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Vaccination Efficacies against Myxomatosis

Evaluation of Various Administration Methods, Vaccine Strains and Vaccination Protocols in Wild, Farmed and Pet Rabbits

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Abstract

This literature review aims to consolidate the most recent scientific findings on the efficacies of different vaccination methods, vaccine strains, and protocols against myxomatosis used in rabbits. The scope of this research includes populations of wild, food-producing industrial, and companion pet rabbits. The review encompasses the pathophysiology of myxomatosis and the mechanisms and efficacies of different vaccines developed since the introduction of the myxoma virus in the 1950s. While occasional references are made to historical research, the main focus is on modern studies published within the last 30 years. Although some studies examine myxomatosis in conjunction with rabbit haemorrhagic disease (RHD), this review primarily focuses on myxomatosis. Conflicting results and controversial pitfalls in published studies are examined, and efforts are made to analyse discrepancies and identify potential gaps and areas of uncertainty for future research.

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List of Abbreviations

IFN-gamma	Interferon-gamma
IL6	Interleukin-6
MV	Myxoma virus
RHD	Rabbit haemorrhagic disease
RHDV	Rabbit haemorrhagic disease virus
SFV	Shope fibroma virus
TNF-alpha	Tumour necrosis factor-alpha

Introduction

This literature review aims to consolidate the most recent scientific findings on the efficacies of different vaccination methods, vaccine strains and protocols against myxomatosis used in rabbits, including populations of wild, food-producing industrial and companion pet rabbits. The scope of this topical research includes scientific knowledge of the pathophysiology of myxomatosis and the mechanisms and efficacies of the different vaccines that have developed since the importation and release of the myxoma virus into Australia and Europe in the 1950s. Occasional references are made to the historical research conducted closer to the initial phase of the artificial viral release into the national territories, with the main focus being placed on more modern studies published within the last 30 years of 2023 (when this review paper is being conducted). Although many studies examine myxomatosis with another highly fatal infectious disease rabbits commonly contract, Rabbit Haemorrhagic Disease (RHD) in joint research (as evident in the most widely used commercial vaccine available in a clinical setting Nobivac that covers both MV and RHD in one injection), and hence it becomes inevitable for this literature review to include data and results related to RHD, myxomatosis remains the quintessential focus within the scope of this work. Following this linear time frame, the emergence of any conflicting results or controversial pitfalls in the published results are examined and scrutinised. Attempts are made to analyse the discrepancies and also to identify potential gaps and areas of uncertainty that will need further research for clarification and advancement as possible future approaches into the field.

Aetiology of myxomatosis

Causative agent

Myxoma virus (MV) belongs to the genus Leporipoxvirus of the family Poxviridae (Walker et al., 2020). The morphologically brick-shaped, large poxvirus contains its own DNA within its nucleocapsid core that encodes for a self-sustainable conservatory of structural and functional proteins associated with fundamental cellular processes and virulence (Kerr & McFadden, 2002). Members of the species MV, are highly adapted for long-term infection in their native rabbit hosts in the Americas, with mild disease manifestations as localised cutaneous fibromas that spontaneously regress. Yet the clinical syndrome of myxomatosis caused by MV appears in European rabbits as an acute fulminant disease, killing up to an estimated 98% of the European rabbit population in Australia when MV was first artificially introduced into the territory as a biological control eradication measure in the 1950s (Kerr et al., 2015). The co-evolutionary relationship between the virus and their hosts has since been noted as an interesting and ever-shifting dynamic for species' survivals that "has resulted in less virulent viruses and more resilient rabbits such that rabbit populations have gradually recovered" (McInnes et al., 2023).

A related virus, the Shope fibroma virus (SFV), also known as the "rabbit fibroma virus", occurs in the eastern cottontail (*Sylvilagus floridanus*). It does not cause serious disease in adult European rabbits; instead, it provides cross-protection as a vaccine against myxomatosis (Kerr & McFadden, 2002).

Transmission

Endemic outbreak patterns of MV on 4 continents – North and South America, Europe and Australia – suggest that the viruses are "in continuous recirculation and peak in association with rabbit breeding seasons in summer and autumn" (Pacios-Palma, 2018).

While the main source of infection is infected individuals and asymptomatic carriers via their urine, saliva, faeces, and secretions from the nose and eyes (Bertagnoli & Marchandeau, 2015; Meredith, 2013), the disease is also passively mechanically vectored by mosquitoes (*Aedes, Anopheles, Culex,* and *Simulium*), stable flies (*Stomyx calcitrans*), and fleas (*Spilopsyllus cuniculi*) (Fenner & Ratcliffe, 1965). Rabbit warrens are a key

element since they provide refuge for several flea species (Osácar-Jiménez et al., 2001). The crucial role of mechanical vectors in the primary spread and survival of MV is highlighted by the trialled but failed release in New Zealand due to a lack of arthropods (Kerr, 2012), although transmission via fomites has also been described (Meredith, 2013; Kerr et al., 2015; Dyce A.l., 1969).

Pathogenesis

Myxomatosis is the devastating consequence of astute immune suppression caused by the myxoma virus in the hosts for the betterment of the virus' survival. After skin inoculation, MV replicates at the inoculation site locally in MHCII+ cells and spreads within leukocytes to the lymph nodes within 24 hours before it further replicates inside lymphocytes to high titres and disseminates via the infected leukocytes to various organs systemically (Kerr et al., 2015). This leads to a progressive impairment of the host immune system that becomes uncontrollably supervened by bacterial or parasitic (e.g. helminths) infections, overwhelming and killing the host (Mykytowycz, 1959). There have been disagreements over what constitutes myxomatosis, as the disease is also known as a syndrome associated with induced toxaemia or cytokine storm following severe immunosuppression and secondary respiratory tract infection, ending in "multi-organ dysfunction" (Fenner & Marshall, 1957; Fenner, 2000; Fields et al., 2001; Moss, 2001).

Induced immune response

Elimination of MV infection is found to be correlated with the activation, stimulation and infiltration of macrophages and monocytes that engulf apoptotic neutrophils, a process which may be essential for the prevention of myxomatosis. As Best et al. (2000) found that "granulocytes predominate in infiltrates of lesions in myxomatosis-susceptible rabbits, while monocytes and macrophages predominate in infiltrates in disease-resistant rabbits", the differences highlight the infection-terminating inflammatory response – of macrophages phagocytising apoptotic and senescent neutrophils (Cox et al., 1995) – as potentiated by pro-inflammatory cytokines IFN-gamma and TNF-alpha (Ren & Savill, 1995) – in protecting tissues from leakage of noxious neutrophil products and presenting ingested antigens to T-cells (Zuniga, 2002). This is further supported by Kerr's suggestion

that innately resistant rabbits are not resistant to infection but are more likely to control the infection and able to make a rapid recovery (Kerr, 2012) due to a strong genetic expression of nitric oxide synthase that activates macrophages quickly (Kerr et al., 2004). By contrast, laboratory rabbits infected with the same virus all died between days 10 and 12 (Robinson et al., 1999) because of immunosuppression by MV (Cameron et al., 2005a; Cameron et al., 2005b).

Clinical signs

In its natural Sylvilagus hosts, MV causes relatively minor diseases as characterised by a cutaneous fibroma at the local site of inoculation, with no systemic spread in immunocompetent adult rabbits. The virus crosses over from the wildlife reservoir via blood-sucking arthropods to laboratory or farmed European rabbits (Kerr & McFadden, 2002), and causes the lethal systemic disease of myxomatosis in domestic and wild European rabbits (*Oryctolagus cuniculus*).

The main sites of pathology in the typical nodular form are in the lymphoid tissues and the skin, resulting in oedematous swelling of the eyelids, face, ears and anogenital area, and blepharoconjunctivitis with serous nasal and conjunctival discharge (Fenner & Ratcliffe, 1965; Moss, 2001).

The "atypical" amyxomatous form is more common in industrial-farmed rabbits (Cavadini et al., 2010). This clinically milder form is characterised by highly reduced cutaneous and respiratory lesions and ocular congestion; it generally causes immunosuppression but is non-lethal (Meredith, 2013).

Virus strains

The severity of myxomatosis differs by the virus strains and the resistance of the infected rabbit hosts.

MV strains are classified into 5 virulence grades (A to E, A being the most virulent and E the most attenuated) based on the mean survival time of rabbits after infection (Fenner & Marshall, 1957). Infection with the original Standard Laboratory Strain (SLS) resulted in "more drastic lymphocyte depletion" than the naturally attenuated Urraria (Ur) strain,

whereas the less virulent Ur strain had a "more robust inflammatory response consisting primarily of mononuclear cells" (Best & Kerr, 2000).

The resultant lethality of a highly virulent strain has, in turn, a harmful effect on the virus's survival in a host that is now deceased, prompting the wild-type strain to weaken its pathogenicity as part of a subsequent host-virus co-evolutionary response to establish "a new, and still-evolving, equilibrium of morbidity and mortality in the wild" (Stanford et al., 2007). This is echoed by Best & Kerr (2000), that "extreme virulence is not favourable for viral transmission, and thus it is not surprising that attenuation of virulence arose in field isolates of myxoma virus" to transition into reduced host mortality. The mutating virus also needs to retain its sufficiency in virulence genes to allow for transmission, as too weak a virulence would be selected against due to its inability to spread from host to host in a very small titre carried by insect vectors (Fenner & Ratcliffe, 1965). Contemporarily, the emerging attenuated field strains can still be highly virulent, but infected rabbits survive for longer, which, in effect, allows for more time for the body's defence system to generate stronger cell-mediated immune and neutralising antibody responses. This is evident in the reduction in disease severity and higher survival rates of resistant wild rabbits (Kerr et al., 2015). This well-equipped genetic resistance, however, is not in wide circulation in the domestic rabbit species, whilst transmission of MV and amplification of clinical signs can still take place from wild rabbits acting as a reservoir of the disease to domestic rabbits, putting them at high risks of myxomatosis.

Prevention

For industrial-farmed rabbits, the prevention is based on thorough exclusion screening and environmental control of arthropod vectors such as mosquitoes and fleas, quarantine of new and/or sick rabbits to contain new outbreaks and vaccinations.

Effective control in pet rabbits is typically achieved by vaccinations and reducing contact with vectors and wild rabbits (Meredith, 2013). Pet rabbits can benefit from ectoparasiticide treatments to control vectors (Singleton et al., 2018), most commonly the cat flea (*Ctenocephalides felis*) infestation.

The significant negative correlation between the risk of myxomatosis in pet rabbits and the distance between a pet rabbit owner's postcode and the closest wild rabbit habitat gives

further support to the hypothesis that wild rabbits are an important source of infection to pet animals. It points out that the shorter the distance a pet owner's home is to the wild, the higher the risk of myxomatosis. This is very highly likely due to the smaller distance blood-sucking arthropods such as fleas and mosquitoes have to travel from the wild into domestic households. The chance of infection rises even higher when pet animals are kept outdoors, but more limited when housed indoors under some degree of confinement (Meredith & Lord, 2014).

Within the same context, entire non-neutered female rabbits were found to be almost twice as likely to contract myxomatosis (Ross et al., 1989; Farrell et al., 2020). Reversely, the risk is found to be heightened in wild male rabbits, for their natural roaming tendency for mating and habituation purposes, thereby increasing their opportunities of coming into contact with infected rabbits and/or vectors at higher frequencies (Ross et al., 1989).

