0.5073	25.1267	-3.072	**0.005**
Autumn	-0.4116	0.4703	19.2672	-0.875	0.392

Table 5: List of samples with variantd MusGHV-1 DNA and host details of tattoo ID, sex, age, natal group, social group and sett visited (obtained from previous capture records (Macdonald et al. 2015; Sugianto et al. 2019))

Tattoo ID	Sex	Sample type	Variant	Age at 2018	Natal group	Social group	Sett visited
1012	M	Oral swab	Common	13	PO	PO	PO, SH
1232	F	Genital swab	Common	10	BB	MT	TR, MT, BB
1283	M	Genital swab	Common	9	JH	JH	JH
1341	M	Oral swab	Common	8	GOO	HC	GOO, GO, HC, CP, KH
1354	M	Genital swab	Common	10	PO	PO	PO, P, JkBr, SH
1359	F	Oral swab	Common	8	JKBR	JH	JKBR, JH, PV
1372	F	Genital swab	Common	8	PO	PO	PO, P, SH
1378	M	Genital swab	Common	8	JH	JH	JH, P
1379	M	Genital swab	Common	>8	CP	CP	CP, MT, Gates
1383	M	Genital swab	Common	8	GOO	GOO	GOO, GO
1445	F	Oral swab	Common	8	TC	TC	TC
1469	F	Rectal swab	Common	5	TC	TC	TC
1478	F	Oral swab	Common	5	JKBR	JH	JKBR, JH, CHO
1487	M	Genital swab, blood	Common	7	MT	JH	JH, MT, PV, M2
1504	M	Genital swab	Common	4	HH	HH	HH, GAH, CLO
1512	M	Genital swab	Common	4	GOO	RC	GOO, RC
1516	M	Genital swab	Common	?	NC	NC	NC
1520	F	Genital swab	Common	4	JH	JH	JHA, BB
1525	M	Genital swab	Common	4	P	P	P
1549	M	Genital swab	Common	4	CP	CP	CP, Gates
1584	M	Genital swab	Common	3	P	P	P
1592	M	Genital swab	Common	4	M2?	M2	M2, JH
1631	F	Genital swab	Common	3	MT	BB	BB, MT
1635	M	Genital swab	Common	3	CP?	TC	OL1, OLX, M2, HH, TC
1641	M	Genital swab	Common	2	CP	CP	CP, OL1
1642	F	Genital swab	Common	2	CH	CH	CH
1644	M	Genital swab	Common	2	GAH	CLO	CLO, GAH, HH
1647	M	Genital swab	Common	2	GAH	GAH	GAH, CLO
1668	M	Genital swab	Common	2	P	P	P, Hill End
1669	F	Genital swab	Common	2	GAH	FB	FB, GW
1678	F	Genital swab	Common	2	CH	CH	CH
1694	F	Genital swab	Common	2	LS	LS	LS
1696	F	Genital swab	Common	2	MT	MT	MT, TR, BB

1699	M	Genital swab	Common	1	JH	JH	JHA, JH
1713	M	Genital swab	Common	1	GOO	GOO	GOO
1720	M	Genital swab	Common	1	SH	SH	SH
1722	F	Genital swab	Common	1	GAH	GAH	GAH, FB, GW
1739	F	Genital swab	Common	0	CP	CP	CP
1742	F	Genital swab	Common	0	BL	BL	McBr
1744	M	Genital swab	Common	0	SW	SW	SW
1745	M	Genital swab	Common	0	SH	SH	SHO
1753	F	Genital swab, blood	Common	0	CH	CH	CH
1756	M	Genital swab	Common	0	FB	FB	FB, RC
1763	M	Genital swab	Common	0	BB	BB	BB
1776	F	Genital swab	Common	0	Hedge	Hedge	Hedge
1777	M	Genital swab	Common	2	FS Farm	FS Farm	OB
1778	M	Genital swab	Common	0	FS Farm	FS Farm	Road
1780	F	Genital swab	Common	0	P	P	P
1784	M	Genital swab	Common	0	GAH	GAH	Ingrid B
1785	F	Genital swab	Common	0	GAH	GAH	GAH, CLO
1787	M	Genital swab	Common	2	CHO	CHO	CHO
1793	M	Genital swab	Common	0	CHO	CHO	CHO
1234X	F	Genital swab	Common	10	BB	MT	TR, MT, BB, LS, JH
1239F	F	Genital swab	Common	10	PO	P	PO, P
1737X	M	Genital swab	Common	0	MM	MM	MM
1435	F	Genital swab, blood	Dual	6	CH	CH	CH, GAH
1498	F	Rectal swab	Dual	5	BL	CH	BL, CH
1751	F	Genital swab, blood	Dual	0	CH	CH	CH
1755	M	Genital swab, blood	Dual	0	CHO	CHO	CHO
1045X	F	Genital swab, blood	Dual	13	CHO	CHO	CHO, Ditch, GAH
1330	M	Genital swab	Novel	8	GOO	RC	GOO, GOA, RC, HC
1622	M	Genital swab	Novel	3	CP	RC	CP, RC,OL64, LS
1746	F	Genital swab	Novel	0	CH	CH	CH
1749	F	Genital swab	Novel	0	CHO	CHO	CHO
1750	F	Genital swab, blood	Novel	0	CH	CH	CH
1754	M	Blood	Novel	0	CH	RC	CH, RC, CC, HSBC

Table 6: Ct values of MusGHV-1 genome by variant in 40 blood samples from badgers of previously known identity (Tattoo ID) and age class

Tattoo ID	MusGHV-1 Variant (genital swab)	MusGHV-1 Variant (oral swab)	MusGHV-1 Variant (Blood)	Age	Age Group	Ct value		
						Spring	Summer	Autumn
1012		Common (spring)		13	Very old		32.56	
1045x	Novel (autumn)			13	Very old	34.30		33.44
1234x	Common (spring, summer)			10	Very old		32.20	34.27
1232	Common (autumn)			10	Very old	34.47		33.99
1283	Common (summer)			9	Very old	36.51	35.90	
1330	Novel (summer)			8	Very old		34.23	
1379	Common (spring)			Unknown	Very old	36.16		33.74
1487	Common (summer)			7	Old	34.97		35.99
1435	Novel (summer)		Dual (summer)	6	Old		31.98	32.36
1478		Common (summer)		5	Old		33.02	
1520	Common (spring)			4	Young	35.31	33.53	
1622	Novel (summer autumn)			3	Young	32.33	30.13	
1694	Common (summer)			2	Young		32.32	33.74
1699	Common (summer)			1	Juvenile		31.88	33.30
1735				0	Juvenile	36.30		
1746	Novel (summer)			0	Juvenile		28.67	
1749	Novel (spring)			0	Juvenile	27.10		
1750	Novel (summer)		Novel (summer)	0	Juvenile		28.74	29.99
1751	Novel (autumn)		Dual (summer)	0	Juvenile	30.77	30.12	30.94
1753	Common (spring)		Common (summer)	0	Juvenile	32.74		
1754			Novel (summer)	0	Juvenile	30.04		
1755	Novel (summer)		Dual (summer)	0	Juvenile	28.87	31.55	31.74
1778	Common (summer)			0	Juvenile		29.97	
1785	Common (summer)			0	Juvenile			30.51

Table 7: Counts of individuals infected with the common or novel MusGHV-1 variant categorized by sex, age class and social groups. There was no significant difference in novel variant prevalence based on sex or age class (Fisher's test: $p>0.05$); however, individuals infected with the MusGHV-1 novel variant all resided in just 3 (of 25) social groups (CH, CHO, RC), producing a marginally significant spatial pattern.

MusGHV-1 variant	Sex		Age group		Social group																								
	Male	Female	Juvenile	Adult	BB	BL	CH	CHO	CLO	CP	FB	Farm	GAH	GOO	HC	Hedge	HH	JH	LS	M2	MM	MT	NC	P	PO	RC	SH	SW	TC
Common	33	22	18	37	2	1	3	2	1	4	2	2	4	2	1	1	1	7	1	1	1	3	1	5	3	1	2	1	3
Novel	4	7	6	5	0	0	5	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
Prevalence of novel variant	11%	24%	25%	12%	0%	0%	63%	60%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	75%	0%	0%	0%
Fisher's test	p=0.1912		p=0.1885		p=0.0518																								

Table 8: List of blood samples tested for MusGHV-1 and variant

Badger ID	Sampling month	Social group	Sex	Age	MusGHV-1 Variant
1045x	Nov-09	CHO	Female	4	Novel+Common
1155	Jun-10	CHO	Female	3	Common
1168	Jun-10	CH	Female	3	Common
1273	Nov-09	CH	Female	0	Common
1332	Jun-10	CHO	Male	0	Common
1335	Jun-10	CHO	Female	0	Novel
1366	Sep-10	CHO	Male	0	Novel
1368	Sep-10	CHO	Female	0	Common
1115	Nov-09	RC	Female	3	Common
1133	Nov-09	RC	Male	2	Common
1165	Nov-09	RC	Male	2	Common
1292	Sep-09	RC	Female	0	Common
1346	Jun-10	RC	Male	0	Common
1342	Jun-10	RC	Female	0	Common

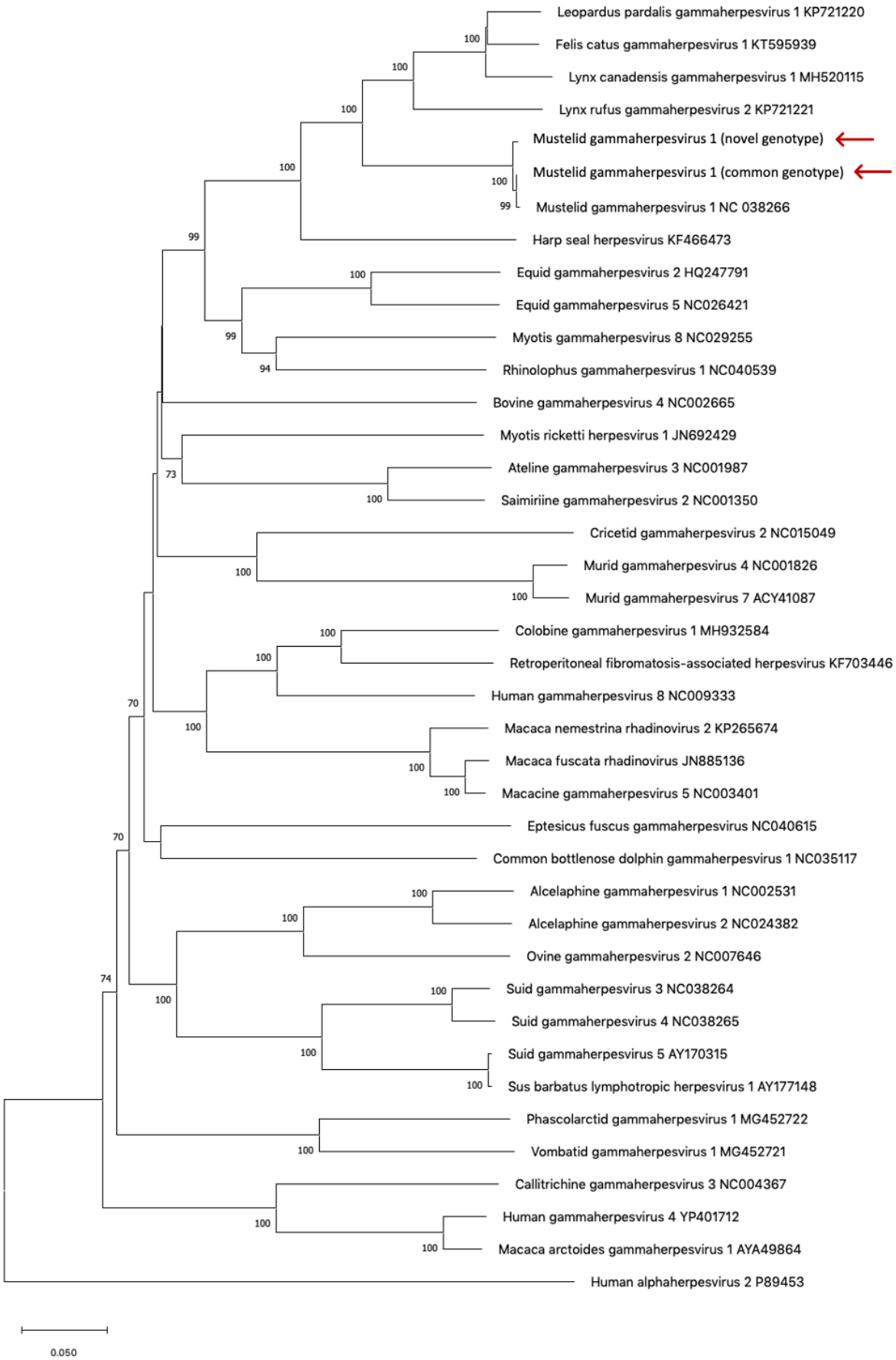

Leopardus pardalis gammaherpesvirus 1 KP721220
Felis catus gammaherpesvirus 1 KT595939
Lynx canadensis gammaherpesvirus 1 MH520115
Lynx rufus gammaherpesvirus 2 KP721221
Mustelid gammaherpesvirus 1 (novel genotype)
Mustelid gammaherpesvirus 1 (common genotype)
Mustelid gammaherpesvirus 1 NC 038266
Harp seal herpesvirus KF466473
Equid gammaherpesvirus 2 HQ247791
Equid gammaherpesvirus 5 NC026421
Myotis gammaherpesvirus 8 NC029255
Rhinolophus gammaherpesvirus 1 NC040539
Bovine gammaherpesvirus 4 NC002665
Myotis ricketti herpesvirus 1 JN692429
Ateline gammaherpesvirus 3 NC001987
Saimiriine gammaherpesvirus 2 NC001350
Cricetid gammaherpesvirus 2 NC015049
Murid gammaherpesvirus 4 NC001826
Murid gammaherpesvirus 7 ACY41087
Colobine gammaherpesvirus 1 MH932584
Retroperitoneal fibromatosis-associated herpesvirus KF703446
Human gammaherpesvirus 8 NC009333
Macaca nemestrina rhadinovirus 2 KP265674
Macaca fuscata rhadinovirus JN885136
Macacine gammaherpesvirus 5 NC003401
Eptesicus fuscus gammaherpesvirus NC040615
Common bottlenose dolphin gammaherpesvirus 1 NC035117
Alcelaphine gammaherpesvirus 1 NC002531
Alcelaphine gammaherpesvirus 2 NC024382
Ovine gammaherpesvirus 2 NC007646
Suid gammaherpesvirus 3 NC038264
Suid gammaherpesvirus 4 NC038265
Suid gammaherpesvirus 5 AY170315
Sus barbatus lymphotropic herpesvirus 1 AY177148
Phascolarctid gammaherpesvirus 1 MG452722
Vombatid gammaherpesvirus 1 MG452721
Callitrichine gammaherpesvirus 3 NC004367
Human gammaherpesvirus 4 YP401712
Macaca arctoides gammaherpesvirus 1 AYA49864
Human alphaherpesvirus 2 P89453
0.050

Figure 1: (A) Neighbour-joining phylogenetic tree of the partial DNA polymerase gene, including the two variants of the MusGHV-1 (red arrows) and 38 other gammaherpesviruses obtained from GenBank. The alignment of 1240 amino acids were produced using default parameters in ClustalW. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Branch support (1000 bootstrap replicates) is indicated at each node. (Felsenstein 1985). Scale bar corresponds to amino acid substitutions per site. Human alphaherpesvirus 2 is used as an outgroup. The tree was constructed using MEGA X (Kumar et al. 2018).

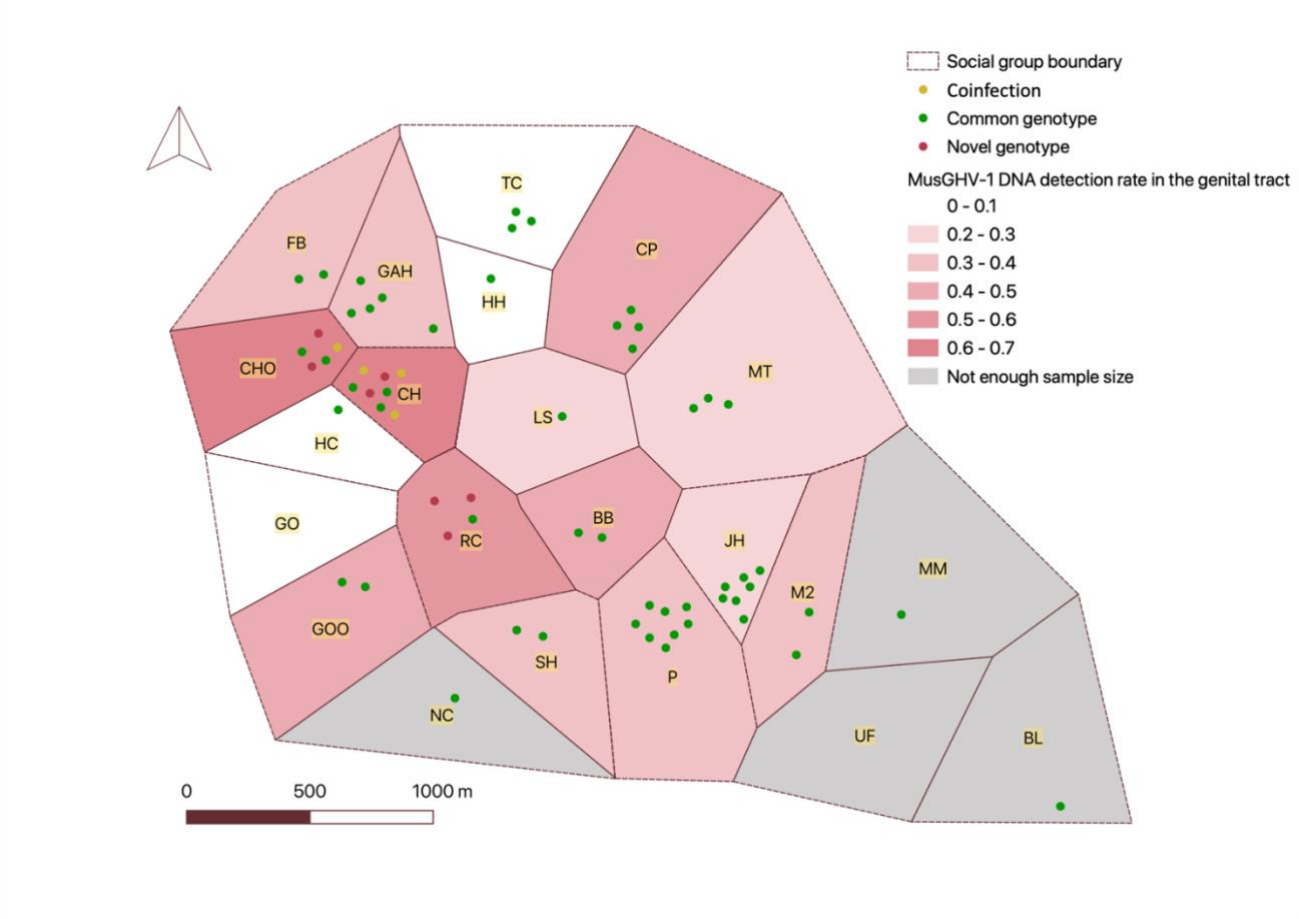


Figure 2: Map of genital tract MusGHV-1 occurrence and MusGHV-1 variants distribution in different badger social groups in Wytham Woods in 2018. The name of each social group is highlighted in yellow. The areas with gray colour indicate sample sizes of less than 3.

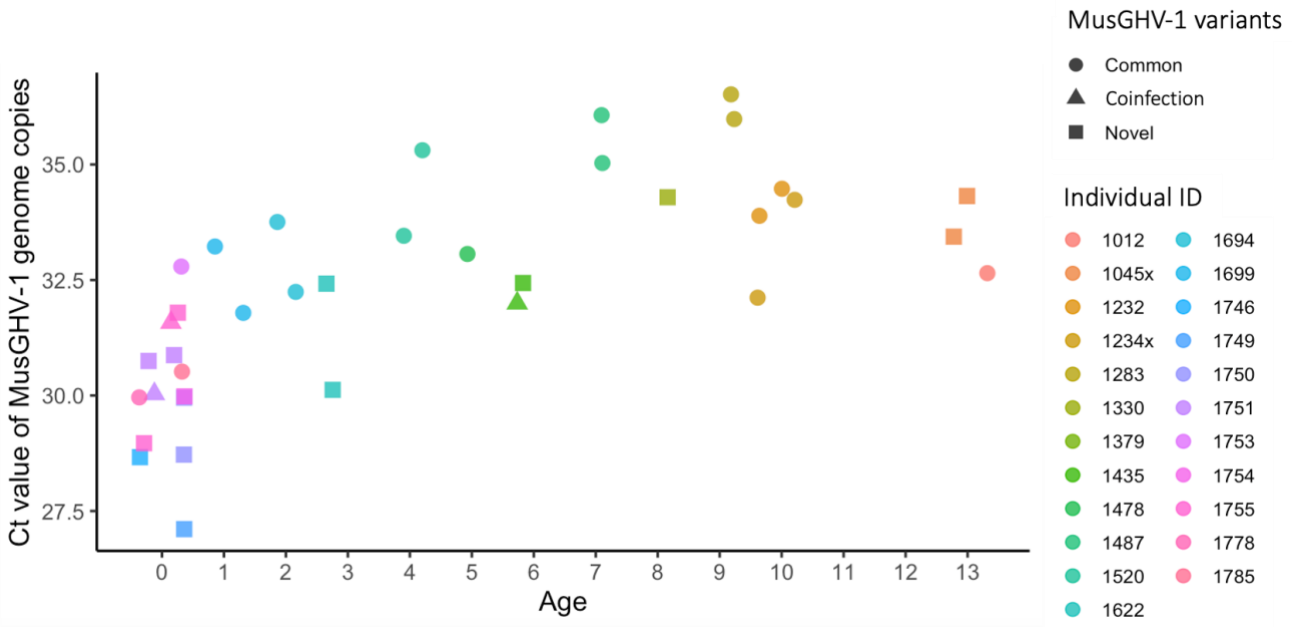


Figure 3: Ct values representing relative blood MusGHV-1 viral load between 39 blood samples collected from 23 individuals in different seasons (see Table 5 for details). The relative viral load was higher in cubs (less than 1 year old) than in other age classes.

Figure 4: Location of main badger setts (communal dens) with associated social group range, in Wytham Woods.

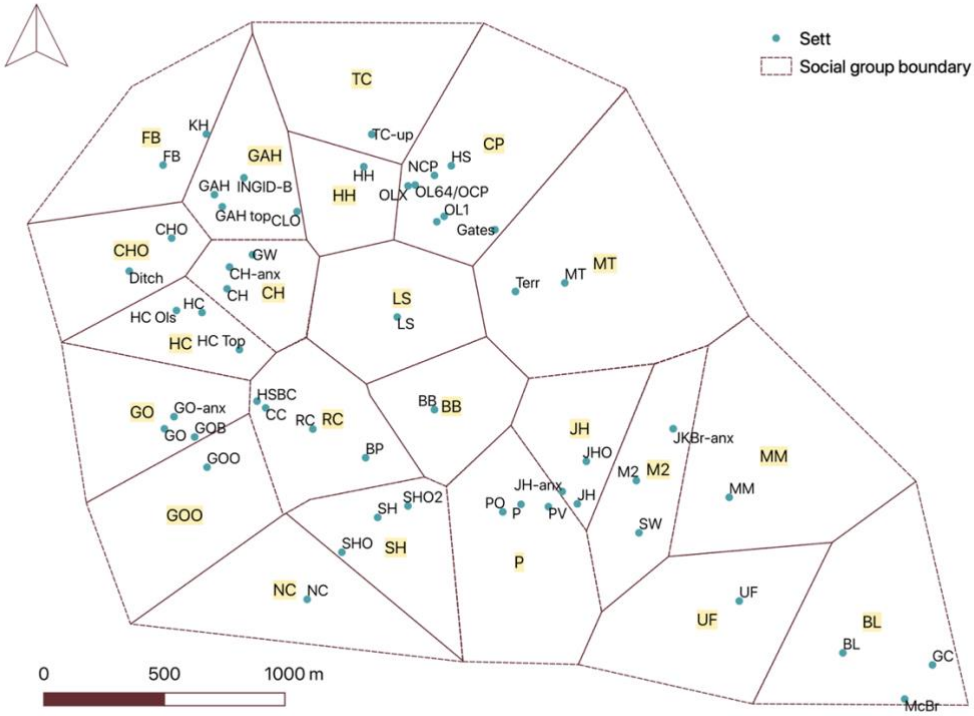
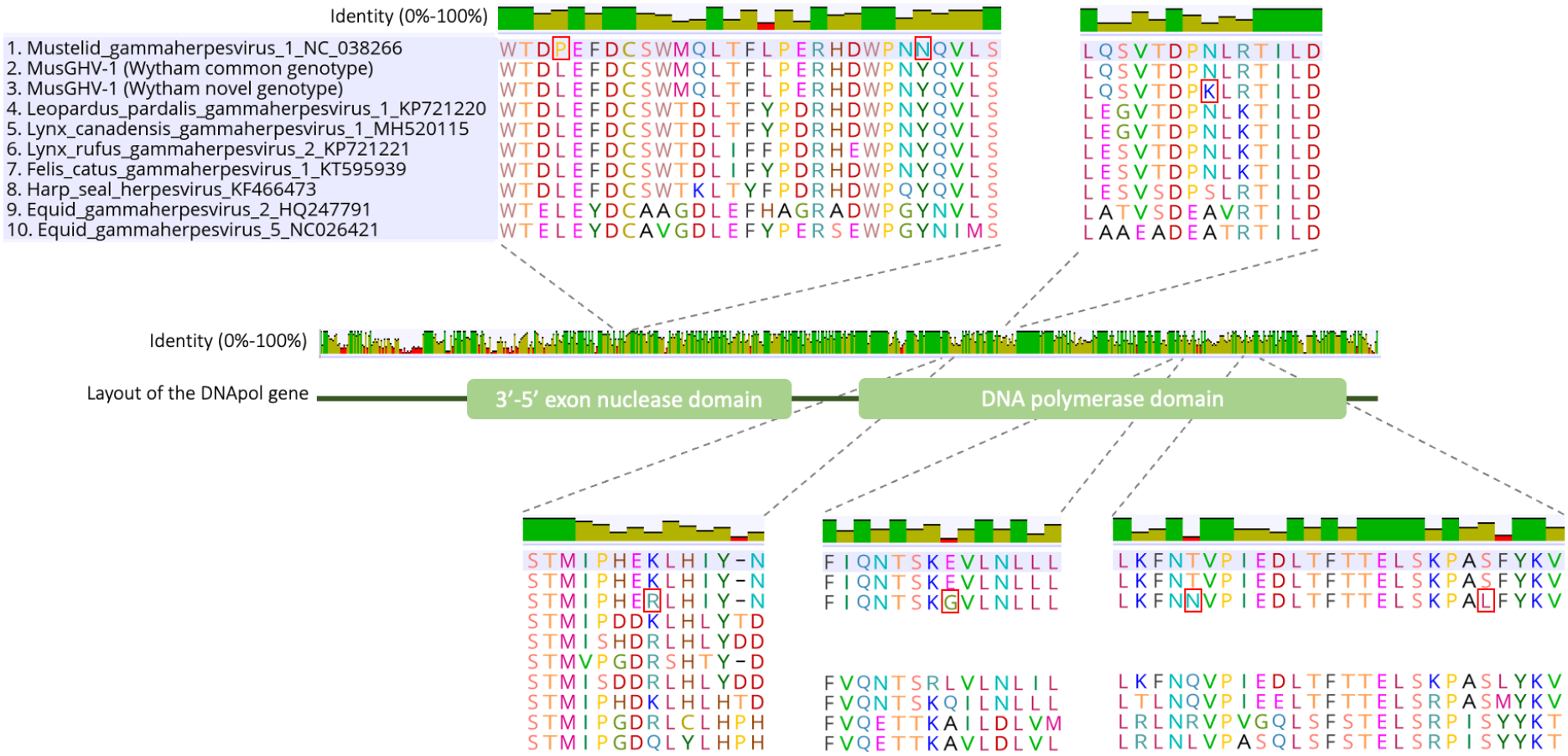


Figure 5: Gammaherpesvirus (genus *Percavirus*) DNA polymerase gene alignment. Amino acid letters wrapped with red rectangle are substitutions between the three MusGHV-1 variants (number 1 to 3 on the list of gammaherpesvirus species/variants).



Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	Infection with a newly discovered novel gammaherpesvirus variant in European badgers, *Meles meles*, is associated with higher viral load in blood
Publication Status	□Published □ Accepted for Publication □Submitted for Publication ■Unpublished and unsubmitted work written in a manuscript style
Publication Details	N/A

Student Confirmation

Student Name:	Ming-shan Tsai		
Contribution to the Paper	Performed field works, sample collection, lab experiments, data analysis, interpretation of data and manuscript writing.		
Signature	Ming-shan Tsai	Date	11th November 2021

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor David Macdonald		
Supervisor comments The candidate took the intellectual lead, and contributed very substantially to this research, which was primarily dependent upon her role.		
Signature	Date	12th November 2021

This completed form should be included in the thesis, at the end of the relevant chapter.

Chapter 6: Adverse weather during *in utero* development is linked to higher rates of offspring later-life herpesvirus reactivation in European badgers, *Meles meles*

Ming-shan Tsai, Chris Newman, David W. Macdonald and Christina D. Buesching

Submitted to *Royal Society Open Science*

Abstract

Maternal immune and/or metabolic conditions relating to stress or nutritional status can affect *in utero* development of their offspring with subsequent implications for later-life responses to infections. We used free-ranging European badgers as a host-pathogen model to investigate how prenatal weather conditions affect later-life herpesvirus genital tract reactivation. In 2018, we applied a sliding window analysis to 164 samples collected from 95 individuals born between 2005 – 2016 to test if the monthly mean and variation in rainfall and temperature experienced by their mother prior to parturition during the 12 months of delayed implantation and gestation subsequently affected individual herpes reactivation rates among these offspring. We identified four influential prenatal seasonal weather windows that corresponded with previously identified critical climatic conditions affecting badger survival, fecundity and body condition. These all occurred during the pre-implantation rather than the post-implantation period. We conclude that environmental cues during the *in utero* period of delayed implantation may affect an individual's developmental programming against later-life infection or viral reactivation in a natural setting. This illustrates how prenatal adversity caused by environmental factors, such as climate change, can impact wildlife health and population dynamics – an interaction largely overlooked in wildlife management and conservation programmes.