This ostensible inconsistency in risk levels between different sexes in wild and pet rabbits is explained by Farrell et al. (2020) as being driven by the mating instincts of sexually active female pet rabbits altering their usually timid behaviour. If sexually active female pet rabbits are attracted to the scents and excrements left behind by wild male rabbits, motivated to leave the more controlled environment of their households, and/or come into direct contact with males in the wild, this would provide the virus more opportunities to infect a new and healthy individual. This postulate is strengthened by the lower risk of neutered female pet rabbits observed in comparison to entire non-castrated males (Farrell et al., 2020). In light of this finding, veterinary practitioners may be inclined to advise owners to make an informed decision on neutering their female pet rabbits to reduce the risk of the deadly contraction of myxomatosis, with or without the use of commercial vaccines.

Interplay of co-infections (Palma-Pacios, 2018)

Because susceptibility to a given pathogen would be affected by the ongoing cytokine response due to a pre-existing infection (Graham et al., 2007), rabbits infected with MV are more susceptible to nematode infection, and rabbits with existing nematode infestations suffer longer MV infections (Cattadori et al., 2007). A high seroprevalence to myxoma virus is found to be inversely proportional to a low coccidian load, while nematode load seems to play a minor role (Palma-Pacios, 2018), whereby the highest MV seroconversion rates would occur in rabbit populations that have lower parasite loads and higher densities. These patterns of co-infection (e.g. nematode-coccidian-MV) could be partially owing to the immunosuppressive effect of MV inducing a decrease in circulating Th cells[1] (Jeklova et al., 2007), as an example of Boag et al.'s hypothesis that that "potential interactions between coccidia, nematodes and myxomatosis result in a substantial reduction of rabbits' fecundity and survival" (Boag et al., 2013).

Helminths

Trichostrongylus, *Graphidium* and *Nematodiroides* are the most common nematodes that parasitise rabbits. Nematode infections have adverse impacts on the growth rate, body weight and fecundity of rabbits (Bull, 1964; Dunsmore, 1980). Similar to coccidia, nematode prevalences are mostly associated with humidity and temperature conditions, so lower nematode intensities are expected in areas with extreme climatic conditions (Blasco et al., 1996).

Coccidia

Coccidian infections have been reported to cause severe body weight losses and consequently compromised overall rabbit's physical condition, in fact even more so than MV infections (Lello et al., 2005), making juveniles with an underdeveloped immunity even more prone to myxomatosis infection. Given the strong cyclic causal relationship

[1] After infection, naïve T helper (Th) cells begin to differentiate into Th1 and Th2 cells. The response triggered by MV (as a microparasite) biases the system toward Th1, but immune defense against nematodes (macroparasites) requires a Th2 response (Cox, 2001; Pedersen & Fenton, 2006). An experimental study showed that if both pathways occur simultaneously, the relaxation of the immune response provokes higher mortality (Kerr et al., 2004).

between coccidia and MV infections, effective control of coccidian loads may minimise the impact of MV outbreaks.

Ongoing antiparasitic treatment against immunosuppressive protozoal enteritis caused by Eimeria spp. (coccidiosis) is highly recommended for both intensively managed food-producing animals and companion pet rabbits from a young age. General hygiene measures and good husbandry maintenance in a stress-free environment include noise and transport minimalisation, regular disinfection of the environment[2], and feeding a diet rich in tannins such as willow, hazelnut, oak, ash, fruit trees and pines which has a marked effect in coccidiosis prevention, washing fresh vegetables before feeding, feeding dry pellets over wet, reducing nutritional stress and feeding off the ground contaminated with faeces with which oocysts are shed. Prevention of consumption of caecotrophs secreted by different rabbits in the same household reduces the risk of coccidiosis, too, as rabbits presented clinically normally and healthy can act as asymptomatic carriers and shed the highly contagious sporozoal infection in their faeces.

The presence of the intestinal protozoal can cause inhibition of the normal function or hypertrophy of the hosting cell, eventually precipitating to epithelial erosion and ulceration. Consequentially, the induced villi atrophy will cement a poor body condition score and general health status as the combined effects of malabsorption of nutrients, hypoproteinaemia, dehydration, electrolyte imbalance and anaemia with a low prognosis of healing. The chronic course of hepatic coccidiosis also induces immunosuppression, predisposing the weakened patient to myxomatosis (van Praag, 2023).

[2] coccidian oocysts need warm temperatures and relatively moist environment for sporulation and survival (Hobbs et al., 1999).

Vaccinations

Vaccination has long retained its pivotal position as a foundational tool for protecting pet animals from myxomatosis (Spibey et al., 2012). "Rabbits between 7 and 365 days of myxomatosis vaccination were almost 10 times less likely to present as a case" in a case-control study (Farrell et al., 2020). Increasing the proportion of vaccine uptake[3] presents as the simplest and most effective solution to tackle the prevalence of myxomatosis in the domestic rabbit population. The importance of vaccination is emphasised when severely compromised patient welfare with a highly fatal outcome[4] is seen in the majority of myxomatosis cases presented to veterinary clinicians.

Efficacies

I. Various administration methods of vaccines

1. Intradermal

Manev et al. (2018) have reported that a "more rapid and potent humoral response was detected in groups with intradermal inoculation in comparison to intramuscular and subcutaneous administration routes", which is consistent with the pathogenesis of MV, which first replicates in the skin at the site of entry before infecting dendritic cells in the dermis and later spreading to the lymphatic system, hence causing a reduction in lymphocytes count systemically. This feature of tissue tropism in the viral replication cycle supports the study's data on intradermal inoculation of myxomatosis vaccine being the administration route of choice for its highest efficacy, as it enables the shortest and most efficient port of entry to the tissue type preferred by an attenuated viral pathogen, evoking a more rapid and potent antibody response. This is reinforced by Sobey & Conolly's (1975) observation of the phenomenon of 100% seroconversion in all rabbits vaccinated

[3] currently limited at (45.8 %) in the UK – less common in veterinary-visiting pet female rabbits (40.3 %) than male rabbits lacking a recorded vaccination history (50.0 %) (Sánchez-Vizcaíno et al., 2017)

[4] The majority of myxomatosis cases identified Farrell et al.'s study (2020) were euthanised at first presentation

intradermally and a further rise in antibody levels when exposed to a challenge later (Dalton et al., 2015).

As the formation of a nodule is considered to be a prerequisite for successful inoculation as an explanatory immune response induced by the vaccine used (Alfonso & Pagès-Manté, 2003), it is postulated that MV's specific tropism for epithelial cells is potentiated by direct intradermal entry into the lymphatic vessels, via which antigen-presenting cells in the regional lymph nodes are stimulated more effectively (Kim et al., 2011). The direct delivery into the epidermis or dermis accelerates a superior and time-efficient immune responses when compared to muscle and subcutaneous tissues (Manev et al., 2018) for the intradermal route allows for longer contact between the antigen and the antigen-presenting cells due to a high number of dendritic cells in the derma compared with subcutaneous tissue (Levin et al., 2014). An analysis of on-farm vaccinations gave similar results to those obtained in laboratory experiments (Dalton et al., 2015), when intradermal vaccinations endowed 100% seropositivity 1 month post-vaccination, while subcutaneous vaccinations gave seroconversion rates of between 16.6 and 54% within the same time frame and 41.6–80% 2 months post-vaccination. As it is shown that the intradermal route of vaccine application produces the most rapid immune response, it is thus recommended that in regions with higher epidemiological risk for myxomatosis infection, the intradermal route may be the preferred way of vaccine inoculation.

2. Subcutaneous

Comparatively, the subcutaneous route is deemed as a subordinary primary location for MV multiplication or antigen presented, as several studies have demonstrated the lower efficacy of the subcutaneous route in inducing immunity compared with the intradermal route (Dalton et al., 2015; Manev et al. 2018). Abade dos Santos et al's study (2022) has shown that conversely, "a 10-fold increase in dose for a viral challenge inoculated subcutaneously (1000ffu) is required to induce fatality" in comparison with the intradermal inoculation route, conferring that the lethal dose inoculated intradermally may be too low a dose for the subcutaneous route to elicit the same pathological response (Psikal et al., 2003; Marshall & Fenner, 1960; Kerr et al., 2004; Kappler-Gratias et al., 2021; Boutard et al., 2015; Bárcena et al., 2000; OIE, 2018; Kerr et al., 2017; Spibey et al., 2012).

Dalton et al.'s farm vaccination trials (2015) have also indicated that "subcutaneous vaccination gives reduced numbers of seroconverted animals when compared to animals using an alternative route of vaccine administration," and hence concluded that when an MV vaccine is administered subcutaneously, a percentage of animals may remain seronegative and at risk of infection with circulating field strains due to the insufficient immune response elicited by the inferior vaccine administration method (Dalton et al., 2015).

In a novel approach, Dalton's 2015 challenge experiment sheds light on the potential of a slight change in vaccination strategy using the same vaccine via the subcutaneous route that can increase seroconversion. By applying the same vaccine dose "in 2 rather than 1 inoculation site" subcutaneously, seropositivity rates improved, and so likely the expected protection levels did too (Dalton et al., 2015). Citing the results of a preliminary study, Dalton proposes the use of this subcutaneous administration protocol as a plausible alternative for farmers with limited access to commercial vaccines, namely the Dermojet, which is a live homologous vaccine licensed to be administered intradermally and has been demonstrated to possess the highest levels of seropositivity (100%) with 1 injection (Dalton et al., 2012). He termed this "the favourable vaccine application method" due to its performance overshadowing the 90% seropositivity detected 1 month after vaccination with 2 subcutaneous injections (Dalton et al., 2012).

Table 1. Seropositivity (%) of vaccinated rabbits 1 month after vaccinaation (Dalton et al., 2012)

Protocol	% seropositivity (RI > 2)
Dermojet	100
1 single Subcutaneous injection	25-80
2 Subcutaneous injections	90

3. Intramuscular

Manev et al. (2018) concludes that with the same antigen dose, “intramuscular injection was found to be inappropriate for myxomatosis vaccination, whereas intradermal and subcutaneous routes proved preferable” as the intramuscular route provokes a weaker response at a slower speed, with lower effectiveness. Comparisons between the subcutaneous, intramuscular and intradermal groups inoculated with the bivalent homologous, lyophilised, attenuated vaccine (Bioveta, Czech Republic) against myxomatosis and RHD have seen the establishment of antibodies titres in intradermal (1//928) a lot higher than in the subcutaneous (1/62.5) and intramuscular (1/136) groups, albeit without statistical differences.

When inoculated with another monovalent, homologue, lyophilised, attenuated myxomatosis vaccine (Bioveta, Czech Republic), the intramuscular application similarly displayed the lowest titre result (Manev et al., 2018). The authors of the paper thus agree with Dalton et al. (2015) that the weak immune responses following intramuscular inoculation of the same vaccine strain hinges more upon the administration route than the valence of the vaccine and concur that the non-conventional intramuscular vaccine administration method can be a factor leading to immunisation failures especially in wild rabbits.

II. Different vaccine strains, as defined in terms of resultant clinical signs and mortality rates following a challenge infection:

1. Inactivated vaccines

Kerr & McFadden (2002) states that "inactivated vaccines have failed to protect rabbits against myxomatosis, even though antibodies may be induced", as Marlier (2010) elaborates that "since a robust cellular immunity is necessary for protection against MV, inactivated vaccines have generally proven unsuccessful" due to their failure to induce multiplication of the virus in the primary injection site.