Introduction

During prenatal development, stressful environmental conditions experienced by individuals can cause non-adaptive disruptions, potentially compromising their future success (Bateson et al. 2004; Ghalambor et al. 2007; Gluckman et al. 2005). For example, animals subject to periods of food scarcity, developing embryos/ foetuses may undergo immediate responses to poor maternal nutrition (Gluckman and Hanson 2004; Bateson et al. 2014), causing impaired neonatal birth weight. Ultimately this can result in offspring being at greater risk of disease and with compromised immune responses as adults (Bateson et al. 2004). This risk is heightened when there is a mismatch between conditions during development and those experienced later in life (Godfrey, Gluckman, and Hanson 2010), giving rise to the Developmental Origins of Health and Disease (DOHaD) paradigm (Barker et al. 1989, 1993; Barker and Osmond 1986; Haugen et al. 2015; Sinclair and Singh 2007). For instance, Barker et al. (1993) noted that inadequate nutrition during gestation reprograms the relationship between glucose and insulin, and between growth hormone and IGF (insulin-like growth factor). In a review of the DOHaD approach, Gluckman et al. (2008) reported the epigenetic modifications of histones and DNA through methylation and acetylation due to developmental stress (see Zhu et al. 2019). Immediately after fertilization, rapid demethylation occurs in both parental genetic contributions to the zygote. Re-methylation (or *de novo* methylation) can occur at the blastocyst (late pre-implantation) stage, with continuing effects throughout gestation (Guo et al. 2014; Marcho, Cui, and Mager 2015; Messerschmidt, Knowles, and Solter 2014), resulting in epigenetic plasticity to environmental cues. For example, sows fed a diet too rich or poor in protein give birth to piglets with relatively low neonatal plasma IgA, altered stress responses until weaning, and inflammation dysregulation (Tuchscherer et al. 2012).

For this study, we selected the European badger (*Meles meles;* hereafter 'badger') as a model species, representative of 130 mammal species that undergo delayed implantation (i.e., embryonic diapause: Renfree and Fenelon 2017; Yamaguchi et al. 2006). Delayed implantation in badgers can extend by ten months, during which *in utero* developmental stress could occur. Badgers live in social groups ranging from 2 to 25 individuals (Macdonald et al. 2015), but around 50% of offspring are conceived from extra-group mating (Annavi et al. 2014). Furthermore, due to superfoetation and superfecundation in badgers (Yamaguchi et al. 2006), additional blastocysts may be conceived during delayed implantation, especially in lower density populations where multiple oestrous cycles provide fertility assurance (Sugianto, Dehnhard, et al. 2021). This can lead to continuous blastocyst turnover (Corner et al. 2015). After implantation, gestation takes around seven weeks, with parturition typically occurring in February.

Badgers are also sensitive to weather-related stressors (Byrne et al. 2015; Macdonald et al. 2010; Macdonald and Newman 2002) that impact the availability of their main food, earthworms (*Lumbricus terrestris*), affecting their body condition (Bright Ross et al. 2021). Badgers suffer thermal stress if they must forage under inclement conditions (Bright Ross et al. 2021; Noonan et al. 2014) instead of remaining in their subterranean burrows, termed 'setts' (Tsunoda et al. 2018). Lower than average rainfall (causing summer drought) is related to higher mortality rates, with cubs also sensitive to excessive rainfall, causing soaking and hypothermal stress (Nouvellet et al. 2013). During the autumn, before winter torpor (Newman et al. 2011), weather conditions are especially critical for badger survival (Macdonald et al., 2010; Noonan et al., 2014), where lower September temperature and higher mean rainfall in October promote weight gain (see Newman et al. 2017 for a complete account). Maternal body condition is also pivotal in determining how early implantation will occur around a median date of 21st December (linked to short-day photoperiod; Woodroffe 1995) and how many blastocysts will implant and develop (Woodroffe and Macdonald 1995). Late implantation and adverse feeding conditions are associated with a cohort sex ratio biased towards females (Dugdale, Macdonald, and Newman 2003), where, mechanistically, the adverse effects of poor weather and an associated lack of food can manifest through oxidative stress and oxidative damage to tissues (Bilham et al. 2018). Such stresses are associated with shorter neonatal (< 1-year-old) telomere length in our study population, which ultimately predicts lifespan, and survival to adulthood (van Lieshout et al. 2019, 2021).

From the disease perspective, we examined patterns of *Mustelid gammaherpesvirus 1* (MusGHV-1) recrudescence, where reactivation (or lytic infection) of latent herpesviruses can be triggered/ sustained by stressful events resulting in immune suppression. Previous molecular screening has found MusGHV-1 occurs with 98% to 100% prevalence in wild badger populations (King et al. 2004; Sin et al. 2014). In humans and domestic animals, primary infection during gestation, or reactivation of herpesvirus that escapes immune regulation, poses a risk to embryonic development through meningoencephalitis, disseminated infections and foetal malformations, ultimately resulting in abortion (François et al. 2013; Smith 1997; Straface et al. 2012; Wathes et al. 2019).

In badgers, reactivation and resultant herpesvirus shedding in the genital tract are associated with a higher rate of unsuccessful pregnancy (absorption of foetuses or abortion) than in females with no detectable virions in the genital tract (Kent et al. 2018; Tsai et al. 2020 or Chapter 2). The reactivation rate is also greater among thinner and older badgers (Tsai et al. 2020 or Chapter 2). This suggests that intrinsic factors such as immuno-competency and sensitivity to physical stress might be instrumental in determining an individual's capability for containing MusGHV-1

replication during pregnancy. In turn, the competency with which MusGHV-1 reactivation is suppressed in later life may be a consequence of prior prenatal programming and/or early life development in that individual (Fagundes et al. 2013; Wright 2010).

Here we used a sliding windows approach (Hindle et al. 2019; van de Pol and Cockburn 2011) to test if weather conditions experienced by mothers during delayed implantation and pregnancy affect the occurrence of genital MusGHV-1 reactivation in offspring later in life; noting the size and direction of any effects. Given that almost all mothers have MusGHV-1 infections during gestation, we predict that those mothers experiencing unfavorable weather conditions during delayed implantation/ pregnancy may suffer nutritional deficiency or increased allostatic load (Olson et al. 2015), causing a stress response-/ immunity-related epigenetic programming (Gheorghe et al. 2010) in their developing foetuses. This food stress cannot be measured reliably through impaired body condition because food scarcity may be compensated by intensive foraging, although this heightened activity also risks stressing pregnant females. Such a stressful *in utero* environment may ultimately result in offspring with different susceptibility to reactivation of chronic infection in adulthood, as has been established for some other diseases in humans and experimental animals such as mice and pigs (Merlot, Couret, and Otten 2008).

Materials and methods

Animals and sample collection

Free-ranging individually identifiable (by tattoo) badgers living in Wytham Woods, Oxfordshire, UK (51◦46'26"N, 1◦19'19"W; see Savill et al., 2010 for details of study site) were trapped over three seasons (spring: 21th May – 3rd June, summer: 3rd – 15th Sep., autumn: 13th – 20th Nov) in 2018 (see Macdonald et al. 2009 for details of trapping methodology). Over our complete dataset 1987-2019, most individuals (1,319/1,823 individuals, 72.3%) were first caught as cubs, and thus their ages were known absolutely. A further 128 individuals (7.0%) were captured shortly after cub-hood, and their age was estimated based on their evident adolescent appearance. For the remaining 376 individuals (20.6%, 100 of which were from the first three years of the study) whose age was unknown at first capture, age was inferred using molar toothwear on a 5-point scale following Bright Ross et al. (2020). We classified sexually mature adults into three age classes: young (2≤ x <5 years old), old (5≤ x <8 years old) and very old (≥ 8 years old) (Sugianto et al. 2020). All sexually immature individuals (i.e., <2 years of age) were excluded from this study.

To test for herpesvirus shedding in the genital tract, we collected 164 genital swabs (using sterile swabs with cotton tops and wooden shafts) from 95 individually identifiable individuals (50

males and 45 females) born between 2005 to 2016. All swab samples were stored at -20°C immediately after collection until processing. We recorded sex, age class, social group, and body condition score (BCS, from 1= emaciated to 5= very fat) for each badger sampled.

Herpesvirus reactivation data

We extracted and purified the DNA from the swabs using the commercial Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's guidelines. We then used conventional PCR targeting the DNA polymerase gene of MusGHV-1 to amplify the viral DNA (for a detailed description of primers and PCR conditions, see Tsai et al., 2021 or Chapter 3). We determined positive results by checking the PCR products using electrophoresis on 2% agarose gel.

Weather data and range of windows

Daily temperature and rainfall (mm) data were obtained from the Radcliffe Meteorological Station (https://www.geog.ox.ac.uk/research/climate/rms/), located within 6 km of the field site. We set monthly windows for the 12 months preceding the 1st February (i.e., typical parturition date) of each individuals' birth year. We computed monthly means of (i) daily temperature, (ii) daily temperature variability (standard deviation), (iii) daily rainfall, and (v) the coefficient of variation (or CV, which is used instead of SD as the rainfall data dispersed a lot and CV can better reflect variation comparing to the annual average) of daily rainfall, following Nouvellet et al. (2013), for each window. These rainfall data were log-transformed to mitigate the high variance caused by days with exceptionally high rainfall.

Most influential weather data window selection

Using the R package *climwin* (developed by Bailey and De Pol, 2016) in R studio, we performed a sliding windows analysis, selecting those windows of weather covariates most influential on later-life MusGHV-1 reactivation rates. The baseline binomial generalized linear model was controlled for the following covariates: age class, sampling season, BCS, and whether the adult was caught in a social group with a high or low percentage of cubs present (threshold set at 30% of group membership) because this was previously proven to have significant effects on MusGHV-1 DNA prevalence in genital swabs (Tsai et al. 2021 or Chapter 3). We tested both linear and quadratic terms for each of the four weather variables to identify the window capturing the greatest reduction in the AIC value of the final model. If the windows identified in both linear and quadratic models were the same, the model with the lowest AIC was selected.

Model diagnostic and performance

Sliding windows can produce random false signals. To mitigate this, we generated randomized window sets with 100 repeats to compare against with the ΔAIC of the most parsimonious models identified and calculated the false positive rate $P_{\Delta AIC}$ (van de Pol et al. 2016). We

considered models with $P_{\Delta AIC}$ less than 0.05 as true signals and unlikely to be artifacts. These models with $P_{\Delta AIC}$ <0.05 indicate that the weather conditions experienced by the mother during this window genuinely affect offspring MusGHV-1 reactivation occurrence in later life. Multicollinearity was detected using the *vif* function in R to obtain the variance inflation factor (VIF) value for each variable included in the final model, where a VIF value >5 is considered problematic (James et al. 2014). After adding the weather metrics into the baseline model as fixed effects and adding the tattoo number as a random effect (to avoid any bias arising from re-sampling the same individual), we performed residual diagnostics using the R package DHARMa (version 0.3.3.0). We then checked the potential to improve the model's diagnostic ability by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) (Hajian-Tilaki 2013; Zweig and Campbell 1993).

Results

Of these 164 genital swabs, 31.1% (51/164) tested positive for MusGHV-1 DNA. Monthly weather conditions from 2004 (i.e., the year in which those oldest individuals in the study were conceived and underwent embryonic diapause) to 2018 (i.e., the year in which the youngest study animals reached sexual maturity) are displayed in Figure 1.

The windows returning the highest ΔAIC for each weather covariate, which are considered as having the greatest effects on MusGHV-1 reactivation in the genital tract when sampled later in life, are shown in Table 1. Interestingly, we found that all influential weather windows occurred at a time prior to that individual's embryonic implantation, i.e., when it was a blastocyst during delayed implantation (Figure 2A). Specifically, increased likelihood of later-life adult occurrence of genital MusGHV-1 was influenced prenatally by:

i) higher temperature variability in spring (April to May) (Figure 2B);
ii) lower or higher rainfall variability in Spring (April) (Figure 2C);
iii) higher rainfall variability in early summer (June to July) (Figure 2D) and
iv) Lower mean rainfall and, to a lesser content, higher mean rainfall in late autumn (October to November) (Figure 2E).
Note: the mean temperature window effect with the highest ΔAIC did not pass the test for false signals ($P_{\Delta AIC}$>0.05) in either the linear or quadratic model.

Adding the weather data from each significant window to the baseline model, including individual tattoo number as a random effect, reduced AIC and improved the AUC for all six models, compared to the baseline model, and passed the diagnostic test for residuals (see Table

2 for details of each model). No multicollinearity was identified in these models. Having detected no effect of sex on MusGHV-1 reactivation (corroborating Tsai et al., 2021 or Chapter 3), we added sex as a fixed effect in each model but still found no sex effect.

Discussion

We identified the monthly environmental conditions as risk factors for MusGHV-1 reactivation using sliding window analysis to corroborate and extend previous findings for this same badger population (Newman et al. 2017). Spring weather conditions outside of an optimum 'Goldilocks Zone' that are either too warm or cool or too wet or dry are associated with lower badger survival rate and reproductive success (Macdonald et al. 2010; Nouvellet et al. 2013), as is a high variation from mean rainfall during early summer. Similarly, low mean autumn rainfall risks poor winter body-condition, unable to sustain torpor (Bright Ross et al., 2021; Noonan et al., 2014). Correspondingly, a very similar set of stressful weather effects appeared to be deleterious to blastocyst development, at least in so far that offspring developing from these blastocysts were more susceptible to active genital MusGHV-1 shedding as adults. Although no herpes-specific disease has been identified in badgers, genital reactivation of MusGHV-1 is associated with poorer body condition and a higher abortion rate in adult badgers (Tsai et al., 2020 or Chapter 2; Tsai et al., 2021 or Chapter 3).

Similar environment-driven *in utero* developmental stress causes phenotypic adaptations in a wide variety of organisms (Bateson, Gluckman, and Hanson 2014). This plasticity generally involves epigenetic changes in DNA expression (Bateson and Gluckman 2012; Godfrey et al. 2007) that increase the probability that the individual's phenotype will be well-matched to environmental conditions, enhancing fitness. In support of maternal effects of nutritional stress, in American mink (*Neovison vison*), another mustelid species exhibiting delayed implantation, kits born to females experiencing a protein-restricted diet during prenatal development exhibit lower birth weight and lower insulin/ leptin related mRNA expression (Matthiesen et al. 2010), with lower protein oxidation in male kits. Nevertheless, there is lab evidence that the effects of low prenatal protein diets can be mitigated by a more balanced post-weaning diet (Vesterdorf et al. 2014). In a further example, Lee and Zucker (1988) discovered that autumn-born vole pups (*Microtus pennsylvanius*) have much thicker coats than those born in spring – cued hormonally *in utero* by the mother experiencing changes in day length – a type of plasticity that Gluckman and Hanson (2004) termed a Predictive Adaptive Response (PAR). Provided the environment remains consistent between the time these developmental adaptations occur and the time the individual goes on to breed, PARs offer selective fitness advantages. An alternative hypothesis is that phenotypes developing during good prenatal conditions (with abundant resources) are

typically more significant, more fecund, and longer-lived, compared to those growing in poor early-life conditions (with scarce resources), irrespective of the adult environmental conditions (Pigeon et al. 2019), and thus experience so-called 'silver spoon' benefits (Grafen 1988; Lindström 1999; Monaghan 2008). Although our study was limited to just one measure of later-life propensity toward MusGHV-1 genital infection, taken in 2018, we included 11 birth years of 94 individuals. Our results indicated that badgers whose mothers experienced better conditions during delayed implantation were at lower risk of MusGHV-1 reactivation in later life, providing support for the silver spoon hypothesis, as well as the DOHaD paradigm (Barker et al. 1989, 1993; Barker and Osmond 1986; Haugen et al. 2015; Sinclair and Singh 2007).

Although sliding window analyses are prone to producing false-positive results, 6 out of 8 of our models passed the false-positive test using repeated randomized model sets (van de Pol et al. 2016). Furthermore, we explored all possible windows without limiting ourselves to predetermined windows of interest. This approach also exposed that some intuitive seasonal weather windows, expected to be influential, were not. For example, very cold winters cause badgers to spend more time in bouts of torpor and to deplete their body-fat reserves (Macdonald and Newman 2002); nevertheless, we did not identify a statistically significant effect of low mean temperature on MusGHV-1 occurrence in later life in this window (December and January), even though it would coincide with -implantation dates and embryonic development. This is despite Bilham et al.'s (2018) proposal that hibernation induced by cold acts to reduce oxidative stress; a process also noted for brown bears (*Ursus arctos*) (Chazarin et al. 2019). This further implies that *in utero* epigenetic programming happens predominantly during the blastocyst stage, pre-implantation (i.e., before mid-December).