2. Shope fibroma virus (SFV)

A live heterologous vaccine is based on the closely related but non-pathogenic leporipoxvirus, SFV. SFV contains the original OA strain, Boerlage strain or other closely related strains. They are employed against myxomatosis due to their phenotypical antigenic resemblance with MV (Marlier, 2010). SFV originates from the Eastern cottontail rabbit (*Sylvilagus floridanus*) and provides considerable cross-protection against MV without causing disease in European rabbits older than 2 weeks (Kerr & McFadden, 2002; Fenner & Fantini, 1999).

The use of the heterologous SFV strain, however, has been remarked by other scientists as being "only moderately effective" (Camus-Bouclainville et al., 2011), "yielded variable results, yet is generally effective" (Fenner & Woodroofe., 1954), "weakly immunogenic and provides only short-term protection for 7 weeks after vaccination" (Marlier et al., 2000b), lasting no longer than 3 months (Jerabek, 1980). Concerns have also been raised regarding the capacity of SFV-based vaccines to completely prevent clinical signs and naso-conjunctival shedding of myxomatosis upon infection (Alfonso & Pagés-Manté, 2003; Marlier et al., 2000b). Marlier's results (2000) confirmed that it was only during the late phase of myxomatosis that a significant reduction in viral shedding was observed in naso-conjunctival exudates in the SFV-vaccinated group when most of the sick animals were already dead.

Although now known to be unsatisfactory, SFV vaccines are used regularly in intensive rabbitries and are still the vaccine most frequently used in some European countries such

as Belgium (Kerr, 2012), perhaps on the basis of conventional public perception, in spite of the progress already made in the development of more effective and safer homologous vaccines.

3. Attenuated homologous SG33 strain

The attenuated homologous SG33 strain is derived from the Lausanne strain of MV by serial passages on RK13 rabbit kidney epithelial[5] and chicken embryo cells at 33°C (Saurat et al., 1978). In this process, genomic deletions of important immunomodulatory genes responsible for pathogenic virulence (Guerin et al., 1998) – such as Serp-2, a protein under the serine proteinase inhibitors family (Petit et al., 1996) – were introduced (Camus-Bouclainville et al., 2004), in addition to a mutation of the M143 virulence gene (Cavadini et al., 2010).

Despite its extensive deployment in France, Belgium and Italy, amongst other European countries, with a relatively long-lasting immunity from 4 days post-inoculation to over 10 months (Saurat et al., 1978), its post-inoculation side effects include immunosuppression in young rabbits and atypical cutaneous reactions such as skin lesions, oedema and rash at injection sites and secondary myxoma lesions (Marlier et al., 2000a; Lemière et al., 2003). Attempts have been made to disperse such concerns, though, as the same author Lemière et al. (2003) also reported "no problems" after vaccination of young Angora rabbits with SG33 strain, which is a rabbit breed known to be particularly sensitive to MV in field conditions (Spiesschaert et al., 2011).

Because of the association between the SG33 strain and immunosuppression in some mass production facilities, the current generally recommended vaccination scheme consists of a primary vaccination with SFV at 3-4 weeks of age, followed by a booster with a homologous MV-based vaccine e.g. SG33, 6 to 8 weeks apart. Booster vaccines with the same SG33 strain should repeatedly ensue every 4–6 months after that, depending on the infection risk (Brun et al., 1981; Vautherot et al., 1997). But on farms at high risk of infection, a 3-week interval can be used without reducing the immunising effect of SG33

[5] "Rabbit kidney epithelial cells (RK13), grown in DMEM supplemented with 10% fetal calf serum and 40 mg/L gentamicin, were used for virus propagation of Cunivax myxoma vaccine (Borghi strain) from Fatro (Ozzano Emilia, Italy), Dervaximyxo SG33© vaccine from Merial (Lyon Cedex, France) or Moses strain (ATCC, VR-116) (Cavadini et al., 2010).

(Vautherot et al., 1997). Likewise, the use of the SG33 vaccine for primary immunisation is strongly discouraged by the manufacturer because of its possible immunosuppressive effect.

In contrast to SFV used alone, the protection conferred by SFV + SG33 vaccination against both clinical signs and mortality has been graded as satisfactory (Saurat et al., 1978; Picavet et al., 1989, 1992; Bertagnoli et al., 1996), as it has also been more recently clarified that SFV+SG33 vaccination significantly reduced viral shedding in naso-conjunctival exudates during both the acute and the late phases of the disease (Marlier et al., 2000b). There were also fewer proliferative cutaneous lesions resultant from the SFV+SG33 vaccinationns than the SFV alone or the naturally attenuated UR strain groups (Marlier et al., 2000b).

However, a worrying trait displayed by the origin of the SG33 vaccine strain is alluded to in several studies. As its present sequence composition points towards an unintended field recombination between a wild-type Lausanne strain and a Californian MSD-derived vaccine strain (Camus-Bouclainville et al., 2004), the biosecurity risk of new recombinant strains formation is elevated by the exposure of a large number of individuals to the mixing of different MV strains. The construct of a new and genetically unstable MV strain can have serious and dreadful consequences in future myxomatosis outbreaks.

4. Attenuated homologous Italian Borghi strain (Cavadini et al., 2010)

The live attenuated Borghi strain is derived from the Californian strain MSD of MV by inoculation in embryonated chicken eggs (161 passages), then processed by serial passages in rabbit kidney RK13 epithelial cell culture[6] (40 passages) until complete loss of pathogenicity was achieved (Saito et al., 1964; Cancellotti, 1985). It is not yet characterised at the molecular level to date; it is speculated that the attenuation process may have induced truncation of Serp-2, M-T4 and/or a mutation in the M121 gene (Cavadini et al., 2010). The advantage of using a homologous MV-based vaccine is the induction of a stronger immune response than any of the current SFV vaccines for slightly

[6] "Rabbit kidney epithelial cells (RK13), grown in DMEM supplemented with 10% fetal calf serum and 40 mg/L gentamicin, were used for virus propagation of Cunivax myxoma vaccine (Borghi strain) from Fatro (Ozzano Emilia, Italy), Dervaximyxo SG33© vaccine from Merial (Lyon Cedex, France) or Moses strain (ATCC, VR-116) (Cavadini et al., 2010).

longer disease protection at a minimum of 4 months after vaccination (Marlier, 2010). The main disadvantages consist of immunodepression in young rabbits after vaccination and the appearance of clinical symptoms in some cases.

5. Attenuated MSD-derived strain developed in California (Saito et al., 1964)

MSD/B was developed in California by serial passage in rabbit kidney cultures for attenuation without sacrificing its immunity (Jiran et al., 1970). Immunisation of rabbits with MSD/B confers immunity for approximately 9 months (Saito et al., 1964) but is later shown to cause mild cutaneous myxomatosis symptoms (Jiran et al., 1970; Jacotot et al., 1967; Camus-Bouclainville et al., 2011).

6. Other attenuated homologous live vaccines

"The antigen in the homologous vaccine is an attenuated myxoma virus strain that induces stable, protective immunity" (Marlier, 2010). Such include the Spanish Poxlap (Marlier, 2010) or BTK/RB/84 strains of MV (Marlier, 2010; Lavazza et al., 2004).

7. Uriarra strain (UR)

The UR strain is a naturally attenuated strain deficient for the virulence gene M063R. It confers long-term immunity similar to those of other heterologous live vaccines but with weaker potency than most homologous vaccines (Adams et al., 2008). Other more potent vaccine candidates have been construed through the deletion of 1 or more virulence genes (of M-T7, M010L and M011L) in the naturally attenuated MV Ur strain (Best et al., 2000; Best & Kerr, 2000; Adams et al., 2008). Vaccination with these vaccines, however, is accompanied by mild clinical symptoms in adult rabbits, thereby making them still too virulent for widespread use as a vaccine. Conversely, when deletions of all 3 virulence genes were enforced simultaneously in the same vaccine to eliminate the occurrence of clinical symptoms in test rabbits, the long-term protection against wild-type MV also subsided as a result (Best et al., 2000; Best & Kerr, 2000; Adams et al., 2008).

8. Plasmid-based subunit vaccines

Spiesschaert et al. (2011) summarise that plasmid-based subunit vaccines are unable to protect rabbits from disease, even though both antigen-specific cell-mediated and humoral immune responses were induced (Adams et al., 2004).

9. Recombinants

MV is genetically modified for the expression of a main structural protein of RHDV to concoct a new recombinant vaccine (Bárcena et al., 2000; Spibey et al., 2012). Abade dos Santos et al. (2022) remark that "the humoral and cellular response against the vaccine strain is expected to be protective against the naturally recombinant virus, taking into account that most of (all) the antigenic epitopes of classic MV are conserved in this recombinant virus."

I. Polyvalent trivalent Nobivac Myxo RHDV PLUS vaccine (Myxo-RHDV2) from Intervet

No vaccine was available to provide protection against myxomatosis and both genotypes of RHDV1 and RHDV2 before the novel trivalent recombinant attenuated myxoma virus vectored RHDV1 and RHDV2 vaccine Nobivac Myxo-RHDV PLUS was licensed in the EU in 2019. The trivalent recombinant vaccine uses the same developmental model as the pre-existing recombinant attenuated myxoma virus vectored RHDV1 vaccine it is based on, vesseled by the same myxoma virus vector as Myxo-RHDV1, with the additional insertion of the capsid protein VP60 of either RHDV1 (a German isolate of classical RHDV) or RHDV2 (a Spanish isolate of RHDV2) into the same position (MGF/M11L locus) of a laboratory-attenuated MV strain using standard laboratory methods for homologous recombination (Reemers et al., 2020). In this manner, 2 live recombinant myxo-vectored RHDV viruses were created in the same myxoma virus vector but containing the VP60 gene of either RHDV1 (strain 009, named after Myxo-RHD1) or RHDV2 (strain MK1899, named after Myxo-RHD2), whilst keeping all the desirable safety features and efficacy properties of its founding model "proven from years of use in the field" (Reemers et al., 2020). "By combining Myxo-RHDV1 and Myxo-RHDV2 in the Nobivac Myxo-RHDV PLUS vaccine, all the desirable properties of Nobivac Myxo-RHDV vaccine were

conserved and protection against disease by RHDV2 was additionally added". Reemers et al. (2020) continue to compliment the design for nullifying the risk of genetically unstable new recombinant formation with the use of 2 separate MV vectored strains, in compliance with the regulations for the construction of a myxoma virus vaccine (*European Pharmacopoeia*, 2017).

Due to the 2 bivalent and trivalent vaccines sharing the same myxoma virus vector, presumptions were made (rather than performing actual testing) on Nobivac Myxo-RHDV PLUS's safety based on previous assessment of the preceding model of Nobivac Myxo-RHDV, which has shown no signs of spread or virus shedding via biting arthropods from vaccinated rabbits to in-contact controls, as indicated by the maintenance of seronegativity of the control rabbits and lack of clinical signs in any of the rabbits, (Reemers, 2020; Spibey et al., 2012; Opgenorth et al., 1992). Moreover, it is claimed that "the frequency of serious adverse reactions to vaccination in companion animals is extremely low and protection provided by vaccination far outweighs these reactions" (Reemer et al., 2020; Day M.J., 2006; Tung et al., 2015; Moore et al., 2010).