This raises an interesting broader question: does prolonged delayed implantation, as documented in 130 species of mammals (Renfree and Fenelon 2017; Yamaguchi et al. 2006), expose blastocysts to a more extended period during which epigenetic changes could occur? Studies are limited; however, working on murine embryos, Nakanishi et al., (2012) reported slightly, but statistically, significantly higher DNA methylation levels in one loci of E3.5 blastocysts with experimentally delayed implantation for four days compared to E4.5 blastocysts, showing that *de novo* methylation during delayed implantation is possible.

In badgers, post-partum effects caused by early-life environmental adversity have been explored by several studies. For example, van Lieshout et al. (2021) found that cubs born in warmer, wetter springs with low rainfall variability had longer early-life (3–12 months old) telomeres, which predicts cub survival to adulthood. Early-life effects also affect the rate at which badgers reach sexual maturity (Sugianto, Newman, et al. 2019), the subsequent pace of life syndromes

(Bright Ross et al., 2020), and later-life telomere dynamics and longevity (van Lieshout et al. 2019).

Conclusion

Our results show that disadvantageous weather conditions during *in utero* development can exacerbate the likelihood of later-life herpes virus reactivation among offspring, exposing strong effects of prenatal environmental cues. We propose that in species with prolonged delayed implantation, the potential for epigenetic programming may be extended and more influential than post-implantation embryonic development. Further studies on prenatal effects on later life immunity, sensitivity to stressors, somatic and reproductive fitness, and metabolic functions are essential to clarify the full extent to which early life adversity caused by environmental factors such as climate change may impact individual health, disease development, and population dynamics, with implications for species conservation management (Champagne 2020).

Table 1: Best weather effect models on later-life MusGHV-1 reactivation selected by sliding window analysis using R package *climwin*.

Weather variable	Function	Stats	ΔAIC	Window period	Beta	$P_{\Delta AIC}$	AUC
Temperature	Linear	Mean	-5.66	May	0.78	0.08	
Temperature	Quadratic	Mean	-4.92	May	0.55	0.16	
Temperature	Linear	SD	-12.81	April - May	1.93	<0.001	0.774
Temperature	Quadratic	SD	-13.08	April - May	2.5	0.01	0.774
Rainfall	Linear	Mean	-7.83	October - November	-8.78	0.04	0.77
Rainfall	Quadratic	Mean	-13.51	October - November	132.93	0.01	0.773
Rainfall	Linear	CV	-11.91	June - July	6.07	0.01	0.773
Rainfall	Quadratic	CV	-11.76	April	270.9	<0.001	0.786

Table 2: Details of the baseline model and final models with effect identified by sliding window analysis added

Model	AIC	BIC	AUC	Fixed effects:	Estimate	Std. Error	z value	p value
Baseline model	195.4	220.2	0.766	(Intercept)	0.3213	1.0881	0.295	0.7678
				SeasonSpring	-0.3591	0.59	-0.609	0.5427
				SeasonSummer	0.6473	0.5194	1.246	0.2127
				AgeGroupVery old	1.5111	0.7096	2.13	0.0332
				AgeGroupYoung	1.6058	0.6851	2.344	0.0191
				BodyCondition	-0.5948	0.239	-2.488	0.0128
				CubPerLow	-1.2308	0.5587	-2.203	0.0276
Baseline model + rainfall mean (linear effect)	188.0	219.0	0.77	(Intercept)	2.9991	1.4857	2.019	0.04353
				SexMale	0.4727	0.413	1.145	0.25232
				SeasonSpring	-0.4723	0.6081	-0.777	0.43736
				SeasonSummer	0.7639	0.5343	1.43	0.15279
				AgeGroupVery old	2.2378	0.8385	2.669	0.00761
				AgeGroupYoung	2.3513	0.8249	2.85	0.00437
				BodyCondition	-0.6527	0.2477	-2.635	0.00842
				CubPerLow	-1.5158	0.5819	-2.605	0.00919
				RainMean	-9.1059	3.2294	-2.82	0.00481
Baseline model + rainfall CV (linear effect)	185.2	216.2	0.778	(Intercept)	-2.0766	1.5233	-1.363	0.17282
				SexMale	0.1544	0.4029	0.383	0.70153
				SeasonSpring	-0.5407	0.614	-0.881	0.37855
				SeasonSummer	0.6633	0.5304	1.251	0.21107
				AgeGroupVery old	1.518	0.8617	1.762	0.07814
				AgeGroupYoung	2.2247	0.9125	2.438	0.01477
				BodyCondition	-0.6351	0.2421	-2.623	0.00871
				CubPerLow	-1.2249	0.55	-2.227	0.02594
				RainCV	5.966	1.8564	3.214	0.00131
Baseline model + temperature Sd (linear effect)	182.4	210.3	0.779	(Intercept)	-3.1117	1.7573	-1.771	0.0766
				SeasonSpring	-0.4751	0.6151	-0.772	0.4399
				SeasonSummer	0.714	0.5302	1.346	0.17815
				AgeGroupVery old	0.73	0.8511	0.858	0.39108
				AgeGroupYoung	0.3628	0.8106	0.448	0.65444
				BodyCondition	-0.605	0.2408	-2.513	0.01197
				CubPerLow	-1.3003	0.552	-2.355	0.0185
				TempSd	1.9373	0.5742	3.374	0.00074

Baseline model + rainfall mean (quadratic effect)	181.5	212.5	0.778	(Intercept)	17.6264	6.2552	2.818	0.00483
				SeasonSpring	-0.526	0.6163	-0.853	0.39342
				SeasonSummer	0.715	0.5348	1.337	0.18126
				AgeGroupVery old	3.3628	1.2777	2.632	0.00849
				AgeGroupYoung	4.7269	1.6063	2.943	0.00325
				BodyCondition	-0.6026	0.2432	-2.478	0.01321
				CubPerLow	-1.5955	0.5709	-2.795	0.00519
				RainMeanQ	-103.9975	40.6844	-2.556	1.06E-02
				I(RainMeanQ^2)	132.9307	55.4751	2.396	1.66E-02
Baseline model + rainfall CV (quadratic effect)	180.8	211.8	0.786	(Intercept)	4.3732	2.0932	2.089	0.036689
				SeasonSpring	-0.5954	0.6166	-0.966	0.334202
				SeasonSummer	0.669	0.532	1.258	0.208556
				AgeGroupVery old	3.6764	1.0906	3.371	0.000749
				AgeGroupYoung	4.128	1.1028	3.743	0.000182
				BodyCondition	-0.6353	0.2429	-2.615	0.008921
				CubPerLow	-1.4582	0.562	-2.595	0.009465
				RainCVQ	-175.8402	43.34	-4.057	4.97E-05
				I(RainCVQ^2)	1171.901	252.2425	4.646	3.39E-06
Baseline model + temperature Sd (quadratic effect)	181.9	212.9	0.783	(Intercept)	4.6441	4.94	0.94	0.3472
				SeasonSpring	-0.4834	0.6267	-0.771	0.4405
				SeasonSummer	0.7747	0.5407	1.433	0.1519
				AgeGroupVery old	3.8992	2.2813	1.709	0.0874
				AgeGroupYoung	3.4567	2.2117	1.563	0.1181
				BodyCondition	-0.6062	0.2437	-2.488	0.0128
				CubPerLow	-1.4389	0.5622	-2.559	0.0105
				TempSdQ	-8.6243	6.6619	-1.295	0.1955
				I(TempSdQ^2)	2.5028	1.5946	1.57	0.1165

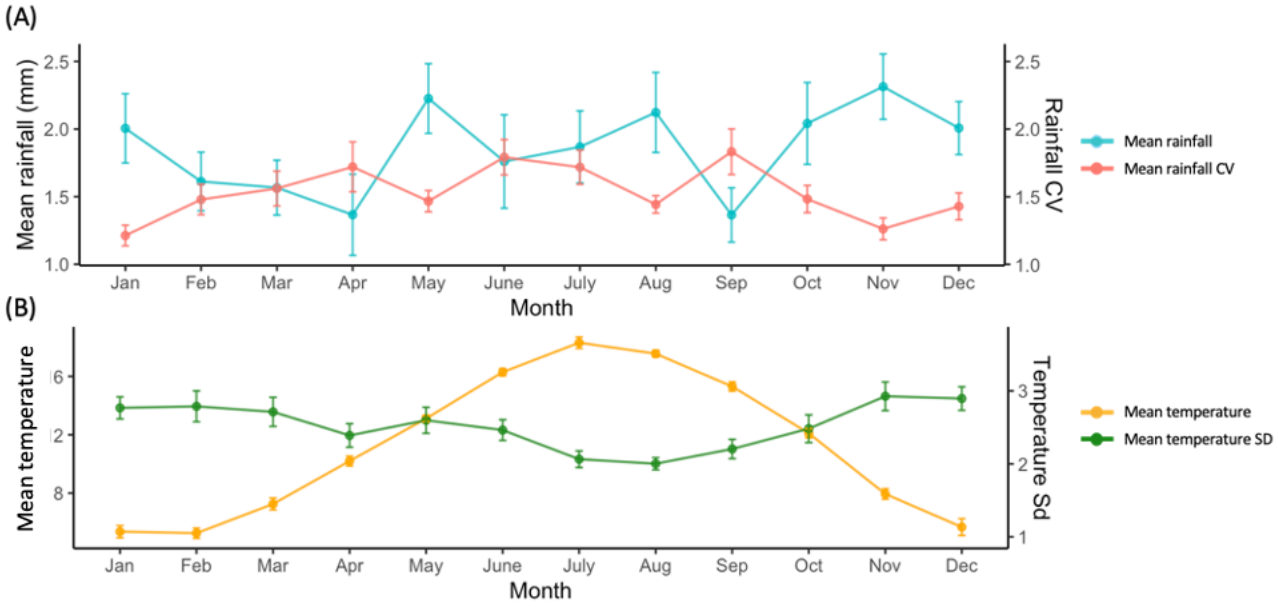


Figure 1: Monthly rainfall (A) and temperature (B) mean and variability from 2004 to 2018 with error bar indicating standard errors across years.

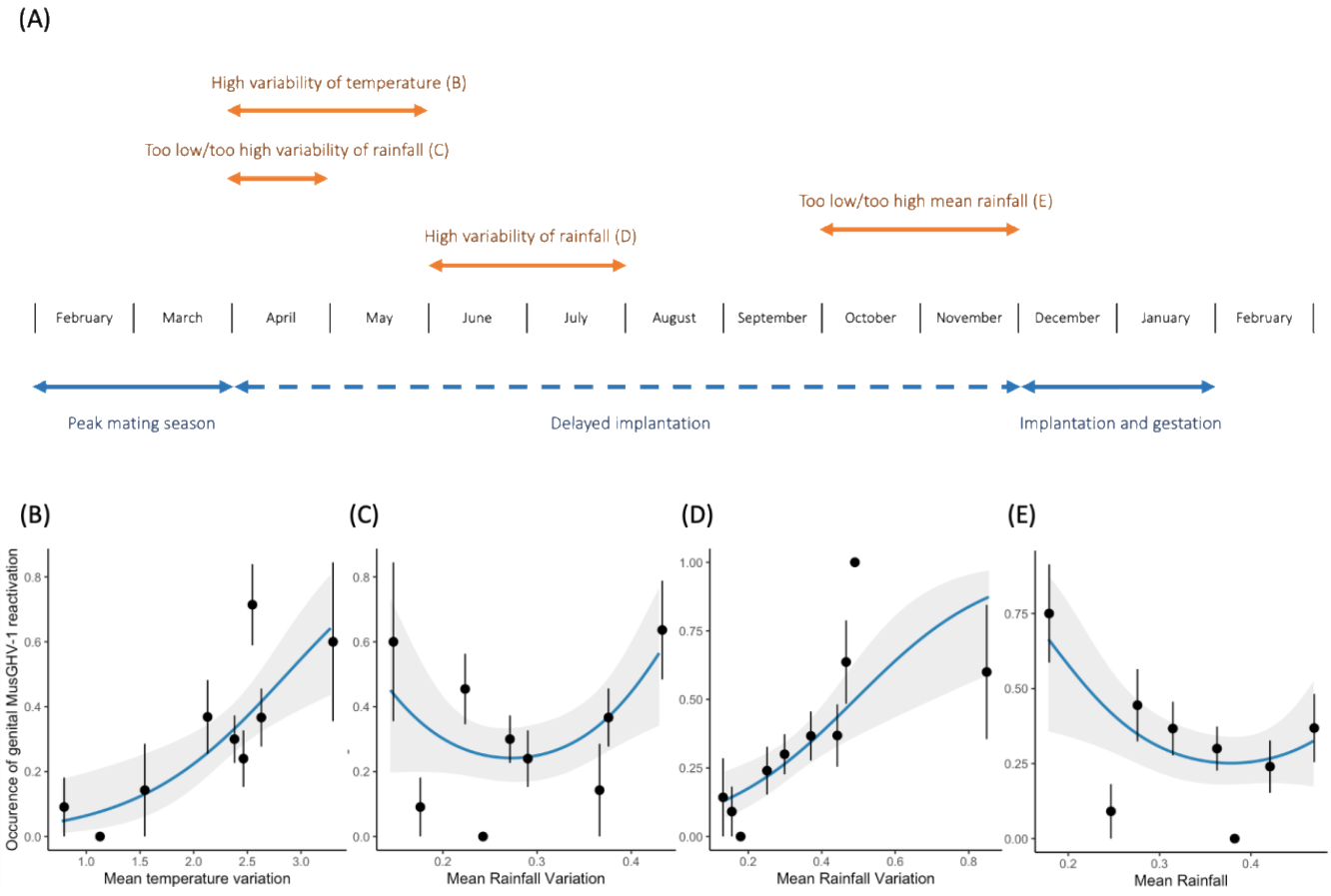


Figure 2: (A) Weather windows in the year prior to birth affect MusGHV-1 reactivation occurrence in adulthood. The blue arrows illustrate badger reproductive events, and the orange arrows indicate the influential windows, with effect extent and direction for each window shown in (B), (C), (D), and (E).

Statement of Authorship for joint/multi-authored papers for PGR thesis

To appear at the end of each thesis chapter submitted as an article/paper

The statement shall describe the candidate's and co-authors' independent research contributions in the thesis publications. For each publication there should exist a complete statement that is to be filled out and signed by the candidate and supervisor **(only required where there isn't already a statement of contribution within the paper itself).**

Title of Paper	Adverse weather during *in utero* development is linked to higher rates of later-life herpesvirus reactivation in adult European badgers, *Meles meles*
Publication Status	□Published □ Accepted for Publication ■Submitted for Publication □ Unpublished and unsubmitted work written in a manuscript style
Publication Details	N/A

Student Confirmation

Student Name:	Ming-shan Tsai		
Contribution to the Paper	Performed field works, sample collection, lab experiments, data analysis, interpretation of data and manuscript writing.		
Signature		Date	11th November 2021

Supervisor Confirmation

By signing the Statement of Authorship, you are certifying that the candidate made a substantial contribution to the publication, and that the description described above is accurate.

Supervisor name and title: Professor David Macdonald		
Supervisor comments The candidate took the intellectual lead, and contributed very substantially to this research, which was primarily dependent upon her role.		
Signature	Date	12th November 2021

This completed form should be included in the thesis, at the end of the relevant chapter.