Although complete immunity takes 3 weeks to form post-vaccination – longer than the previously mentioned vaccines, a single dose of Myxo-RHDV PLUS offers the longest one-year-round challenged protection against myxomatosis, RHDV1 and RHDV2, as proven by challenge infections that produced similar serological responses to MV after vaccination with Nobivac Myxo-RHDV, quoting "all vaccinated rabbits, which were tested seronegative beforehand, turned seropositive for both MV and RHDV after vaccination" (Reemers et al., 2020). Provided that the immune response to infection is highly complex, the immunogenicity of vaccine candidates should be evaluated to give a more accurate prediction of the vaccine accuracy. However, no data was shown to substantiate this claim made by the vaccine manufacturing company; this is information given based on license approval.

The vaccine is recommended for safe use from 5 weeks of age onwards in healthy and pregnant does[7], as well as in farm animals for its convenient 0-day withdrawal period. It

[7] "No significant difference in body temperature was observed between vaccinated and control animals in both terms of the pregnancy. Analysis of the gestation length and litter size revealed no differences between vaccinates and controls. Furthermore, there were no abortions, no deformities and normal sized progeny in all groups." (Reemers et al., 2020)

also has the additional welfare benefit of harming no live rabbits as it is produced using in-vitro culture.

On the contrary, Manev et al. (2018) have cautioned against the high levels of maternal antibodies against MV and RHDV circulating simultaneously in the body of juveniles as a potential limiting factor of the vaccine's effectiveness. It is noted that "rabbits that have previously been vaccinated with a different myxomatosis vaccine or naturally infected with myxomatosis 3 times may not produce an adequate immune response against rabbit haemorrhagic disease"; and in this case, these animals should be vaccinated from 70 weeks onwards to avoid the functionally mutually inhibitory overlapping of passive and active immunity and ensure full duration of protection (Manev et al., 2018).

II. Recombinant attenuated vectored RHDV1 vaccine Nobivac Myxo-RHDV

The recombinant Nobivac Myxo-RHDV vaccine is "composed of an attenuated MV vaccine strain in which the VP60 gene of RHDV1 is inserted using homologous recombination" (Spibey et al., 2012), wherein the artificial insertion of a foreign viral gene removes the viral virulence of the 2 MV genes (MGF and ML11), achieving further attenuation of the virus (Opgenorth et al., 1992; Graham et al., 1992). This is the pre-existing recombinant vaccine model on which the infrastructure of the later trivalent recombinant Nobivac Myxo-RHDV PLUS vaccine was built.

Spibey et al. (2012) have stated its "extensive proven safety and efficacy profile against myxomatosis and RHDV1 from years of use in the field", with its provision of full-year protection in addition to the advantage of null vaccine virus dissemination or spread. It produces a strong serological response to myxomatosis and RHDV1 but a much weaker challenged protection to RHDV2, which is a new genotype that emerged in 2010 in the field with only limited cross-protection with the classical RHDV1 strain (Reemers et al., 2020). Analogous to the trivalent Nobivac Myxo-RHDV PLUS spawned from it, the bivalent recombinant vaccine strain can be cultured in-vitro, preceding the use of live rabbits for recombinant vaccine production.

There are many potentially problematic data and conclusions drawn in the study conducted by Reemers et al. (2020) nonetheless, which is a research funded by MSD Animal Health that was also employing all the authors penning the paper. The conflict of interests

overlying this investigation has produced very positive outcomes in supporting stance of the commercial product, which may be seen as a tool of marketing advertisement hired by the vaccines manufacturing company to produce an evidence-based study with a biased premise.

Firstly, the study problematically used a very small sample to represent the whole population of different varieties, breeds, ages, and health for different purposes and dismissed other anecdotal evidence and the established scientific consensus[8] over the post-vaccination adverse reactions to draw purported conclusions on safety and efficacy. Only 35 vaccinated and control rabbits in total were used to test for the efficacy and safety of the Nobivac Myxo-RHDV2 vaccine. A similar case is made for safety for use in pregnant animals when only 30 vaccinated and unvaccinated controls in total were used to evaluate safety prospects in the first and second terms of pregnancy for the entire rabbit population. Furthermore, the enhanced claim on safe use of the vaccines in pregnant rabbits was made on 2 mated but non-pregnant rabbits, the post-mortem examinations of which showed "no abnormalities and normal follicular development of the ovaries". Using the results from these 2 rabbits, the paper made a bold statement that "treatment within this study was unlikely to be the cause" of the infertility of these rabbits but "likely to be related to the overall 90% pregnancy success rate of the supplier," which is insinuating the responsibility to be falling under the 10% incidental failure rate category. The deployment of a very small sample size to back up the important lack of correlation between the lack of harmful negative effects of the vaccines and the health status of fertility, abortion and pregnancy in vaccinated rabbits appears to be more farfetched than what would normally be accepted on a reasonable scientific standard. The number of animals used is not justified in this study, and neither is the power of biostatistical analysis of between-group differences strengthened by a clearly defined quantitative measure of a meaningful confidence interval.

Despite the small sample size, macroscopically visible tissue lesions displaying possible myxomatosis symptoms and local reactions in the injection sites were observed and recorded at the end of the study (on day 35), such as swelling of the eyelids, nose, anus, mouth, external genitalia and ear base, abnormal breathing, reduced appetite, abnormal

[8] "Inoculation of such rabbits with myxoma virus was sometimes followed by the development of a local lesion at the inoculation site, and in these rabbits, the titre of complement-fixing antibody rose, but there was no alteration in the neutralizing power of the serum. In other animals, no lesion developed, and there was no change in the antibody titre" (Fenner et al., 1953).

attitude and ocular discharge in the vaccinates[9]. However, apparent contradictory conclusive remarks were listed instead – "clinical examination, macroscopic analysis and histological analysis of the injection sites from animals indicated no abnormal reactions to vaccination other than injections site reactions within the normal limits upon subcutaneous injection" and "during clinical monitoring, no clinical signs of myxomatosis were observed in any of the rabbits in either safety study (data not shown)." The shortcomings of this data analysis strategy include the untimely late examination of lesions (on day 35) for acute onset of local reactions and suspected myxomatosis symptoms, lack of precise microscopic investigation of the morphology and cell count of lesions to confirm the presence or absence of infected dendritic cells and/or lymphocytes, and not submitting essential data where it is significant.

The incidental failure to provide sources of knowledge and data in Reemers et al.'s paper (2020) is seen on more than one occasion, as referred to above. Another instance transpires when the authors of the paper claim "there were no differences in results between a laboratory breed rabbit and pet breed rabbit in either the vaccine safety or efficacy study using Nobivac Myxo-RHDV PLUS", whereas no data has been provided or proof attached in the paper to cite the origin of the aforementioned study. The authors conclude from this, nonetheless, that for Nobivac Myxo-RHDV PLUS, both the laboratory and pet breeds were compatible matches to prove vaccine safety and efficacy for each other in their defence against the contention around the extrapolation quality of vaccines safety and efficacy results obtained in tested laboratory breed rabbits as representative for pet breed rabbits' reactions. The commonalities of immune systems and comparable susceptibility to MV infections of laboratory breed and pet breed rabbits were not investigated in details or referred to a wealth of other literature otherwise by Reemers et al. (2020).

Another study (Selleri et al., 2014), coupled with anecdotal clinical evidence reported by veterinarians[10] (for instance, van Praag, 2023), is in slight disagreement over the degree of

[9] "Small, transient swellings up to 1.5 cm diameter and lasting up to 7 days were seen at the injection site in many cases following vaccination. These could be slightly larger (up to 2 cm diameter) and more persistent (up to 9 days) following vaccination at 10× overdose. Thickened skin could also be palpated at the majority of injection sites for up to 10 days. Furthermore, following overdose vaccination, a slight swelling of the local lymph node could be palpated for 1 day."

[10] "Bucks, a ±3 years old male non-castrated dwarf rabbit, suffered from eye infections. It was unsuccessfully treated with Gentapolycort eye drops and Baytril (enrofloxacin). Bucks was then vaccinated against myxomatosis (Lyomyxovax). He apparently developed an allergic reaction to the vaccine. Ten days after the vaccination, a huge lump appeared on his back, and he became blind.

"low" incidence of cutaneous lesions development caused by the inoculation of the recombinant vaccine strains against a widely cited research by Spibey (2012) – which is also cited by Reemers et al. (2020) – promoting the efficacy and safety of the then-novel bivalent recombinant Myxo-RHDV vaccine. By examining formalin-fixed skin biopsies from lesioned skin on "the ear pinna, dorsal aspect of the nose, vulva and conjunctiva", the embedded tissue and affected surface crusts were confirmed by real-time polymerase chain reaction (RT-PCR) for myxoma virus in Selleri et al.'s 2014 research. The histological findings also corresponded to the clinical signs typically exhibited by myxomatosis patients, which included "severe ulcerative, necrotising dermatitis and intralesional cytoplasmic inclusion bodies in myxoma cells". The results rectify the arguably incorrectly underestimated or understated "extremely low"[11] (Reemers et al., 2020) possibility of rabbits inoculated with the live attenuated bivalent recombinant Myxo-RHDV and the trivalent recombinant Myxo-RHDV PLUS developing post-vaccination adverse reactions and lesions, and emphasise the more accurate grading of "low" (Selleri et al., 2014) in the possibility of such occurrence.

The heavy reliance on the use of serological data in Reemers et al.'s study (2020) also underlies a high degree of negligence towards the debate within the scientific community over the appropriateness of antibodies used as a comprehensive diagnostic tool for myxomatosis and measurement of immune protection conferred by the vaccines against myxomatosis. This literature review acknowledges the somewhat minor participation of antibodies in the host's immunity against myxomatosis, whilst also assessing Reemers et al.'s 2020 study critically for its full integration of qualitative approach into its analysis of what constitutes seropositivity and seronegativity. "A titre of <6 (log 2) was regarded as negative"; whereas above <6 (log 2) would land a positive result – rather than specifying quantitatively what amount of titre or folds of increase in antibodies would be considered to offer what varying degrees of protection, especially with the lack of challenge infection to follow through after vaccines inoculation. This attracts scepticisms over the true sensitivity and specificity of the seronegative and seropositive statuses as reported in its

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Blisters appeared around his eyes. Crusty dermatitis developed. As his condition kept worsening, Despite dedicated care by the veterinarian and the owners, his condition kept deteriorating, and he was humanely put to sleep." (van Praag, 2023)

[11] "Furthermore, the frequency of serious adverse reactions to vaccination in companion animals is extremely low and protection provided by vaccination far outweighs these reactions," quoting Reemers et al. (2020).

results and hence accuracy of its data and conclusions[12]. It would be worthwhile to consider using conventional virological methods such as PCR, cell culture isolation and negative electron microscopy staining to improve the controversial credibility of the results obtained solely by serology in this case.

Conversely, Manev et al.'s experiment (2018) produced partially conflicting results using the same recombinant myxo-RHDV vaccine, witnessing a lower level of humoral response to MV instead. The authors wrote in a forthcoming confession that "it is quite difficult, without performing challenge experiments, to determine whether such titres are indicative or not of a complete protection against high virulent myxoma virus [, and] in fact, it is well known that antibody titres are not the only parameter correlated to protection against myxomatosis" (Manev et al., 2018). Manev et al. also reference the predominant role cell-mediated immunity plays in clearing poxvirus infections and providing long-term protection from myxomatosis as transcendent over the significance of humoral response in this stead. This doubtful undertone over the role of antibodies is resonated by, for instance, Kerr & McFadden (2002), who state that "pre-existing antibodies may provide some limited protection against infection, but neutralising antibodies developed during lethal infection are probably not critical for survival".