General discussion

Sexually transmitted infections have been largely overlooked in studies of wildlife disease, noticeably amongst musteloids, as reviewed in Chapter 1. Nevertheless, my explorations of the literature revealed STI-related reproductive disease, and associated adverse outcomes, are often reported in captive wildlife, and many endangered species are susceptible to STIs. Using European badgers as a wildlife model, the research for my thesis has involved a systematic survey at the individual - as well as at the population level of - a sexually transmitted herpesvirus. My wider purpose was to achieve a more profound understanding of STIs in wildlife in general. In this concluding overview, I reflect on the most crucial findings of the thesis, and in particular draw comparisons between my results and previously proposed theories relating to STIs. Specifically, I explore the association between herpesvirus infection and stress, interpreting the effects of co-infections and variant differences, and discuss why STIs can be of wildlife conservation concern.

Does MusGHV-1 match the characteristics of an STI as predicted by theory?

Isolation of herpesviruses from the genital tract is commonly reported in animals, but sexual contact sometimes plays only a minor role in the spread of such infection. For example, oral transmission is epidemiologically important for HSV-1, while sexual transmission is the main route of HSV-2 spread in humans. Therefore, it is essential to determine the role of sexual transmission of MusGHV-1 in badgers before interpreting this host-pathogen model in terms of STI dynamics. Although manipulative, strictly controlled experiments can directly explore transmission mechanisms in laboratory animals, it is generally impractical to conduct such experiments in free-ranging wild animals; aside from legal restrictions of various wildlife laws (e.g., Protection of Badgers Act, 1972 (Fitzgerald 2014)) the impracticalities include the need to prioritise animal welfare, the necessity to limit disturbance, and the challenge posed by controlling numerous environmental and individual variables. Despite these limitations, we can use indirect evidence to judge if sexual transmission is an important route of MusGHV transmission. A convincing line of evidence is the higher prevalence of the virus found in the genital organs of badgers, compared to humans and other mammals (as detailed in the General Introduction). A second line of evidence comes from the extent of a match between the features of MusGHV-1 and those listed as characteristic of STIs by Lockhart et al. (1996).

When I began the research for my thesis, it was already known that MusGHV-1 had the following characteristics of an STI: relatively low mortality, prolonged infection, and high host specificity. However, these are all characteristics shared by most herpesvirus species. The critical test was whether MusGHV-1 exhibited the remaining STI characteristics, including relatively low

fluctuation in prevalence and host sterility. Testing these thus became a focus of my research. My studies with population-wide screening in Ireland and the population in Wytham, UK, answer this question. Through postmortem examination of badgers sampled in Ireland, I found that genital reactivation of MusGHV-1 is linked to impaired female reproductive performance (i.e. fewer successful pregnancies) but not to male fertility (sperm abundance and testes weight). This corresponds with the findings by Lockhart et al. (1996) that viral STIs are less likely to cause male sterility. Furthermore, although MusGHV-1 shedding in the genital tract is prevalent across all sampling seasons, I found seasonal patterns of genital MusGHV-1 reactivation. In brief, the prevalence in summer tended to be higher than in spring or autumn in the Wytham population. Unfortunately, data from winter were unavailable, as it would have been illegal to trap badgers when they had dependent young. But when I compare the prevalence between winter and spring, the periods when the Irish badgers were sampled, I found that, in both seasons, adult males had significantly higher genital MusGHV-1 prevalence than females.

Although I did not find such a sex-based prevalence pattern in spring, summer, or autumn in the Wytham population, I found that females there had an overall higher prevalence than males or juveniles. This finding is tantalizing, but hard to interpret without more information. According to results from the murine experimental model, after initial nasal inoculation with the virions, sexual transmission of MHV-4 (also a gammaherpesvirus) is only possible from female to male mice but not vice versa (François et al. 2013). This raises the possibility that also in badgers, females may be more active in spreading MusGHV-1 across the population than males. Nonetheless, in juveniles, if corrected for sex-specific effects, good body condition was positively correlated with genital MusGHV-1 prevalence. Considering that males who enter puberty earlier have higher body condition scores (Sugianto, Newman, et al. 2019), this precocity may expose them to a higher risk of MusGHV-1 infection in the genital tract through copulation. This would also explain why adult males have exceptionally high genital MusGHV-1 prevalence during the peak mating season. Although whether virus shedding in these males was caused by reinfection with MusGHV-1 virions from the female vagina or reactivation of resident latent viruses remains undetermined.

In summary, the findings that MusGHV-1 reactivation is associated with female reduced fertility and minimally fluctuating prevalence, which followed a seasonal pattern, suggests that MusGHV-1 meets all the criteria previously established as defining an STI. In addition, the higher prevalence in males during the peak mating season suggests sexual transmission plays an important role in the spread of MusGHV-1. The male-biased prevalence found in winter and female-biased prevalence in juveniles also reveal that the direction of sexual transmission is likely from females to males.

Does stress correlate with MusGHV-1 reactivation?

Stress is often found to be a trigger of opportunistic herpesvirus reactivation from latency, as a result of increased corticosteroid levels causing immunosuppression, which can act as a sensitive indicator for stress-induced immune dysregulation (Glaser and Kiecolt-Glaser 1994; Rooney et al. 2019). Stress hormones such as glucocorticoids can cause lymphocyte apoptosis and theoretically induce residential latent viruses such as gammaherpesviruses to replicate and release (Duke, Chervenak, and Cohen 1983). I thus expected MusGHV-1 reactivation might be linked to stress in badgers. However, I did not have the facilities to test the cortisol level in faeces, a commonly used non-invasive method to measure stress levels in wild animals. Therefore, as an alternative approach, in Chapter 3 I explored the MusGHV-1 reactivation patterns I identified and their link to stress in the light of previous studies about a variety of known badger stressors.

Interestingly, the seasonality of MusGHV-1 reactivation (samples collected in summer have a higher MusGHV-1 detection rate than those from spring and autumn) in the Wytham population reflect seasonal stress patterns analysed by George et al. (2014). They found higher faecal cortisol levels in summer than in other seasons. They attributed this result to badgers in summer facing lower earthworm availability due to summer drought (Macdonald and Newman 2002). Such nutritional stress can also be reflected in body condition, which was also negatively linked to cortisol level in badgers (George et al. 2014b). Interestingly, in my own research, I found body condition score had a robust negative correlation with genital MusGHV-1 prevalence in adult badgers (≥ 2 years old). However, I could not confirm the underlying causal relationship to distinguish whether low body condition induces herpesvirus reactivation or herpesvirus reactivation results in thinner body condition. However, since herpesviruses rarely cause disease in immune competent animals, and reactivation generally occurs when the host is experiencing immune-suppression, I deduce that that MusGHV-1 reactivation is probably a response to the same stressor that caused a decrease in host body condition.

Individuals at different ages may respond differently to stress, as aging can reduce resilience to stress, and this effect can in turn cause higher cortisol levels in old individuals (Lavretsky and Newhouse 2012). George et al. (2014) did not find an effect of age on badger faecal cortisol levels, but their result may be questionable as they only separated the tested individuals into cubs (< 1-year-old), yearlings (between 1 and 2 years old) and adults (more than two years old); this categorization thus combined all mature individuals into a single age-group. In contrast, I found a strong effect of age on MusGHV-1 shedding rates, which are higher in cubs and yearlings, then decrease to less than 20% at badgers aged between 5 and 6 but increase subsequently until reaching 100% prevalence at age 13. Aging in badgers, which can result in the individual becoming sensitive to stress and prone to immunosuppression, clearly acts as a risk factor for MusGHV-1 reactivation. Nevertheless, the high prevalence in juvenile badgers is more likely due

to primary infection, as reported in the the murine MHV-4 model (François et al. 2013), and in the human cytomegalovirus (CMV) blood viral load (Parry et al. 2016). I also found MusGHV-1 viral loads in juvenile badgers' blood to be significantly higher than in adults (Chapter 5), and genital MusGHV-1 prevalence was positively correlated with body condition (Chapter 3), indicating a weak association between MusGHV-1 reactivation and physical stress also in juveniles.

Other stressors identified by George et al. (2014) include shedding of *Mycobacterium bovis* in faeces and living in grasslands rather than in woodlands. We cannot test either stressor as the Wytham population has particularly low *M. bovis* prevalence and the badgers sampled all lived in woodlands. Although the Irish population has 20-27% prevalence of *M. bovis* across the nation (Aznar et al. 2018), my attempt to culture *M. bovis* in my samples of Irish badgers was cancelled due to unforeseen constraints on the central Irish lab during the Covid-19 pandemic, where my collaborators were required to prioritise other aspects of *M. bovis* culturing for the National Bovine Tuberculosis eradication program (O'Keeffe 2006).

Another critical stressor for badgers is weather, which influences food availability, individual body condition, and survival (Bright Ross et al. 2021; Macdonald et al. 2010; Macdonald and Newman 2002). I therefore investigated the effect of weather conditions on MusGHV-1 reactivation in the Wytham population, using a sliding window analysis (van de Pol et al. 2016) to test all windows (weekly or daily) with relevant weather metrics (here I tested mean rainfall) to see if dry conditions prior to sampling can influence MusGHV-1 reactivation. The best windows returned from each analysis, however, was not consistent, and the percentage of false-positive was high in all analyses ($p=0.15\sim0.68$), from which I conclude that the windows indicated by the analysis cannot reliably be distinguished from results produced by randomized data. I therefore rejected this analysis as invalid. This means that four interesting questions remain unanswered: i) what duration of drought can trigger MusGHV-1 reactivation, ii) how long does it take from the stress trigger to viral shedding in the genital tract, iii) how long can viral shedding persist, and iv) do all badgers have the same tolerance to physical stress? I suspect that an experimental approach will be needed to answer these questions. Furthermore, and with reference to the last question, based on the effect of age identified on MusGHV-1 reactivation, I anticipate that tolerance to stress may be different at different life stages.

Nevertheless, it is well established that somatic functions or phenotypes of an individual are reliably determined by genetic coding inherited from the parents, but differences such as susceptibility to disease can nonetheless be observed in monozygous twins (Fraga et al. 2005). This may be due to epigenetic modification that contributes to phenotypic differences after fertilization. Although epigenetic changes can happen throughout an individual's lifetime, the

most critical period is during the *in utero* stage. The foetus is particularly sensitive to environmental cues and epigenetic modification (through methylation or histone wrapping across the genome), which can play a vital role in permanent phenotype shaping such as immunity or tolerance to stress (Van den Bergh et al. 2020; Thorsell and Nätt 2016). This is also known as foetal programming (Godfrey and Barker 2001). Since weather conditions are an important factor for badger survival, I deduce that they may also be relevant to later life susceptibility to stress or MusGHV-1 reactivation through foetal programming. In Chapter 6, I applied the sliding window analysis on the weather metrics before the birth of the tested animals to investigate the effect on genital MusGHV-1 reactivation as an adult. Importantly, I found several significant signals indicating that adverse weather conditions experienced in utero can contribute to a higher incidence of MusGHV-1 in the genital tract, illustrating that the susceptibility to MusGHV-1 reactivation (or tolerance to stress) is indeed dependent on foetal programming.

So far, my research has added strong evidence that herpesvirus reactivation is highly correlated to physical stress, which can be linked to seasons and body condition. Furthermore, these associations can be enhanced when an individual ages and/or its mother experienced adverse weather conditions during the critical stage of foetal programming. However, high viral loads in juveniles are more likely associated with an increased extent of virus replication in the early stage of infection, rather than with stress.

What is the effect of coinfection between MusGHV-1 and other pathogens? What are the differences between different MusGHV-1 strains?

Several studies have reported substantial effects of coinfection with herpesvirus and other pathogens on disease progression (Dai, Qiao, et al. 2020; Donofrio et al. 2005; Nordengrahn et al. 1996). This prompted me to explore further evidence for this phenomenon in order to better understand the impact of MusGHV-1 on badger health. Although my intention to test the coinfection relationship between herpesvirus and *M. bovis* was thwarted by the impact of the Covid-19 pandemic on our collaborators' lab, in Chapter 4 I succeeded in investigating the co-occurrence of MusGHV-1 and *Clostridium perfringens*. This zoonotic food-borne bacterial pathogen can cause enteric diseases in humans and other animals. I confirmed that genital MusGHV-1 reactivation, associated with physical stress as discussed above, can be a predisposing factor for *C. perfringens* overgrowth. Interestingly, I also found badgers infected with a specific MusGHV-1 variant, which has higher relative blood viral load, to be at greater risk of *C. perfringens* proliferation.

Based on the sequencing results of MusGHV-1 DNA polymerase gene PCR products in Chapter 3, I found two distinct variants of MusGHV-1 circulating in the Wytham population. I named them

the common variant and the novel variant, as the former is also identified in Irish populations, while the latter has been found only in the Wytham population. Multiple pathogen strains derived from the same host population can result from host-pathogen coevolution driven by host resistance or changes in viral virulence. Thus, different pathogen strains may have different virulence, or the host may have different susceptibility to different pathogen strains. In Chapter 5, I explored the genetic and phenotypic differentiation of the 2 MusGHV-1 variants and tested for demographic differences between hosts infected with either variant. I found significantly higher blood viral loads in badgers infected with the novel variant, indicating that it may exhibit higher virulence than the common variant. It was also the novel variant that was associated with a higher risk of *C. perfringens* detection (see Chapter 4, above). For future research, a priority will be to test whether this novel MusGHV-1 variant is associated with a different progression of disease development or a higher risk to female reproductive health. These questions were beyond the scope of my research because I did not identify any obvious MusGHV-1 associated symptomatic disease in badgers, and in the Irish population, where I have data on female reproductive performance, only the MusGHV-1 common variant occurred. I consider it is important to explore further the potential interplays between herpesvirus abundance/ variants and the structure and composition of the entire microbial (bacterial) community (Sin, Buesching, et al. 2012), using metagenomic approaches.

Although I could detect no difference in host demographics, with respect to either sex and/or age, between the two MusGHV-1 variants, I did document that badgers infected with the novel variant were clustered in only 3 neighbouring social groups, located in the northwest of the woods. Although sampling efforts differed somewhat between social groups, this observation is unlikely to be an artifact of sampling intensity as the novel variant was not identified in any of the most intensively sampled social groups. This suggests that the novel variant has thus far been circulating only between these three social groups and/or the badgers in these groups are particularly susceptible to this variant. I deduce that the first proposition is less likely because, although permanent dispersal between groups is infrequent, badgers frequently mate between groups, and more than 40% of cubs (268/655) were fathered by extra-group males (Annavi et al. 2014). Furthermore, I identified the novel MusGHV-1 variant also in earlier blood samples collected back in 2009 and 2010 (i.e., a decade earlier than my own sampling efforts). It is implausible that this strain had no opportunity for transmission beyond these three groups during the intervening years. Therefore, I tentatively conclude that the different distributions of the two variants reflect differences in viral variant-specific susceptibility to infection between badgers (and their groups). This difference could arise from immune-related genes, such as the MHC genes. In fact, specific badger MHC haplotypes have been confirmed to coincide with higher or lower-than-average MusGHV-1 blood viral loads (Sin et al. 2014; Sin, Dugdale, Newman, Macdonald, et al. 2012; Sin, Dugdale, Newman, MacDonald, et al. 2012). Therefore, I propose

future research to investigate whether MHC haplotypes can act as a risk factor of susceptibility to specific MusGHV-1 strains.

Another way to explore the phenotypic differences in these two pathogen variants is to compare their genomes and perhaps reveal regions with higher variance; this would identify genes under higher positive selection. Such genes can be considered critical to virulence and are typically associated with genes involved in viral replication or entry to cells. However, only partial genomes compromising the DNA polymerase gene and the glycol protein B gene are currently available on the GenBank. I therefore attempted to send two extracted blood samples containing the highest level of viral DNA of either MusGHV-1 variant (as determined by qPCR results) for sequencing using both Illumina and nanopore technologies to obtain their complete genomes. However, due to lack of specific equipment and facility for virus culturing, I could not purify sufficient genetic MusGHV-1 material from host blood samples (i.e., the samples were highly contaminated with host DNA) to successfully sequence the full-genomes of both MusGHV-1 isolates. The fact that the gammaherpesvirus resides inside lymphocytes in low volume in immune-competent individuals makes virus isolation from host DNA through filtration particularly challenging. Even under ideal conditions, only a small volume can be recovered (Dollard et al. 2013). In future, a possible solution might lie in virus culture using mustelid cell lines for viral particle enrichment in preparation for sequencing the MusGHV-1 full-length genomes (Sinzger et al. 1999).

In summary, the one bacterial pathogen I investigated was sufficient to demonstrate important coinfection dynamics with MusGHV-1, and doubtless such interactions occur with a wide diversity of bacteria. The predisposing position of MusGHV-1 reactivation that I discovered raises the probability that MusGHV-1 may play an important role in the complex dynamics of multi-pathogen coinfection and host immune regulation. Furthermore, my discovery that the two MusGHV-1 variants that I identified in my thesis have different effects on *C. perfringens* proliferation, and on different blood viral load levels, has added another layer of complexity to the interplay of co-infections. The difference in the prevailing dominant variant in different badger social groups could indicate that host-specific differences, likely in immune-related genes (such as MHC genes), could be a selection pressure promoting the emergence of the novel variant in Wytham.

Why are STIs of concern in the context of wildlife conservation?

Although many modelling studies predict the theoretical ability of STIs to cause extinction (Antonovics et al. 2011; Best et al. 2012; Ryder et al. 2005), such events are rarely observed in nature (Brearley et al. 2013). In fact, STIs commonly occur at stable prevalence in animals (Lockhart et al. 1996). This is likely because endemic wildlife pathogens coevolve with their host

species and maintain a balance between host survival and pathogen transmission as the outcome of a genetic arms race. However, this harmony is open to disruption by anthropogenic interference, such as human development and human induced rapid environmental change (HIREC) (Sih 2013). In the worst-case scenario, it can in principle cause disease-driven local extinction of wildlife populations. I thus conclude that the impact of STIs has been largely underestimated in wildlife studies. The present thesis demonstrated that a ubiquitous STI could affect female reproduction in apparently healthy animals. Such an effect could be enhanced when the host experiences stress or other predisposing factors such as aging or prenatal epigenetic modification/ programming. Finally, as reduced fecundity is commonly found in wildlife populations affected by human-modified landscapes (Brearley et al. 2013), future studies should be mindful of a possible resurgence/ increase in STIs, as demonstrated in this thesis. I conclude that clarifying the impacts of STIs on population dynamics is a priority for vulnerable wildlife populations and should be kept in mind when formulating long-term wildlife management plans.

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Appendix I: An overview of pathogens found to be sexually transmittable in musteloid species

Viruses

1. Herpesvirus

Herpesviruses are common across mammals, comprising numerous host-specific species and strains, a number of which have been proven to be sexually transmittable (e.g., herpes simplex virus 2 in humans, equid herpesvirus 3 in horses (Ferreira et al., 2010), suid herpesvirus 1 in feral swine (Romero et al., 1997) , bovine herpesvirus 1 in cattle (Muylkens et al., 2007) and canine herpesvirus in domestic dogs: Decaro et al., 2008), and some studies have confirmed cross-species transmission, e.g. of suid herpesvirus (a pseudorabies virus) from swine to mink and raccoons (Marcaccini et al., 2008; Mettenleiter et al., 2019; Thawley & Wright, 1982) through consuming infected swine organs. One of the common consequences of herpesvirus infection is reproductive failure (abortion, still birth, infertility, neonatal disease/ death), which has been well-studied in humans and domestic animals (Nigro et al., 2011; Smith, 1997), but remains under-investigated in wildlife species.

In carnivores, occasional reports from clinical cases, carcasses and field sampling over the past few decades indicate that herpesvirus may be widespread in this order (Cabello et al., 2013; Gaskell & Willoughby, 1999). In musteloids, most of the identified herpesviruses belong to the *Gammaherpesvirinae*, which include MusGHV-1 commonly found in European badgers (*Meles meles*) in the UK and Ireland (Kent et al., 2018; King et al., 2004), *fisher herpesvirus* (confirmed in a male captive fisher (*Martes pennanti*): Gagnon et al., 2011), but also several species of alpha-, beta-, and gammaherpesviruses in martens (*Martes americana*) (Dalton et al., 2017), *mustelid gammaherpesvirus 2* in Northern sea otters (*Enhydra lutris kenyoni*) (Tseng et al., 2013) and a gammaherpesvirus in Asian small-clawed otters (*Aonyx cinerea*) (Cabello et al., 2013). In addition, ferrets are confirmed as a natural host of bovine rhinotracheitis virus (Kiupel & Perpiñán, 2014), a member of *Alphaherpesvirinae*.

Despite of the high prevalence, there is a notable lack of reports in musteloids of lesions caused by herpesviruses, although suspected herpesvirus-associated lesions were found post-mortem on the tongue of stranded Northern sea otters (Tseng et al., 2013) and the skin of a fisher (Gagnon et al., 2011). Both animals experienced stressful events (oil spill, followed by changes in their captive rehabilitation environments) before their deaths, substantiating that also in musteloids pathogenicity and reactivation of herpesviruses are likely related to psychological and/ or physical stress as previously confirmed in other host families (Glaser & Kiecolt-Glaser,

2005). Nonetheless, none of the above reports suggest herpesvirus infection to affect host mortality rates.

Although none of the above mustelid herpesviruses have been proven to be sexually transmittable, MusGHV-1 DNA has been isolated using conventional PCR from genital swabs with 55% prevalence (54/98) in a free ranging badger population (Kent et al., 2018), suggesting a strong potential for horizontal transmission between mating partners, and the confirmed infection of young, prepubescent cubs indicates the likelihood of vertical infection before or during birth.

2. Parvovirus

Parvovirus infection is an important infectious disease in carnivores. In dogs and cats, canine and feline parvoviruses can cause fatal systematic diseases, and both have been proven to infect many other Carnivora (Steinel et al., 2001).

Parvovirus species that infect musteloids include the Aleutian mink disease virus (AMDV), which belongs to the genus *Amdoparvovirus*, reported in American mink and several other domestic and feral mustelid species; *mink enteritis virus* (MEV) in American river otters (*Lutra canadensis*), skunks (*Mephitis mephitis*) and ferrets; *feline panleukopenia virus* (FPV) in raccoons and coati (*Nasua nasua*); and *canine parvovirus 2a* (CPV-2a) in stone marten (*Martes foina*) (Steinel et al., 2001).

Among the above species, AMDV poses the most serious threat to both farmed and free-ranging populations, due to its high pathogenicity and ability to cause reproductive failure (Reichert & Kostro, 2014). Although asymptomatic infection has also been observed (Pennick et al., 2005), AMDV typically causes chronic, persistent and severe immune dysregulation disease (Eklund et al., 1968; Storgaard et al., 1997). First described in domesticated American mink in 1956 (Porter et al., 1980), it was likely spread to the wild by escaped farmed mink (Nituch et al., 2011). Seropositive results and molecular detection of AMDV have been reported in ferrets (Canuti et al., 2015), European mink (*Mustela lutreola*) (Fournier-Chambrillon et al., 2014; Mañas et al., 2001), European polecats (*Mustela putorius*) (Fournier-Chambrillon et al., 2014; Knuuttila et al., 2015), stone martens (Fournier-Chambrillon et al., 2014), pine martens (*Martes martes*) (Fournier-Chambrillon et al., 2014), common genets (*Genetta genetta*) (Fournier-Chambrillon et al., 2014), European badgers (Knuuttila et al., 2015), Eurasian otters (*Lutra lutra*) (Mañas et al., 2001), North American river otters (*Lontra canadensis*) (Farid, 2013), short-tailed weasels (or stoats, *Mustela ermine*) (Farid, 2013), striped skunks (Farid, 2013; Giannitti et al., 2017) and raccoons (Farid, 2013).

Other amdoparvoviruses that are closely related to AMDV include *skunk amdoparvovirus* (SKAV) infecting free-ranging striped skunks (Canuti et al., 2017; Glueckert et al., 2019), *and red panda amdoparvovirus* (RpAPV) infecting captive red pandas (*Ailurus fulgens*) (Alex et al., 2018). Both, SKAV and RpAPV can cause subclinical, mild or fatal diseases in the infected host species.

Parvoviruses are predominantly transmitted through the faecal-oral route and are not usually considered as a sexually transmitted pathogen. However, the porcine parvovirus is frequently isolated from wild boar semen, and has been proven to be transmittable through artificial insemination (Maes et al., 2016). It has also been isolated from the urine of a domestic ferret and transmission through urine is confirmed in farm mink (Gorham et al., 1964; Pennick et al., 2005), indicating a potential transmission risk during mating. Vertical transmission of AMDV through the placenta has been confirmed experimentally in farmed mink (Broll & Alexandersen, 1996; Farid et al., 2018).

AMDV can cause significant reproductive failure in naturally infected asymptomatic farmed mink with significantly higher rates of infertility, still birth, abortion and neonatal mortality (Reichert & Kostro, 2014); in addition, litter size and litter weight are substantially lower in AMDV seropositive females. Pathogenicity and impact of AMDV in other free-ranging susceptible hosts, however, remain largely unknown.

3. Canine distemper virus

Canine distemper virus (CDV), a Morbillivirus, is considered one of the most important viruses infecting carnivores and can cause severe and acute respiratory, gastrointestinal and neurological diseases (Deem et al., 2000). Likely all musteloids are susceptible to CDV infection, but susceptibility appears particularly high in black footed ferrets (*Mustela nigripes*) (Williams et al., 1988). CDV infections have also been confirmed in domestic/ feral ferrets (Trebbien et al., 2014), American mink (Trebbien et al., 2014), fishers (Brown et al., 2006), raccoons (Roscoe, 2013), European badgers (Di Sabatino et al., 2016), Asian small-clawed otter (Bosschere et al., 2005), European mink, European otters, North American river otters , southern sea otters, American badgers (*Taxidia taxus*), Chinese ferret badgers (*Melogale moschata subaurantiaca*), striped skunks, stoats, least weasels, Siberian weasels (*Mustela sibirica*), pine martens (*Martes martes*), stone martens, Japanese martens (*Martes* melampus), European polecats, greater grisons (*Galictis vittata*), lesser grisons (*Galictis cuja*), wolverines (*Gulo gulo*), South American coatis (*Nasua nasua*), Kinkajou (*Potos flavus*), Cozumel raccoons (*Procyon pygmaeus*) and red pandas (Martinez-Gutierrez & Ruiz-Saenz, 2016).

CDV transmission typically occurs through exposure to aerosol or excreta, although all body fluids can act as potential transmission media, including urine (Elia et al., 2006). Venereal

transmission of CDV has not been recorded, but transplacental infection is documented in domestic dogs, and is typically associated with abortion and/ or neonatal death (Krakowka et al., 1977). Vertical transmission has also been reported in an infected wild ferret by molecular isolation of CDV from its foetus (Trebbien et al., 2014). A case of localized epizoonotic CDV outbreak causing mortality to four fishers in California recorded CDV infection in the urogenital tract, where immunohistochemistry (IHC) confirmed CDV presence and inclusion bodies in uterus, vagina, epididymis, skin, lung and urothelium (Keller et al., 2012).

Bacteria

1. Campylobacter spp.

Campylobacter jejuni, originated from chicken and other avian species, but also infects mammals and is now responsible for considerable economic losses in the livestock industry. Campylobacterosis is an important food-borne zoonotic disease that can cause enteritis and abortion in humans and domestic animals (Horrocks et al., 2009; Young et al., 2007). *C. fetus* is highly adapted to conditions in genital tracts and known to be sexually transmittable within livestock herds (Truyers et al., 2014). It causes venereal campylobacterosis in *Bovidae* that results in poor reproductive performance, abortion and embryo mortality. Sheep are also susceptible to *C. fetus* and related reproductive failure, although venereal transmission is rare in this species (West, 2002). Venereal transmission of *C. jejuni*, however, has been documented in homosexual men through faecal-oral contact (Arsalani-Zadeh et al., 2018).

In wildlife, occurrence and prevalence of *Campylobacter spp.* are poorly understood. A survey in Denmark identified a wide distribution of *C. jejuni* in wildlife, including 3 European badgers (Petersen et al., 2001). A survey in California has confirmed the presence of several *Campylobacter spp.* in the faeces of Southern sea otters' (6.5% prevalence, n=244). In captivity, campylobacterosis outbreaks in mink farms typically result in severe enteritis in kits caused by *C. coli* and reproductive failure in females by *C. jejuni*, where *C. jejuni* was also isolated from the liver of an aborted foetus, suggesting potential vertical transmission. Abortion caused by *C. jejuni* infection has been proven experimentally in mink and ferrets. (Bell & Manning, 1990b, 1990a; Hunter et al., 1983, 1986).

2. Brucella spp.

Brucellosis is one of the major domestic animal diseases associated with reproductive failure. It has been well-studied in cattle, goats, sheep, dogs and pigs and can be transmitted orally and/ or sexually depending on the host and the respective brucella species involved (Corbel et al., 1997; Hayward, 1974; Kim et al., 2006; Maes et al., 2008; West, 2002).

Brucellosis is well known to also affect wildlife species, particularly wild ruminants (Godfroid, 2002; Godfroid et al., 2013), causing reproductive disorders such as abortion, inflammation of reproductive organs and infertility. Although less often reported in carnivores, *Brucella canis* is known to cause abortions in domestic dogs and pose a considerable zoonotic risk to humans, and the knowledge on potential hosts of *Brucella spp.* is expanding rapidly focussing particularly on brucellosis transmission dynamics in the wildlife-livestock-human interface. A systematic review study conducted by Kosoy & Goodrich (Kosoy & Goodrich, 2019) has listed several musteloid species that tested seropositive, PCR-positive or cultural-positive of *Brucella spp.*, including Northern sea otters, Southern sea otters (Hanni et al., 2013), Asian sea otters (*Enhydra lutris keyoni*), wolverines, Eurasian otters, Asian badgers (*Meles leucurus*), stoats, European mink, American mink, American badgers (*Taxidea taxus*), striped skunks, Western spotted skunks (*Spilogale gracilis*), brown-nosed coati (*Nasua nasua*) and raccoons (Kosoy & Goodrich, 2019; Martino et al., 2017; Miller et al., 2017). Captive European badgers have also been confirmed experimentally to be susceptible to *B. abortus* (Corbel et al., 1983).

The pathogenicity and epidemiology of brucellosis in wild musteloids, however, still lack systematic study. A detailed pathology report of a stranded Southern sea otter infected with a *Brucella spp.* that was shown to be closely related to the marine strain commonly found in other marine mammals and humans, was published in 2017 (Burgess et al., 2017), where the authors observed multiple granulomas in the otter's joints and spinal cords, which matched *Brucella* lesions in other animals (Miller et al., 2017). A different study isolated *Brucella spp*. by culture from the internal iliac lymph node of a Eurasian otter, but no strain differentiation was conducted (Foster et al., 1996). Abortion, neonatal death and smaller litters associated with *B. abortus* infection have been reported in farmed mink, where successful isolation from foetuses suggests (also) vertical transmission (Prichard et al., 1971; Tessaro, 1986).

3. Salmonella

Salmonella enterica is another important zoonotic food-borne pathogen known to cause serious enteritis and reproductive diseases. There are 6 *Salmonella ssp.* with over 2,600 serotypes which can infect a wide range of vertebrate hosts. In birds, Salmonella enterica is a confirmed STI(Lombardo, 2016), and although no direct evidence for horizontal sexual transmission has been recorded in any musteloid, vertical transmission was suspected in mink in an outbreak in several Danish farms causing host mortality, abortion and neonatal death (Dietz et al., 2006).