Contentious use of serology for diagnosis of myxomatosis:

The use of ELISA still lies within a contentious area of discussion on its diagnostic value for myxomatosis in the present scientific community, as myxomatosis is a disease counterattacked by the host's cell-mediated immunity (predominantly lymphocytes and monocytes) rather than serological antibodies. Below is a compilation of exemplary quotes in opposing stances for and against serology for the accurate detection of myxomatosis and vaccine protection:

[12] For instance, "serological data showed that all rabbits were seronegative for myxoma virus and RHDV at the start of the study. After vaccination, all vaccinated rabbits were seropositive for both myxoma virus and RHDV. With regard to virus shedding, none of the in-contact control rabbits that were housed together with the vaccinated rabbits became seropositive to either myxoma virus or RHDV."

For	Against
- "Immunoglobulins possess significant importance in resolving poxvirus infection" (Panchanathan et al., 2008). - "Antibodies are used as biomarkers for successful vaccination" (OIE, 2014). - "The gold standard for the serological evaluation post-infection or post-vaccination is enzyme-linked immunosorbent assay (ELISA)" (Kerr, 1997; Lavazza et al., 2004; Dan, 2014). - Fenner & Marshall (1954) demonstrated that passively administered antibodies could modify the course of virulent virus infection in young kittens from seronegative mothers, indicating that antibody alone can provide some protection from natural infection and that maternal antibody could alter the epidemiology of myxomatosis, particularly with attenuated viruses.	- "A number of results infer that the cell-mediated immune response is critical. For instance, the relatively minor importance of serum antibody in recovery and protection from myxomatosis suggests that the cell-mediated immune response is most critical." (Kerr & McFadden, 2002) - "neutralising antibodies did not protect from death in virulent infections, even though the titres were similar to rabbits that survived infections with attenuated virus" (Kerr & McFadden, 2002). - "However, in a separate study, no advantage of maternal antibody was found in kittens exposed to MV by fleas or contact compared to animals with no antibody" (Sobey & Conolly, 1975; Kerr, 2012).

Seropositivity to MV can have both advantages and disadvantages for rabbits, whether they are inoculated via vaccinations or natural infections. While seropositivity may confer higher immunity to the specific pathogen of MV, it can also have an immunosuppressive effect, making the MV-seropositive rabbits more susceptible to co-infections with other pathogens (Cattadori et al., 2007, 2008). This is notably dangerous in the presence of the amyxomatous form, which has a significant immunosuppressive property that can exacerbate the clinical severity of other secondary bacterial diseases commonly infecting

rabbits. In particular, young rabbits show lower survival rates when they are seropositive for MV, as their immune systems are not yet fully mature (García-Bocanegra et al., 2011; Villafuerte et al., 2017). The production of antibodies in young rabbits can be detrimental to their health, making them more susceptible to other diseases. This vulnerability among juveniles is well-documented in previous studies, which have observed lower MV seroprevalence among young rabbits (García-Bocanegra et al., 2011; Villafuerte et al., 2017). Interactions with environmental, epidemiological and individual factors likely influence this shifting balance of health benefits and hazards.

10. Monovalent attenuated Bioveta Myxoren

Myxoren is a subcutaneous injection recommended for animals at 10 weeks or above, to be repeated every 6 months as it provides a 6-month immunity with each dose. It should not be given to sick or immunocompromised rabbits or does in their last phase of gestation period due to the risk of abortion.

Unsurprisingly, "vaccination with the monovalent live vaccine against myxomatosis singularly induced a higher antibody titre than a bivalent attenuated vaccine against myxomatosis and rabbit haemorrhagic disease" (Manev et al., 2018), as inoculation of a single type of pathogen one at a time enables fewer other pathogens competing for adequate immune response. The immune system is less likely to be overburdened by a wide range of pathogens with differing binding receptors and virulence factors, hence equivocally more likely to produce sufficient antibodies of a good quality against one type of pathogen to facilitate recovery of health.

11. Polyvalent Bioveta Pestorin Mormyx

Mormyx contains an inactivated RHDV strain in a liquid component and an attenuated Myxoma virus strain in the freeze-dried component. Industrial farm animals commonly receive the vaccine every 6 months but are usually given more frequently than every 6 months in reality. Likewise with monovalent attenuated Myxoren, Mormyx as a live vaccine should not be given to sick or weak rabbits or does in their last week of pregnancy due to the risk of abortion.

In difficult epizootic situations requiring earlier immunisation, rabbits may be given the monovalent Myxoren vaccine at 4-6 weeks old then the Pestorin Mormyx vaccine at 10 weeks, with another dose of Pestorin Mormyx given at 14 weeks. Immunity against myxomatosis is achieved on day 9 post-vaccination, lasting for 6 months; and on day 10 in the case of RHD, which will cover for a year (Reemers et al., 2020).

However, this combined use of different attenuated MV vaccine strains underlies a dangerous risk of chance recontamination between different pathogenic viral strains. For instance, by genetic reassortment during viral replication (Muller et al., 2009) or in-vivo co-infection with various attenuated vaccine strains consecutively used for the prophylactic treatment of myxomatosis in industrial rabbits, for which it is common practice to vaccinate 3 to 4 times annually. Although concerns for such recombination events have been purportedly downplayed as "purely speculative", a more recent study has enlightened the cause for a more thorough review on the mix use of different vaccines with the example of the SG33 strain, whose dual origins were revealed in genome sequencing as an unintended and unforeseen product of in-vivo field recombination between a virulent wild-type South America (Lausanne) strain and a California MSD-derived vaccine strain, probably because several attenuated vaccine strains have been widely circulating in the industrial rabbits sector (Cavadini et al., 2010; Camus-Bouclainville et al., 2011). This finding calls for scrutiny of the safety of inadequately attenuated viruses, especially those used for recombinant vaccination strains. For instance, a consequence of the insufficiently attenuated Saito strain widely used before the 1970s – which was later shown to cause myxomatosis symptoms in rabbits – is hypothesised to be recombining with certain disseminating wild-type strains (Jiran et al., 1970; Jacotot et al., 1967). Efforts were prompted to further attenuate the Saito strain as a result (Jiran et al., 1970), which has gifted the industry with subsequent productions of derivative vaccine strains used throughout Europe nowadays, like the Borghi (Cancellotti, 1985) and MAV strains (Górski et al., 1994). The increased safety level of these further attenuated and stable vaccine strains has eased their integration into conventional European vaccination protocols, as "no event of virulence recovery was ever reported" (Camus-Bouclainville et al., 2011). Nonetheless, more extensive and rigorous scientific research in the field of (re)engineering recombinant viruses is warranted before any premature discernment of valid safety concerns and hesitation for switching between uses of different attenuated vaccine strains in the same animals.

As the choice of vaccines and their administration route are paramount factors to consider for judicious immunisation success in the target population, a general protocol has been initiated and recommended by Manev et al. (2018) to use monovalent vaccines against myxomatosis and RHD for prophylaxis in zootechnical rabbits – on the basis of their separate vaccination schedules presenting a lower risk of infection and higher seropositivity conversion; and vice versa, using single bivalent recombinant MV-RHDV vaccines in wild animals in field conditions or pet rabbits in a clinical setting, to achieve higher efficiency in endemic areas and reduce the handling stress imposed on animals unaccustomed to humans' manipulation or veterinary interventions (Manev et al., 2018).

Vaccination protocols for farmed, pet and wild rabbits

I. Farmed rabbits

The unique dimension of vaccines development for farmed animals is the utmost importance of preventing viral shedding from the tissues of vaccinated animals (i.e. tissue infection) in post-vaccination infections since trades of live breeding stock and semen are prevalent in the commercial rabbit industry. The evaluation of the efficacy of a vaccination protocol for farmed rabbits hence hinges on this parameter.

SFV, the SG33 strain and/or the combination of the aforementioned 2 vaccines are the most widely adopted vaccines against myxomatosis on farms. Both vaccination schemes – SFV and SG33 separately – respectively show promising results in reducing the number of virus-positive eyelid samples in post-vaccination challenge infections. However, the combination of SFV and SG33 stands out most as the only protocol that succeeded in reducing the number of virus-positive testicular samples. This underlines the potency of this protocol in the view that "the testis is an immunologically privileged site, and even attenuated strains of MV induce intense pathology in the testis with persistence of viral DNA for long periods" (Kerr & McFadden, 2002).

Additionally, the presence of the infectious amyxomatous viral strains in the testes of recovered animals has been detected up to 100 days after infection. This illustrates the probability that "rabbits vaccinated with SFV and, to a lesser extent, those given SFV+SG33 would, if introduced into unvaccinated rabbitries, spread the virus" (Marlier et al., 2000b). This is of considerable importance due to the routine practice of artificial insemination in industrial rabbitries (Prigent, 1989; Contera et al., 1994; Dagaut, 1994) – infection of does inseminated with infected semen (Castellini et al., 1994) transported in from a distant farm can introduce the deadly virus into a previously clean facility, potentially causing a devastating eruption of an epidemiological event in the other region.

Using seropositivity investigation, Dalton's 2012 challenge experiment concludes that "the current vaccines used can protect with high levels of antibodies against one of the currently circulating strains of myxoma virus of the maximum virulence grade A (the Granada-05/09 strain)" (Dalton et al., 2012). However, the existence of an outlier result displaying clinical signs post-challenge raises alerts concerning the inevitably uneven distribution of seroconversion amongst individuals in an industrial rabbitry flock following vaccination. In very controlled laboratory conditions where variable factors were subjected to strict adherence in order to ensure high accuracy of experimental results, 1 "outlier" result in a relatively small sample may be extrapolated to a significant portion of rabbits in field conditions on a mass production scale where circumstances are far less controlled and more autonomous. This is synonymous with the meaning that an unpredictable and perhaps large percentage of rabbits may fall unprotected even after a conventional vaccination procedure has been carried out on them. A high proportion of vaccinated rabbits would be susceptible to infections if a highly pathogenic wild myxomatosis viral strain breaks through biosecurity on the farm attributable to a delay of seroconversion caused by a less effective vaccine protocol used, which in turn provides a greater window of opportunity for pathogenic invasion. Moreover, Dalton et al. warn against the inappropriate use of emergency vaccines in ongoing outbreaks that are causing more immunosuppression in young rabbits and ending up doing "more harm than good" (Dalton et al., 2012). In this circumstance, the vaccination programme would be seen in a negative light as unjustifiably costly for the farm managements in terms of the under-delivery of promised health benefits and extra (if not redundant) economical costs incurred with the use of vaccines.

The extent of seroconversion based on serological antibody quantification may not be directly correlated with the level of protection vaccinated animals have enlisted – as

clearance of MV from the host's system is performed predominantly by cellular-mediated immunity with the assistance of neutralising antibodies playing a minor role. However, appreciating the fact that the only commercially available method of decrypting how successful vaccination programmes are on farm holdings is by detecting antibodies in sera, Dalton et al. (2015) advise the management of production units with the accommodating expandable capacity to test the levels of seroconversion 1-2 months following application of vaccines. Dalton advocates that when immunity is fully developed 3 weeks post-vaccination, it would be beneficial to monitor how successful the seroconversion is, identify any trends that would indicate an insufficient or delayed immune response to the vaccines currently in use, and ultimately evaluate the efficacy of the current vaccination protocols (Dalton et al., 2015).

The same group of scientists (Dalton et al., 2012) have proposed that the possible causes of recurrent failures to prevent myxomatosis in farm rabbits in Europe are likely to be associated with the emergence of new myxoma viral strains that can evade the protective immune responses generated by vaccinations, as well as the improper execution of vaccine strategies on farms. In spite of the extensive coverage of the otherwise effective live homologous attenuated MV and live heterologous SFV vaccines currently in use, discrepancies in frequency, administration methods and employment of the homologous or the less effective heterologous vaccines from farm to farm have dampened protective efforts and contributed to a stagnant vaccination strategy on rabbit farms.

II. Pet rabbits

The clinical relevance of vaccination programmes is solidified by statistics confirming that vaccinations of pet rabbits account for 5.1% to 24.5% of the reasons for veterinary visits (Ziętek et al., 2022). Since the licensing of the trivalent recombinant vaccine Nobivac Myxo-RHDV PLUS in 2020 in Europe with year-long immune protection on offer, increased interest in vaccines inducing longer immunity has been registered on records, and in fact, 23.7% of all vaccines administered belong to the polyvalent recombinant vaccine. Some owners perceive the once-per-annum vaccines a good solution for their novel additional protection against RHDV2 viral antigen despite the higher retail prices, whilst others habitually prefer the semi-annual vaccines for their 2 to 3 times lower

financial costs, which are recommended by rescue organisations and chosen by commercial breeders for similar economic reasons. Thus, the popularity of the semi-annual vaccines immunisation protocol has remained steady with no palpable drastic downturn, despite the rise in interest in the recombinant vaccines (Ziętek et al., 2022).

III. Wildlife

A very recent study (García-Vicente et al., 2023) conducted in the Iberian Peninsula has noted the ineffectiveness of the current control measures mainly based on vaccination campaigns in the conservation and recovery of native wild rabbits population as an ecologically vital species on the island. The continuous plummet in the wild rabbits population has led to "major repercussions" in a down-spiralling phenomenon of chain effects on biodiversity, spanning from inadequate establishment and maintenance of vegetation cover due to lowered degrees of warrens digging and seeds dispersing to knock-on dwindling populations of predator species, including endangered species such as the Iberian lynx (*Lynx pardinus*) and the Imperial eagle (*Aquila adalberti*) (Lees & Bell, 2008; Delibes-Mateos et al., 2014; Moreno et al., 2007). The persisting issue with conservation efforts not dislodging their intensive efforts in success despite the rigorous vaccination programmes in place calls into question the efficacy of the current MV vaccines and their uses.

Vaccination campaigns against myxomatosis and RHD are continued in the Iberian Peninsula on the assumption of their usefulness to revitalise the wild European rabbit population under the population loss scenario (Cabezas et al., 2006). Although Abade dos Santos et al.'s very recent study (2022) has shown that 2 of the commercially available vaccines, Mixohipra-H[13] and Nobivac Myxo-RHDV PLUS[14] are fully protective against a naturally attenuated strain of myxomatosis (ha-MV) infection in wild rabbits (Guitton et al., 2008), there are also mounting evidence stacking up against the negligible effect of the blind double-vaccinations programme against myxomatosis and RHD (Cabezas et al., 2006;

[13] (HIPRA Headquarters, Amer, Girona, Spain; lot 05D7G), a live homologous vaccine containing attenuated myxoma virus

[14] (MSD Animal Health, Boxmeer, The Netherlands; lot A003B02), a live homologous vector vaccine containing two attenuated recombinant myxoma virus vectors expressing the VP60 gene of RHDV or RHDV2 (Reemers et al., 2020).

Calvete et al., 2004a, Calvete et al., 2004b). As Ferreira et al. (2009) have elucidated, the success of vaccination campaigns in the field can be affected by a multitude of factors (Fig. 1). Such factors include "the general low density of wild rabbit populations, the cost of capturing animals (Delibes-Mateos et al., 2008), the individual physiological condition (Cabezas et al., 2006; Calvete et al., 2004), the presence and density of vectors (Rosell et al., 2000) or the time the immunisation takes place (Calvete, 2006)".

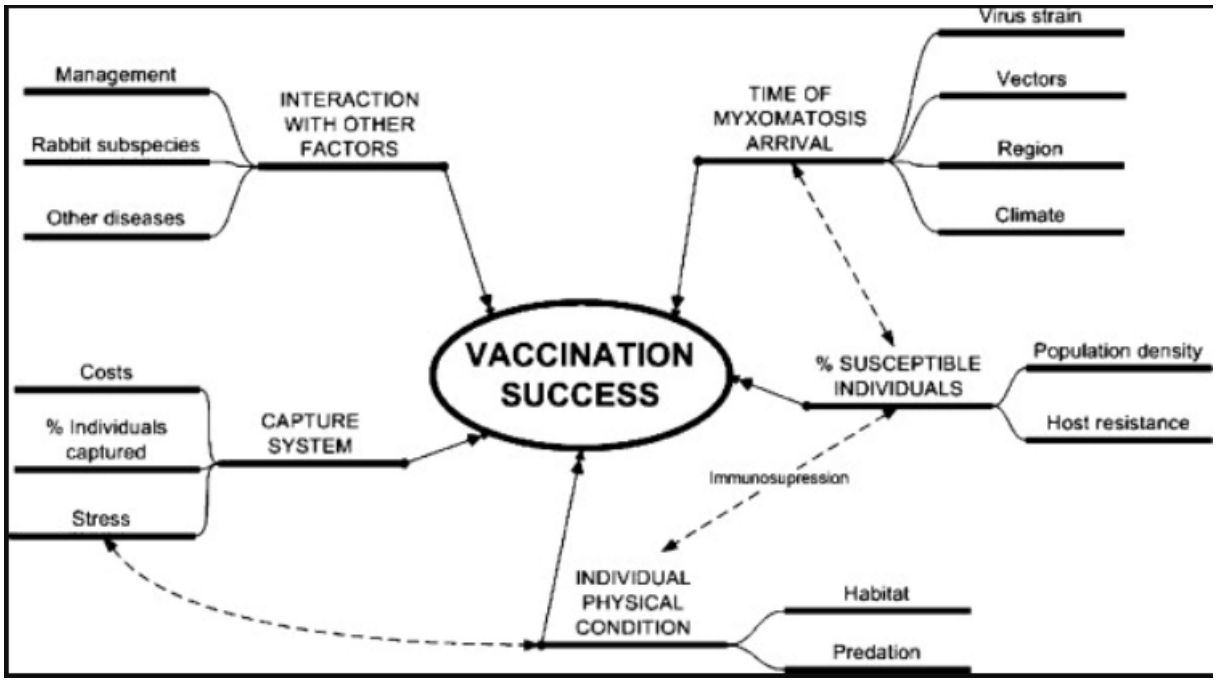

Figure 1 "Factors affecting the success of wild rabbit vaccination against myxomatosis." (Ferreira et al., 2009)

For one, conservationists will first have to consider the impractical aspect of some commercial vaccines, such as POX-LAP, which must be freshly prepared and immediately applied to animals within 15-20 minutes of preparation to sustain their efficacy and prevent loss of potency in field conditions. This limitation alone has excluded an unsuitable range of vaccines available for use in wildlife at the initial planning stage.

Furthermore, a traditional capturing method by ferreting as conventionally used by hunters only has a maximum efficacy of 36% in low-density populations (Cowan, 1984), inferring that it would be implausible for the proportion of translocated (captured, directly vaccinated and released) rabbits to reach herd immunity at above 70% of the whole population. In light of the low success capturing rate, Ferreira et al. (2019) has remarked

that “under low-density conditions, juvenile immunisation against myxomatosis due to vaccination would be, to say the least, impractical.”

To overcome the imposingly limiting obstacle of individual parenteral injections needed for vaccines administration (Marlier, 2010; Ferreira et al., 2009), some scientific researches have ventured into the exploratory praxis of developing transmissible vaccines to spread horizontally amongst rabbit individuals after the initial direct inoculation of a small number of rabbits, to expand the reach of immunised protection in a wild population potentially exponentially (Angulo & Bárcena, 2007; Bárcena et al., 2000). One of the naturally attenuated circulating strains originated from the Lausanne MV strain, isolate 6918, has been identified to possess potentially compatible characteristics for safe inoculation and immunisation, as a result of several frameshift mutations that severely disrupted different important virulence factor-encoding genes (Blanie et al., 2010; Barrett et al., 2007; Morales et al., 2009). The promising prospects were unfortunately hampered by unsatisfactory results, which revealed the relatively low horizontal transmissibility of the severely attenuated strain inducing seroconversion in only 50% of the non-inoculated rabbits (Torres et al., 2000, 2001). Despite its potential functionality, the instrumental flaws in limited horizontal transmissibility and the resultant failed goal of achieving herd immunity would be macroscopically expanded when the scope of its use is extended to the vast geographically sparsely populated areas in the wild (Ferreira et al., 2009).

Research has revealed that the impact of vaccination also varies depending on the time when it is administered (Ferreira et al., 2009). Myxomatosis outbreaks tend to peak in summer in the wild shortly after the birth of a new generation of juveniles (Calvete et al., 2002), gradually manifesting a rapid increase of antibodies in these young rabbits just after the outbreak, which translates into a high prevalence of antibodies in the surviving population of adult rabbits later (Calvete et al., 2004a). Blind vaccinations after the annual outbreak before the breeding season are likely to generate nullifying neutralisation of the vaccines’ immunisation effect since a high prevalence of natural antibodies against myxomatosis in the adult rabbits population has already been developed after natural infection by the circulating strains in an enzootic region. As “wild populations of rabbits are characterised by age-dependent increases in the prevalence of antibodies to myxomatosis” (Calvete et al., 2002; Cooke et al., 2000; Cabezas et al., 2006; Calvete et al.,

2004a) therefore, the older the animals receive vaccinations, the higher the probability of natural antibodies interference will happen.

Substantial and costly efforts have been incorporated into wildlife conservation in recent decades to reduce mortality from fatal diseases, including myxomatosis and stimulate the recovery of wild rabbit populations by translocating wild rabbits into areas where populations are low and undertaking vaccination campaigns before release (Angulo & Villafuerte, 2003). In most translocation programmes, for supposed economic and logistic constraints, wild rabbits are vaccinated in a non-systemic and blind approach without prior assessment of the immunological status, sex, heath or age of the population (Cabezas et al., 2006; Ferreira et al., 2009). Blind vaccinations (Cabezas et al., 2006; Calvete et al., 2002) in field conditions where vaccines are typically administrated only once to individuals of any age class (Angulo, 2003), regardless of their serological status and the highly variable epidemiological pattern of myxomatosis in a specific region annually (Villafuerte et al., 2000), have considerable drawbacks. Conflicting evidence has been presented on such an immunisation regime's effectiveness in improving rabbit survival chances and restoring population abundance (Cabezas et al., 2006; Calvete et al., 2004a; Calvete et al., 2004b). As the course of amyxomatous myxomatosis is arguably more difficult to predict in wild rabbits, as though wild rabbits are genetically more resistant against myxomatosis than domestic rabbits, and amyxomatous myxomatous generally produce milder clinical signs, Marlier et al. have shown that the mild manifestation of the same amyxomatous strains in specific-pathogen-free laboratory rabbits grows in pathogenicity when transferred to conventional rabbits (Marlier et al., 1999, 2000b; Marlier, 2010). This is postulated to be related to the higher burden of parasitic- and bacterial-origin pathogenic loads in wild animals, such as *Pasturella multocida*, an opportunistic commensal commonly causing rabbits' respiratory tract infections, which could aggravate the virulence of MV strains and amyxomatous viruses (Kerr, 2012). This makes the profiling of serological status before and after wildlife vaccination programmes all the more important in ensuring successful and effective immunisation for the purposes of increasing the vaccinated animals' chance of survival and re-establishing population abundance.