Salmonella infection has been confirmed in several *Musteloidea*. Some of those were asymptomatic, including a study in Italy that isolated *Salmonella* from lymph nodes and/ or faeces by microbiological culture in wild badgers, stone martens, martens and polecats and showed an average prevalence of 11.3% (25/221), while several similar studies in the UK

reported a 7.2% (350/4881) prevalence of *Salmonella* in wild badgers (Botti et al., 2013; Euden, 1990) and 75% (18/25) prevalence on badger social group level(Wilson et al., 2003); 3% (1/30) prevalence in Southern sea otters (W. A. Smith et al., 2002); 47% (n=132) prevalence in captive raccoons in a zoo environment, 27% (n=30) prevalence in an rural site and 65% (n=20) in an urban site (Jardine et al., 2011); and also confirmed *Salmonella* in one hooded skunk in a zoo (Silva-Hidalgo et al., 2012), and ferrets in captivity (Morris & Coburn, 1948). Another one reporting 63.6% (n=44) seropositive rate in free-ranging white-nosed coatis (*Nasua narica*) and 67.9% (n=56) in raccoons in Mexico (De-La-Rosa-Arana et al., 2016).

Salmonella was isolated also post-mortem from a Eurasian otter that died from hepatitis(Capber, 2007), a Libyan striped weasel (*Poecilictis libyca*) that died from enteritis (Fiennes, 1961), an American river otter that died of unknown causes during rehabilitation (Hoover et al., 1985), and was isolated from liver tissue in a skunk, although no symptoms were reported in this case (Kinde et al., 1996). One study reports *Salmonella* sp. infection in a free-ranging stoat in Switzerland (Akdesir et al., 2018).

4. Clostridium spp.

The genus *Clostridium* is common in natural environments such as soil, water and marine sediments. It can cause infection and disease in animals through wound contamination or ingestion. In humans, *C. difficile* can be transmitted sexually and verticaly (Tabaqchali et al., 1984) and is commonly cultured from patients attending venereal disease clinics (Hafiz et al., 1975), and asymptomatic infection in neonates (Jangi & Lamont, 2010). It is also transmittable to neonates during birth via the vaginal tract (Tabaqchali et al., 1984).

Two large scale *C. limosum* outbreaks have been reported affecting multiple mink fur farms in Denmark and Finland (Biström et al., 2016; Hammer et al., 2017). Both events reported mortality and metritis in pregnant females, and infected animals presented with abortions, reduced litter sizes, ruptured uterus, haemorrhagic vaginal discharge and/ or dead foetuses in the peritoneal cavity.

Although *Clostidium* is considered as one of the normal microbes in the genital tract of female dogs (Graham & Taylor, 2012), some *C. spp* have been reported to cause disease as well as asymptomatic infection in several Musteloids, namely American river otters (Kimber & Kollias, 2000), Eurasian otters (Simpson et al., 2008), Southern sea otters (Miller et al., 2010), Asian small-clawed otter (Watanabe et al., 2019), European badgers (Di Sabatino et al., 2016), ferrets (Powers, 2009), black-footed ferrets (Schulman et al., 1993), stone martens (Akdesir et al., 2018), raccoons (Bondo et al., 2015; C. M. Jardine et al., 2013), South American coatis (Silva et al., 2016) and red pandas (Philippa & Ramsay, 2011).

5. Opportunistic bacteria or endogenous bacterial infection

All body sites, including the reproductive tracts, harbour a wide variety of microbiota (Ursell et al., 2012), particularly bacteria, These often comprise also potentially harmful species, such as *Escherichia coli*, *Staphylococcus spp.*, *Streptococcus spp.*, *Proteus spp, Plesiomonas spp.* and *Enterococcus spp.* (Graham & Taylor, 2012), that are part of the urogenital microflora in healthy animals, and are thus not normally pathogenic. Horizontal transmission of these bacteria is common between sexual partners, as well as vertical transmission to neonates through the vaginal tract during birth (Mändar & Mikelsaar, 1996).

Nevertheless, in case of predisposing circumstances (e.g., physical trauma/ injury (Von Graevenitz, 1977), changes in the normal microbiome composition (Eloe-Fadrosh & Rasko, 2013), primary viral infections (Dezzutti et al., 2012) etc), pathogenetic strains can invade the genital tract from the intestine and cause opportunistic infection and diseases such as pyometra, metritis, mastitis, vaginitis, prostatitis, abortion or neonatal septicaemia (Graham & Taylor, 2012; Järvinen, 1980).

Infections and diseases of reproductive organs caused by opportunistic bacteria have been reported in several musteloids, and are commonly found during systematic pathogen surveys in wildlife or veterinary records from zoos, rehabilitation centres and fur farms (Aarestrup, 2001; Akdesir et al., 2018; Britton et al., 2017; Counihan-Edgar et al., 2012; Di Sabatino et al., 2016; Escobar-Páramo et al., 2006; Fiennes, 1966; Kimber & Kollias, 2000; Mallon et al., 2002; M. Oliveira et al., 2008; Orcutt, 2003; Rewel, 1948; Schulman et al., 1993; Simpson, 1997; Fiennes, 1961; Vulfson et al., 2001, 2003; White et al., 2018; Williams et al., 2012).

Pathogenic *E. coli* and *Proteus mirabilis* (a member of the normal vaginal microbiome in dogs: Groppetti et al., 2012) have been isolated post mortem by culture from a ferret vagina (Marini et al., 2004), and from a dead farmed mink with uterus rupture (Graham & Taylor, 2012; Yang et al., 1995). *P. mirabilis* was also isolated by culture from the mucopurulent vaginal discharge of two American river otters, one of which also tested positive for *E. coli* (Hoover et al., 1985). *Streptococcus phocae*, known to cause diseases in marine mammals (Higgins, 2000), was isolated from oral and genital swabs as well as lesions of stranded Southern sea otters (Imai et al., 2009), and severe neutrophilic inflammation of the vaginal mucosa in a free-ranging raccoon was confirmed by culture to be caused by *Streptococcus canis* (Rentería-Solís, 2017). *Staphylococcus intermedius* was found in the vagina and the milk of lactating American mink females with kits that suffered from adenitis of the cervical apocrine glands (Hunter & Prescott, 1991), and a case of pyometra caused by heavy infection of *Enterococcus faecalis* has been reported in a captive Siberian steppe polecat with suspected infertility (Johnson et al., 1999). Finally, a case reported

an aborted foestus from Eurasian otters found in the field and *Plesiomonas shigelloides* has been isolated from the foetus by culturing (Weber & Roberts, 1989).

Protozoa

1. Toxoplasma gondii

Toxoplasma gondii is a globally distributed intracellular zoonotic and pathogenic protozoan that can infect most vertebrate cells, although felids are the only definitive hosts; i.e., *T. gondii* can only reproduce sexually in felid intestines and oocysts are then excreted in cat faeces to be ingested by an intermediate host (including humans). In the natural world, intermediate hosts will be consumed by predatory felids to complete the sylvatic (i.e., epizootic) cycle. Infections with *T. gondii* are mostly asymptomatic (Tenter et al., 2000).

Aside from ingestion of oocysts, *T. gondii* can also be sexually between intermediate hosts. Horizontal transmission by semen and vertical transmission from AI infected female dogs to their offspring via the transplacental route are experimentally confirmed in domestic dogs (Arantes et al., 2009). Confirmed sexual transmission of *T. gondii* has been raising concerns in human health as unprotected sexual intercourse can induce congenital toxoplasmosis, the most severe form of infection of *T. gondii* to pregnant women and new-born babies (Flegr et al., 2014).

T. gondii has also been reported in many musteloid species, including American mink(Barros et al., 2018; Burrells et al., 2013), black-footed ferrets (Burns et al., 2013), Southern river otters(Barros et al., 2018; Gaydos et al., 2007), Southern sea otters (Hanni et al., 2013; Miller et al., 2010), Northern sea otters (White et al., 2018), Eurasian otters (Sobrino et al., 2007), spotted-necked otters (*Hydrictis maculicollis*)(Cano-Terriza et al., 2020), European mink (Shamaev et al., 2020), polecats (Akdesir et al., 2018; Burrells et al., 2013), stoat (Burrells et al., 2013), stone martens (Sobrino et al., 2007), pine martens (Sobrino et al., 2007), fishers (Philippa et al., 2010), ferrets (Burrells et al., 2013; Powers, 2009; Sobrino et al., 2007), ferret badgers (Chen et al., 2017), Eurasian badgers (Akdesir et al., 2018; Burrells et al., 2013; Sobrino et al., 2007), raccoons (Dubey et al., 1992, 1993, 2008), coatis (Minervino et al., 2010), white-nosed coatis (Torres-Castro et al., 2019), great grisons (Torres-Castro et al., 2019), striped skunks (Dubey et al., 2008) and red pandas (Cano-Terriza et al., 2020; Yurong Yang et al., 2019). Particularly farmed mink and farmed ferrets are prone to *T. gondii* outbreaks where symptoms typically include some form of reproductive failure: For example, congenital toxoplasma infection was confirmed to be the cause of large-scale neonatal ferret deaths in a New Zealand fur farm (30%, n=750: Thornton & Cook, 1986), and a total of 10,408 abortions and high neonatal death rates were attributed to *T. gondii* infection in an American mink farm (Frank, 2001). Isolation of *T.gondii* in neonate organs evidences the potential of vertical transmission (Śmielewska-Łoś & Turniak, 2004; Thornton, 1990).

Other musteloid pathogens that have potential to be sexually transmittable

1. *Mycobacterium* spp.

Being responsible for around 1.3 million human deaths annually and affecting 7.6% herds of domestic cattle in the United Kingdom, with increasing incidence and mortality also in wildlife species, tuberculosis or mycobacteriosis caused by *Mycobacterium spp.* has become a major health concerns in humans and animals (Perrin et al., 2019; Schrenzel, 2012; WHO, 2016). Bovine tuberculosis caused by *M. bovis* results in substantial annual economic losses to the dairy industry, where European badgers have been implicated as a self-sustaining natural wildlife reservoir in the UK and Ireland (Phillips et al., 2003). Other musteloid hosts susceptible to *Mycobacterium spp.* include polecats (R. J. Delahay et al., 2007), stoats (Coleman & Cooke, 2001; Delahay et al., 2007), least weasels (Coleman & Cooke, 2001), ferret (Coleman & Cooke, 2001; Delahay et al., 2002), American mink (Richard J. Delahay et al., 2002), Eurasian otters(Matos et al., 2019), Asian small-clawed otters (Lee et al., 2015), raccoons (Bruning-Fann et al., 2013; Corn et al., 2005) and skunks (Corn et al., 2005).

Although many studies focus on *Mycobacterium spp*. (especially *M. bovis* and *M. tuberculosis*), surprisingly, *M. spp.* epidemiology and symptomatics are still poorly understood in wildlife species. In *M. bovis* and *M. tuberculosis*, transmission is mainly through droplet infection and aerosol inhalation of contaminated excretions (Genewein et al., 1993; Menzies & Neill, 2000). In humans, both species can cause urogenital tuberculosis and infertility, and the pathogens can be transmitted through sexual intercourse (Jacob et al., 2008; Kulchavenya, 2014; Namavar Jahromi et al., 2001; Neonakis et al., 2011).

No evidence of sexual transmission has been recorded in badgers thus far, but vertical transmission from parents to cubs could play an important route to maintain tuberculosis in the population (Anderson & Trewhella, 1985; Cheeseman et al., 1988). Although rare, in cattle, vertical and pseudo-vertical (calves infected by pathogens excreted in the genital tracts while giving birth) transmission of *M. bovis* from mother to calves have been recorded, and sexually transmission is possible (Menzies & Neill, 2000). In addition, it is well established that bulls infected with *M. avium ssp. paratuberculosis* (Johne's disease) can shed these pathogens in semen and may thus act as a source of infection to the breeding herd (Münster et al., 2013). In ferrets, aggressive mating behaviour has also been suggested to promote horizontal transmission of *M. bovis* (Qureshi et al., 2000) in an experimental study.

2. Circovirus

Circoviruses are emerging infectious pathogens in domestic and wild carnivores (Zaccaria et al., 2016). Horizontal transmission of porcine circovirus, an important disease in swine, occurs predominantly through body fluids and vertical transmission through uterine infection (Caprioli

et al., 2006; Madson & Opriessnig, 2011; Patterson & Opriessnig, 2010). Presence of the virus has been reported in boar semen, and sows can potentially get infected through artificial insemination (AI) using contaminated semen, potentially resulting in reproductive failure (Larochelle et al., 2000; Madson et al., 2009; Madson et al., 2009), although infection risk appears to be highly dose dependent.

Mink circovirus (MiCV) was discovered in 2013 in a Chinese fur farm and has been associated with frequent outbreaks across several farms resulting in acute fatal gastroenteritis in mink (Lian et al., 2014). In the same farms, cross-species infections were also confirmed between mink, raccoon dogs (*Nyctereutes procyonoides*) and foxes (*Vulpes vulpes)* (Yang et al., 2018). Circovirus epidemiology, prevalence, pathology and transmission are still largely unknown. A molecular epidemiological survey in Italy, however, reported that 1 out of 10 European badgers (=10% compared to 9/34= 28.5% of free-ranging wolves) tested positive using circovirus-specific PCR, where the amplified sequence was closest to the canine circovirus (DogCV) (Zaccaria et al., 2016).

3. Leptospirosis

Leptospirosis caused by *Leptospira interrogans sensu lato* is a globally distributed zoonotic disease that can infect a wide range of vertebrate hosts. The symptomology of leptospirosis infections varies from asymptomatic to lethal pulmonary haemorrhage. Transmission occurs predominantly through direct or indirect contact with urine from reservoir hosts (Bharti et al., 2003). but *Leptospira* is also commonly isolated from the reproductive organs of cows, sheep, sows, horses, and confirmed to be sexually and vertically transmittable (Director et al., 2014; Hamond et al., 2013; Heinemann et al., 1999; Kim et al., 2006; D. Maes et al., 2008).

Many musteloid species are reported to be susceptible to *Leptospira* infection: high seroprevalence rates have been found in European mink, stone marten, pine martens, common genet and American mink (Moinet et al., 2013), and North American river otters (Gaydos et al., 2007); a low seroprevalence rate was found in the Southern sea otter (*Enhydra lutris nereis*) (Hanni et al., 2013), and leptospirosis was confirmed as the cause of death in several Northern sea otters (*E. lutris kenyoni*) (White et al., 2018). In a molecular epidemiological study, *Leptospira spp.* has been isolated from the kidneys of European badgers, stone martens, European pine martens, European polecats, least weasels (*Mustela nivalis*) and stoats (Ayral et al., 2016), although in these species no symptoms have been linked to *Leptospira spp*. infection thus far.

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Appendix II: Reference list for Table 1 of Chapter 1

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Appendix III: Post-mortem report of badger 1469

On 14th March 2020, a badger was reported dead on a pasture adjacent to the River Thames, about 320 meters from the edge of Wytham Woods (Oxfordshire), c. 770 meters away from its sett of residency (Figure 1A). The carcass was very fresh and collected on 16th March (average maximum outdoor temperature between 14th - 16th March was 11.7˚C and the minimum temperature was 2˚C, with no precipitation). This badger was identifiable from the tattoo on its inner thigh as belonging to the long-term study population monitored continuously at Wytham Woods since 1987 (Macdonald et al. 2015). This tattoo, '1469' (Figure 1B), enabled the female to be matched to its previous trapping record: Since her birth in 2013, this female had been captured 7 times in total during her life, each time at the same social group. She showed signs of lactation in 2016 and 2018, but with no offspring assigned by genetic pedigree. A relatively large-bodied female, this badger had exhibited consistently thin body-condition throughout its capture history, although when last caught on 20th Nov 2019 it was very fat, with a maximum body-condition score, weighing 9.9kg, with a minor healed wound on its rump. It was administered an intramuscular dose of BCG vaccination (Sofia) against bovine tuberculosis (bTB) infection.