Against blind vaccinations

The immunosuppressive outcome of vaccination with live attenuated viral strains (Marlier, 2010) inoculated during an epidemiological outbreak is shown to have a negative impact on vaccinated individuals in a test who suffered from a shorter survival (8%) than unvaccinated juveniles (16%). When also considering the short-term detrimental impacts of vaccinations on juveniles (Calvete et al., 2004b) alongside fever, secondary myxomas formation and anaphylactic reactions (Rosell et al., 2000), which are likely to exacerbate the health status and body condition of vaccinated wild rabbits under high physiological stress, the preferred timing of wildlife vaccinations is fixated to be after the breeding season and not during an outbreak.

In fact, Teixeira et al.'s study (2007) has found that adult wild rabbits did not benefit from improved survival in the short and long terms after a blind vaccination campaign. Rather, the high level of physiological stress that translocation processes to a new and foreign environment and manual handling such as capturing, weighing, sexing and earmarking with numbered metal tags (Rouco et al., 2016), generated the target species was likely to have shortened their life span in the short run (Teixeira et al., 2007) and decreased the vaccine's protective potency (Cabezas et al., 2006), especially for young or weak individuals (Calvete et al., 2004b; Cabezas et al., 2006). Female rabbits seem to be receiving the worst brunt of the accumulated effects of these artificial immunisation programmes, as they have an overall lower survival rate reported due to the compounded stress from translocation and depletion of body condition during gestation and infant nursing period (Kontsiotis et al., 2014).

Further data has shown that regardless of whether rabbits were vaccinated against the disease prior to the outbreak, all juvenile animals turned seropositive at the end of the experiment. This uniform seropositivity results and the unanimous lack of difference between the monthly vaccinated and unvaccinated juvenile rabbits under study showing myxomatosis symptoms have led the author of the paper to the interpretation that, after the annual disease outbreak, virtually every animal in the population that has not succumbed to the disease will have developed antibodies against the disease as a result of natural exposure and hence become immune to following infections (Teixeira et al., 2007). Nonetheless, it must be declared that this author's pre-emptive recognition of the patterns may not be an accurate representation of the real functionality of the vaccines in preventing

deaths caused by MV and that seropositivity, whether at a high or low level, is not an automatically straightforward synonym for (in)effective protection against myxomatosis, as it is cell-mediated immunity involving monocytes and macrophages that participate in the major defensive role combatting myxomatosis.

All in all, these published data suggest that a single, blind, non-systemic vaccination campaign in a low-density rabbit population neglecting the time when the outbreak occurs has resulted in undesirable immunisation performance that may have actually made the species more vulnerable to extinction (Calvete, 2006). In light of the results, Ferreira et al. (2019) advise against the implementation of vaccination campaigns against myxomatosis as a prophylactic measure, as they are "generally not functional tools" to improve population abundance without meticulous planning, long-term monitoring and management. Rather, "the typical blind vaccination would have compromised any well-intentioned attempt to recover rabbits population" (Ferreira et al., 2019). It is argued that "this measure should be avoided, and instead, efforts should be made to understand the immunological status of the population and other crucial parameters such as body conditions" (Cabezas et al., 2006; Calvete et al., 2004).

For vaccinations in wild rabbits

In the opposing stance, a different group of scientists promoting the beneficial role of wildlife vaccinations for the purpose of population conservation have suggested that monthly vaccinations against myxomatosis of free-living, naïve, wild juvenile captive rabbits have expedited improved individual survivals in challenge infections (Calvete et al., 2004; Guitton et al., 2008) and are considered almost completely effective (Rosell, 2003). Radio-tracking data has shown that juvenile rabbits vaccinated before outbreaks benefited from higher survival rates (31%) and longer lifespans compared to unvaccinated individuals (14%). Likewise, vaccinated juveniles showed a "1.9-fold higher overall survival rate than unvaccinated ones, respectively 0.39 (±0.08SE) and 0.21 (±0.08SE), although these differences were not quite statistically significant" (Rouco et al., 2016). Statistical analysis points to an increased mortality rate of 18% in overall unvaccinated juveniles compared to vaccinated juveniles participating in systemic vaccinations before

the epizootic event and after breeding season when there was greater juvenile availability (e.g. Gonçalves et al., 2002).

Nevertheless, Rouco et al. (2016) still advise the utilisation of critical thinking skills and caution when reflecting on the costs and benefits relationships of different vaccination protocols. It is concluded in their study that in spite of the lack of detrimental effect of vaccinations on short- and long-term rabbit survival, no clear evidence in support of the beneficial effects is found either. Given the hefty consequences of myxomatosis and RHD in rampant areas, even "if it is not feasible to assess the immunological status of the donor population, the most conservative option is to carry out blind vaccination campaigns to ensure that translocated rabbits are at least protected against infection after release, particularly if juveniles are being translocated since they generally have lower antibody prevalence" (Guitton et al., 2008). In the particular circumstances of adults translocation, however, results have indicated that "blind double vaccinations may increase the economic cost without any benefit of significantly increased survival" (Rouco et al., 2016) when carried out without previous yearly assessments of the immunological status of the donor population, as the high prevalence of natural antibodies against myxomatosis in an already seroconverted fraction of the rabbit population would render any time-overlapping vaccinations "worthless" (Arenas et al., 2012; Parkes et al., 2008; Santoro et al., 2014).

Factors to consider in wildlife vaccination programmes

Due to the interactive panoply of factors that can elicit paradoxical effects at individual levels (Rouco et al., 2016), the crux of wild rabbits population protection against myxomatosis (and other deadly diseases) can be described as "one of the chimaeras of rabbit's management and conservation with few easy and visible short-term solutions" (Rouco et al., 2016). Challenging as it is, summarising the crucial factors and potential changes needed to be taken into account when establishing sound and well-balanced vaccination protocol to be undertaken against myxomatosis in the field to mitigate the enigmatic uncertainty of all possible outcomes and maximise the campaign's effectiveness in endemic areas:

- The timing of vaccination with respect to the seasonality of epidemics in a specific location varies from year to year (Guitton et al., 2008). This would be ideally supplemented with a health monitoring programme of the wild rabbit populations, if its logistic constraints and costly implications can be overcome. Vaccinations would be most efficiently performed in the 2-month window before the appearance of the yearly epidemic – when herd immunity will be at its lowest – and after breeding season – when there is a wealth of susceptible non-immune kits recently came into existence (Guitton et al., 2018). Precisely, vaccination on 1 single occasion from days 21 to 80 after the birth of several cohorts of rabbits naïve to myxomatosis (Ferreira et al., 2009) is proposed to be the best time for the introduction of a new attenuated MV strain (Merchant et al., 2003).
- In view of the distinctively variable spatial-temporal pattern of the causative agent i.e. MV (Villafuerte et al., 2000) – such as the fluctuating yearly variations of the onset of epidemics (Guitton et al., 2018) as previously mentioned – avoidance of vaccinations before breeding season or during disease outbreaks is recommended, as the success of immunisation would be compromised otherwise. This is a frequently neglected component of conservation programmes management due to the difficult practicality of enforcement without a pre-existing rigorous population monitoring scheme (which is absent most of the time).
- Several monthly vaccinations or otherwise structured in a systemic manner throughout the pre-, during and post-phases of a myxomatosis outbreak (Calvete et al., 2004) to guarantee immunisation of the largest feasible proportion of juveniles, versus a single vaccination campaign that can only offer limited protection to a mix bag of seronegative kits and already seropositive adults. However, care must be taken to reduce the level of handling stress experienced by wild rabbits that are not accustomed to high vaccination frequencies, as a high level of physiological stress can contribute as a negative survival factor, causing deterioration of individual physical conditions and immunities in the interplay of individual-population-disease dynamics (Calvete et al., 2004; Ferreira et al., 2014).

Vaccine breaks

The prevalence of vaccine breaks rises in the event of line production problems such as quality control assurance failures that render batches of vaccines ineffective, or when changes in the pathogen, host and environmental factors (Knight-Jones et al., 2014) have registered an effect on the host-pathogen relationship. Problems with regard to production are prone to arise as a result of erroneous manual handling or procedures application, for instance, failure to maintain a stable range of temperature in the cold chain, inappropriate dosing or improper execution of vaccine strategies. More specifically, evolutionary changes of the pathogen lead to the re-emergence of old strains or the introduction of new strains with an accompanied renewed pathogenicity and increased resistance to the original vaccine doses or have developed a new adaptability to evade the protective immune responses generated by vaccination. As immunity conferred by vaccines has a large individual variability and unpredictable duration in time, individual or specific flock factors including genetic resistance and health status of rabbits, population densities and exposure rates, should be taken into account when incorporating an effective vaccination strategy to protect industrial and domestic farm, laboratory and pet rabbits from myxomatosis. Naturally, the frequency of booster vaccinations to guarantee a constant high level of immune protection, and the pros and cons of the use of live attenuated homologous, heterologous, recombinant or monovalent vaccine strain should be thoroughly examined and considered to reinforce an effective protocol.

Conclusion on efficacies of different vaccination protocols

Reiterating the similarities in disease prevalence and control dynamics, the severity of myxomatosis outbreaks under the influence of various factors shares a similar mechanism with the objective assessment of vaccine efficacies, as the disease itself and the preventive vaccines used against it are equivocally affected by a multifactorial caveat in a permanently shifting background. The intrinsic virulence of the virus[15], genetic resistance of the local rabbit populations, and external factors such as weather conditions are among some of the paramount parameters in determining the success of a vaccination protocol. For instance, the nutritional status and age of the vaccinated rabbit and the presence of other diseases or parasites in the host can also affect the clinical severity of the disease and the success of immunisation (Joubert et al., 1982; Cattadori et al., 2007; Marlier et al., 2000b). These factors, amongst others, need to be weighed in when evaluating the efficacy of different vaccination methods, strains and protocols, as they are constantly fluctuating in a multifaceted and ever-changing environment.

[15] Cold temperatures can increase the effective virulence of MV, while high temperatures can ameliorate its impact (Marshall, 1959).

Future directions

1. General

As myxomatosis retains its vital role in wild rabbit populations regulation, the direction of further research can be steered towards investigating under which conditions and circumstances will ascertain the success of systematic vaccination campaigns and will vaccines then be proven as a useful management tool (rather than have its negative consequences outweigh the advantages). This can include analysis of the minimum threshold of wild rabbits required to be vaccinated to ensure effective herd immunity (Ferreira et al., 2009), and whether double vaccinations against both myxomatosis and RHD simultaneously have a mutually inhibitory or potentiating effect on the production of specific antibodies.

2. Effective vaccination of wild hares (*Lepus europaeus*)

A concerning deficiency to address would lie in the urgency to develop a robust novel vaccine and/or establish effective dosages[16] of the currently commercially available MV vaccines for use in the Iberian hare (*Lepus granatensis*), in order to prevent the contagious transmission of myxomatosis[17] jumping from the more resistant species of hares (*Lepus linnaeus*) to the less resistant[18] but more endangered wild European rabbits species[19]. No

[16] application of a higher dose of Mixohipra-FSA vaccine alone may induce protection and could possibly be used to counteract the accelerated decrease of wild hare populations due to ha-MYXV emergence. 2 out of the 3 hares vaccinated with a 10-fold higher dose as recommended for domestic rabbits induced a satisfactory humoral response against MV, with no signs of general or local (inoculation site) reactions. Furthermore, the haematological and biochemical data of the groups of hares vaccinated with the lower doses revealed no differences in globulins, contrasting with the decrease in the albumin/globulin ratio in the case of hares vaccinated with the 10-fold vaccine doses (Abade dos Santos et al., 2022).