A simple dissection and postmortem examination were conducted in the field to examine lungs, liver, kidneys, heart, stomach, intestine and reproductive system and ascertain possible cause of death. Uterine scars were observed, indicative of the animal having given birth to 3 cubs in the preceding spring (Figure 2A). The animal's body condition was very poor with no subcutaneous fat. The stomach was completely empty, and no food content or nematodes were found throughout the rest of the gastrointestinal tract. The intestine, especially the ileum, was enlarged and loss of structural integrity, filled with gas, necrotizing and heamorrhagic on the surface (Figure 2B & 3C). Fresh samples of liver, lung, kidney and ileum were collected and stored at -20˚C for later laboratory examination. Part of the ileum was also preserved in 10% formalin for histological examination.

Microscopic examination of a gram stain slide of ileum mucosa revealed a predominance of gram-positive bacilli (Figure 3). Clinical presentation and bacterial morphology caused us to suspect that this heamorrhagic necrotic enteritis was caused by *C. perfringens*. We therefore performed a multiplex PCR to identify the variant(s) (primer list in Table 1) of the pathogen involved, which confirmed presence of *C. perfringens* type A, where only the α toxin gene *cpa* was successfully amplified by PCR but no other toxin genes. We also performed PCRs to detect and differentiate other potential enteric pathogens including *Aleutian mink disease virus* (AMDV)

(Jensen et al. 2011), *canine circovirus* (primers designed using canine circovirus replication associated protein gene), *mustelid gammaherpesvirus 1* (MusGHV-1) (King et al. 2004), *Clostridium difficile* (multiplex PCR to determine pathogenicity via detection of toxin gene A and B) (Persson et al. 2008), *Brucella spp.* (Mukherjee et al. 2007), *Salmonella spp.* (Sin et al. 2014), *Campylobacter spp.* (Yamazaki-Matsune et al. 2007) and *Mycobacterium spp.* (multiplex PCR to detect *M. tuberculosis* complex species, or other *Mycobacterium* species) (Mokaddas and Ahmad 2007). Only MusGHV-1 and *C. difficile* returned positive results, but *C. difficile* was determined to be non-pathogenic due to the absence of toxin genes A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes (Figure 4). The PCR product of MusGHV-1 was then sent for genotyping using Sanger sequencing and classified as the common variant.

The microscopic examination of histology shows cell autolysis of the villus and epithelium indicating severe postmortem changes (Figure 5, H&E stain), and large amount of gram-positive bacilli present in the edge of lysed cells (Figure 5, gram stain).

Figure 1: The female badger with tattoo number 1469 (B) found dead on a grassland near the River Thames (red arrow) about 770 meters from her sett of residency (yellow star) (A)

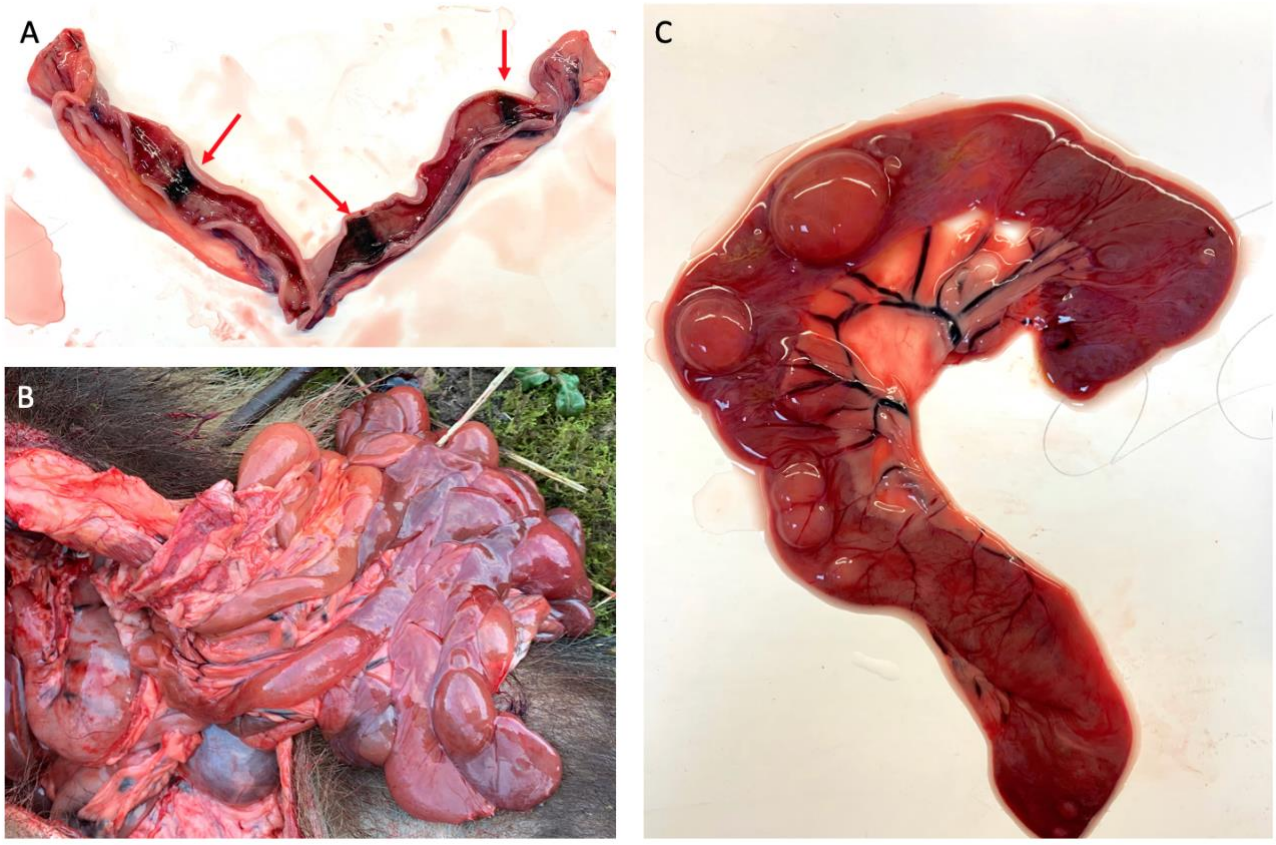

Figure 2: The fresh scars (red arrows) present in uterus of badger 1469 suggests that she had recently given birth to 3 cubs (A). The intestines of the badger were severely necrotic, enlarged and filled with gas (B). A closer look of the ileum (C).

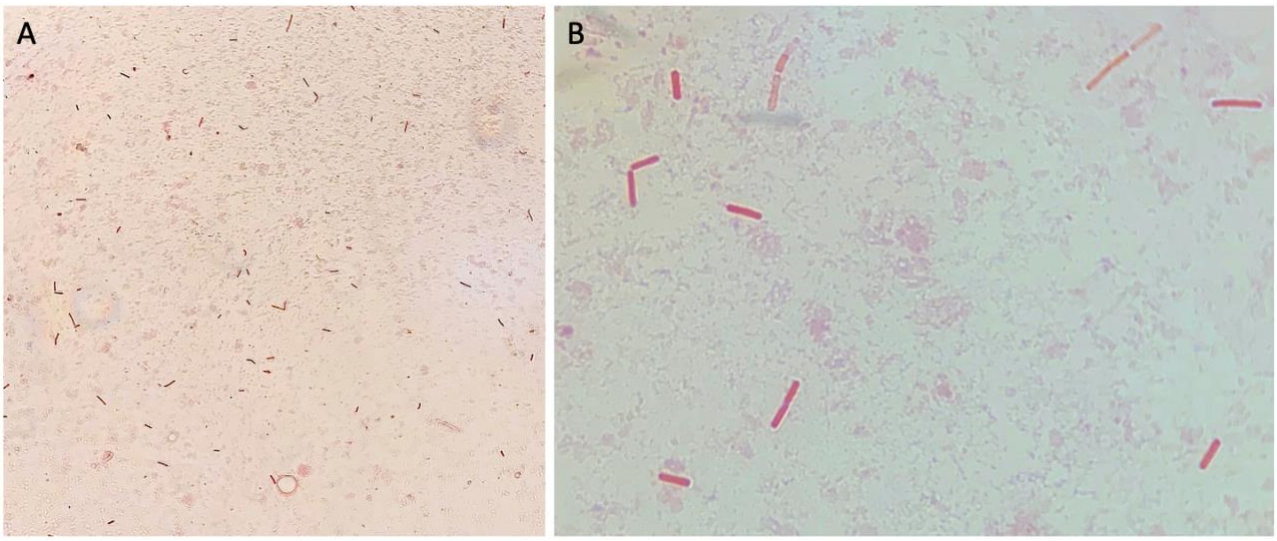

Figure 3: Gram stain revealed large amounts of rod-like gram-positive bacteria in the ileum mucous tentatively identified as *C.perfringens* under microscopic magnification of 40x (A) and 100x (B).

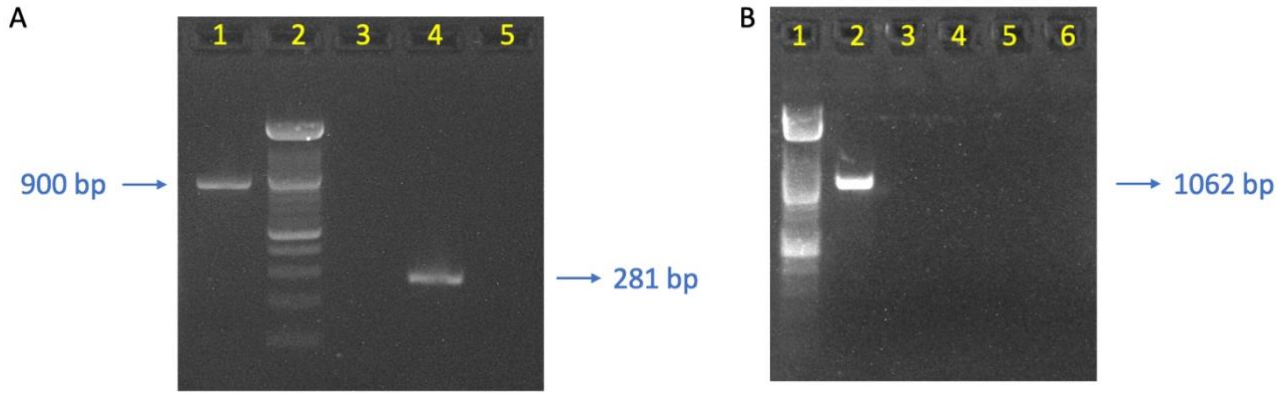

Figure 5: Gel images of the PCR amplification results of DNA extracted from 1469's ileum tissue sample targeting various pathogen genes. (A) Lane1: multiplex PCR of *C. perfringens* toxins, only amplification of *cpa* gene is detected; lane 2: Ladders of DNA with 100 bp as interval; lane 3: amplification of canine circovirus *rep* gene; lane 4: amplification of MusGHV-1 DNA polymerase gene; lane 5: negative control with nuclease-free water as template. (B)Lane1: DNA ladder, lane 2: Multiplex PCR of *C. difficile* 16srDNA gene and toxin gene, only the *C. difficile* specific 16s rDNA gene is detected; lane 3: amplification of *C. perfringens NetB* toxin gene; lane 4: amplification of C. perfringens *NetE* toxin gene; lane 5: amplification of *C. perfringens NetF* toxin gene; lane 6: amplification of *C. perfringens NetG* toxin gene

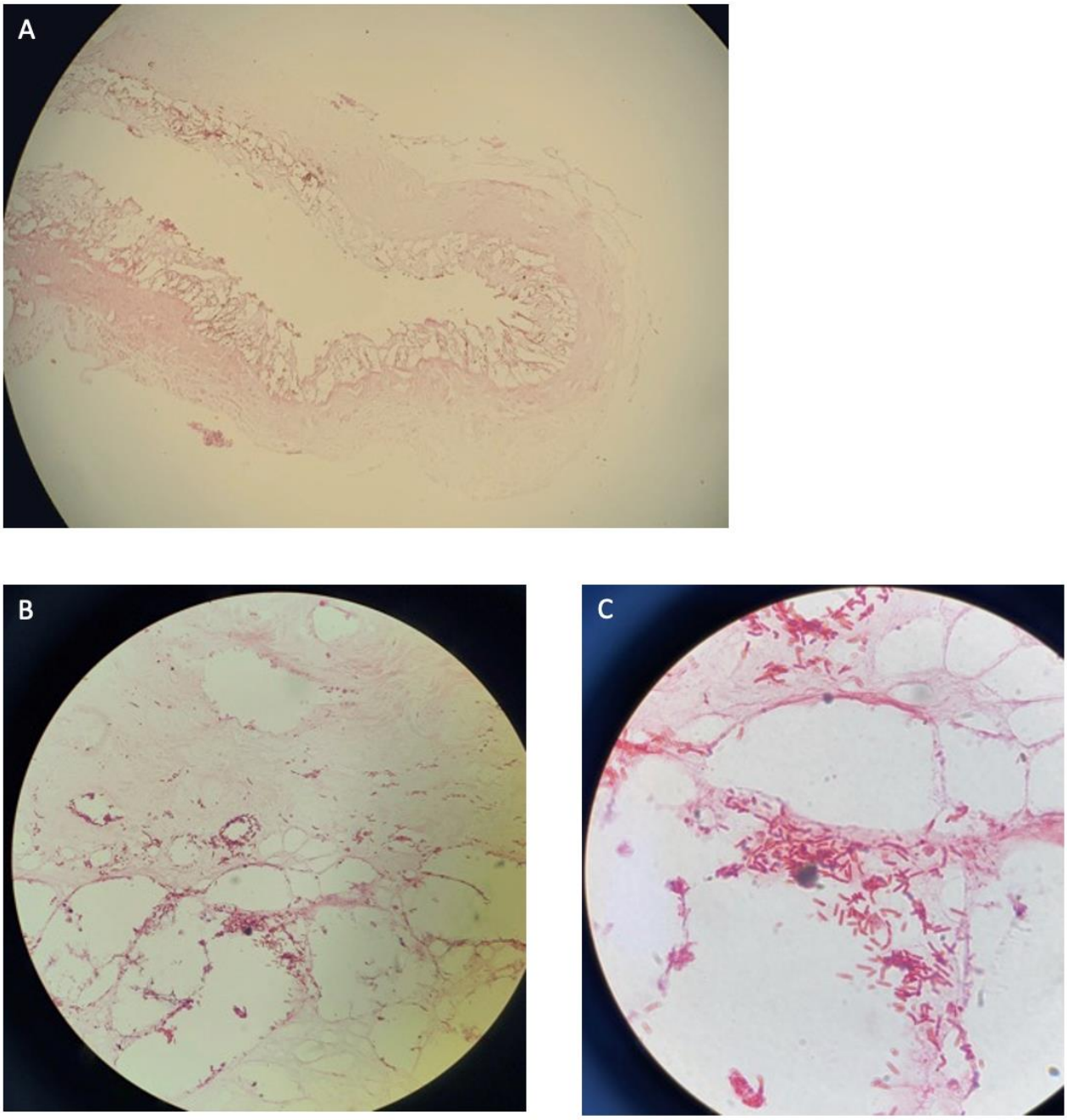

Figure 6: The histology pictures of the formalin-fixed ileum tissue at 4x (A) and gram-positive bacilli attached on lysed cells at 400x (B) and 1000x (A)

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