[17] Although ha-MYXV was initially detected only in Iberian hares, it was later (mid-2020) reported in wild and domestic rabbits (Abade dos Santos et al., 2022). The recognition that ha-MYXV affects not only hares but also the European rabbit (*Oryctolagus cuniculus*), indicates that hares may be contributing to the spread of ha-MYXV in the wild European rabbit (*Oryctolagus cuniculus*).

[18] An important difference to highlight is the higher viral load (10–100× higher titres) in the lungs of rabbits compared to hares, which may be related to the fact that the clinical course in rabbits was more acute and deadly, and also elucidate the amplification of severe clinical signs in a host species with weaker innate resistance after contraction of the same viral strain from a more resistant host (Abade dos Santos et al., 2022).

protection[20] was afforded to hares against the natural recombinant ha-MV strain in a challenge infection after vaccinations with a commercial dose of Mixohipra-FSA[21] or Mixohipra-H[22] (Abade dos Santos et al., 2022), which is alarming for the conservation of the wild rabbits population as it pertains to the possible transmission of myxomatosis between wild hares and wild rabbits and the subsequent magnification of clinical signs in the less resistant wild rabbits.

3. Immunostimulants supplementation

Reverting into the context of farmed and companion pet rabbits that are under more delicate human care, despite all the triumphant successes of the current vaccines in use against myxomatosis reported by a wide array of scientific studies, myxomatosis specifically has remained a perpetrating challenge for farm rabbitries revenue protection throughout Europe (Farsang et al., 2003; Kritas et al., 2008; Marlier et al., 2000a, 2000b; Marlier, 2010; Belsham et al., 2010) in spite of the extensive coverage of vaccination schemes and control measures (Arthur & Louzis, 1988). In light of this failure, scientists have a vested interest in exploring other alternative non-conventional epidemiological measures, utilising current knowledge of the structure and properties of MV to turn the table in the war for the conservation of endangered wildlife species and the economic interests of growing farm rabbits. One such option is the incorporation of immunostimulant supplementations into oral feeds alongside the current implementation of vaccination protocols to boost immunity against viral pathogens and reduce excessive pro-inflammatory responses in host animals.

MV is characterised by the possession of virulence factors encoded in a peripheral region, allowing for attack on and escape from the host's immune response whilst modulating the

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[19] a species given the status of "Endangered of Extinction" by the IUCN in 2019 (Villafuerte & Delibes-Mateos, 2019; Soriguer & Carro, 2019).

[20] Hares vaccinated with Mixohipra-FSA and Mixohipra-H in the conditions recommended for rabbits did not seroconvert robustly. Nor did the vaccinated hares gain protection against a very low dose challenge with ha-MYXV (100 ffu). In fact, with this standard vaccination protocol, all vaccinated hares developed severe disease after challenge, similarly to the non-vaccinated controls (Abade dos Santos et al., 2022).

[21] (HIPRA Headquarters, Amer, Girona, Spain; lot 12M9J), a live heterologous attapulgite adjuvanted vaccine containing Shope fibroma virus.

[22] HIPRA Headquarters, Amer, Girona, Spain; lot 05D7G), a live homologous vaccine containing attenuated myxoma virus.

secretion of cytokines (Yua et al., 2022). The onslaught of cytokine storm is a biological process inhibited in the naturally resistant host, the tapeti or jungle rabbit (*Sylvilagus brasiliensis*), which is able to resolve the disease into the manifestation of only mild lesions. In contrast, when the virus jumps hosts and infects the less resistant species of the European rabbit (*Oryctolagus cuniculus*), increased production of pro-inflammatory cytokines, especially IL6 and TNFα, arises as a result of the host's lack of genetic materials to encode for inhibitory biochemical pathways. The infection is thus able to progress into more severe forms of myxomatosis with higher mortality rates in the less resistant hosts (Chong & Sriskandan, 2011). Taking into account the interdependent relationships between cytokines activation and the occurrence of myxomatosis, the reduction of pro-inflammatory cytokines level is posited at the core of combative strategies in mitigating the fatal clinical syndrome of excessive cytokine storm as triggered by myxomatosis. Dietary supplementation with postbiotics could potentially offer a hopeful branch of solutions aimed at restoring the homeostatic cytokine balance (Foligne et al., 2007; Herfel et al., 2011) by increasing anti-inflammatory cytokines while downgrading pro-inflammatory cytokines, as successfully demonstrated in studies in other vertebrate species (Humam et al., 2022; Izuddin et al., 2018; Wang W., 2017; Yang et al., 2015).

A study was conducted in 2023 (García-Vicente et al., 2023) to quantify the immunomodulatory effect of postbiotic supplementations on the growth parameters of rabbits vaccinated against myxomatosis, in an attempt to materialise novel and more effective ideas in preserving species abundance in the wild. Blood samples collected from all animals immediately before vaccination and 30 days later were analysed to evaluate the gene expression for cytokines IL6 and TNFα in animals on postbiotic supplements. Despite the negative report on the lack of improved body indices and mean daily gain of the vaccinated subjects on postbiotic supplements under study (García-Vicente et al., 2023), the results reveal "a relative under-expression of the gene" encoding for TNFα. The positive result shines a glimpse of optimism on the benefits of postbiotic supplements as a down-regulator of pro-inflammatory cytokines, capable of mediating and alleviating severe lesions in rabbits infected with immunosuppressive myxomatosis (Jeklova et al., 2007). The causality between dietary postbiotics supplementation and the under-expression of TNFα may be harnessed to lower the risk of adverse effects in animals vaccinated with live vaccines as well as lesions severity in naturally infected animals.

The positive result of the use of dietary supplements as a complementary immunostimulant to increase the efficacy of full vaccination programmes against myxomatosis is supported by another study (Volosyanko & Popova, 2016). The findings on the cytological characterisation of thymus in rabbits vaccinated against myxomatosis suggest that the protective immunity conferred by commercial vaccines upon vaccinated animals can be potentiated synergistically by the administration of Ribotan as an immunostimulant (Volosyanko & Popova, 2016). Myxomatosis vaccination followed by an injection of Ribotan may reduce signs of inflammation such as oedema and hyperaemia, and enhance activation and proliferation of immune cells. The mechanism is conceptualised as the functional modulation of the thymus as a central immune system organ that produces thymic hormones, the role of which is to promote maturation and differentiation of T-lymphocytes. A "fast and adequate immune response" to viral antigens as reflected by a tremendous T-lymphocytosis in blood following vaccination, may signify a good degree of immunisation against myxomatosis (Vasicek et al., 2014). This may have useful implications as an aide to facilitate the development of an adequate and good-quality immune response in young, old, pregnant and immunocompromised animals. Nevertheless, T-lymphocyte count is not a suitable parameter alone to be solely dependent on for the evaluation of the efficacy of vaccination programmes against myxomatosis, as changes in T-cells count do not exclusively answer to MV. A high T-lymphocyte level in the blood may be swayed by interference of other viral pathogens, such as the rabbit haemorrhagic disease virus (RHDV), which is used in the recombinant vaccines with the myxoma virus.

Considering the ease of administration in animal feed, even in a large-scale industrial setting, and the potential health benefits supplementary postbiotics may bring along to naturally infected animals, further research should aspire to prove or debunk dietary immunostimulant supplements as a valuable tool for disease control.

4. **Anti-cancer therapy**

Numerous discoveries and attenuation processes of vaccine strains have been accomplished regarding the high genetic variability and immunomodulatory capabilities of MV "elegantly subverting the host immune system through mimicry, stealth and avoidance" since the sequencing of the full MV genome has opened the door to careful genetic manipulation of the virus using different primer sequences and analysis.

One of the most prominent areas of limelight interest is the leasing of MV constructs in anti-cancer therapy (Lalani, 1999). The stenotic host range of MV (i.e. non-pathogenicity to other vertebrate mammals) combined with its innate potent oncolytic "tropism for many human tumour cells" (Marlier, 2010), has fostered enthusiasm in the clinical prospect of improving treatment outcomes (Liu et al., 2010). Studies have so far shed light on the effective translational applications of MV as a live oncolytic virus in infecting tumour tissue in-vivo and "curing" mice from brain tumours in-vitro (Langland & Jacobs, 2002), whilst remaining harmless to the host mammals. A recombinant MV with attenuated virulence in rabbits and enhanced oncolytic capacity by insertion of selective anti-cancer therapeutic genes (Liu et al., 2009) can augment and improve its safety and potency features in-vivo (Kim et al., 2009; Lun et al., 2005, 2007). MV's comprehensive capability to counterattack malignancies such as murine melanoma lung metastasis in a mouse model, when systemically administered with chemotherapeutic rapamycin (Stanford et al., 2008) to promote the regression of tumours, is reported to have no detectable side effects even in immunocompromised mice. Its ability to substantially purge cancer cells from human bone marrow transplants has also been described by Rahman et al. (2010).

5. **Anti-inflammatory disorder treatment**

MV's mechanisms in mitigating inflammatory responses have also garnered research interest in its potential therapeutic power in treating inflammatory-based diseases in humans. Continuity of these researches to acquire a more detailed understanding of the behaviour, adverse effects and pathogenesis of MV in both permissive (i.e. rabbit) and non-permissive (especially human) hosts is of utmost importance if their useful immunomodulatory qualities are to be extrapolated to clinical applications in the future (Spiesschaert et al., 2011) – "to date, the first of these virus-derived drugs (Serp-1) has

behaved particularly well in humans, with essentially no clinical adverse side effects, in Phase I and II clinical trials" (Spiesschaert et al., 2011).

6. **Mist vaccine**

The hypothesis that amyxomatous myxomatosis, as an adaptive response to increase survival in the absence of vectors, occasionally transmits via respiratory and conjunctival secretions (Joubert et al., 1982; Kerr & McFadden, 2002) could virtually open a new vision in vaccines development – into feasible opportunities for the development of a mist-spray mass vaccine that can be administered via aerosols into the nose and/or eyes. This would be largely useful in an industrial setting if the effective vaccination dosage could be inferred from a known infective viral titre via these routes, too.

7. **New assay or PCR diagnostic tool**

It would be very helpful if a new assay[23] or PCR method could be developed to generate a fast diagnosis with high sensitivity and specificity for acute and chronic myxomatosis – which may or may not present typical lesions and appear inconclusive in histology (Krogstad et al., 2005; Wunderwald et al., 2001) – by taking a nasal, ocular or genital swab. If developed, this diagnostic tool could easily be incorporated into the swift enactment of epidemiological containment measures within the vicinity of a disease outbreak.

[23] Kwit & Rzezutka has proposed the alternative employment of "novel methods such as microarray, next-generation sequencing, and mass spectrometry (MALDI-TOF MS)" for their greater output in screening samples for viral pathogens (Kwit & Rzezutka, 2019).

Conclusions

In general, the success of myxomatosis vaccinations is influenced by multiple factors, including the virulence of the virus, appropriate vaccination methods, viral strains used in the vaccines, frequencies of vaccinations, the age and the health status of the animals to be vaccinated, and various environmental conditions. Seropositivity to MV can have both positive and negative effects on rabbits, with age playing a significant role in susceptibility. Further research is needed to understand better the complex interactions between vaccination protocols, seropositivity, and the overall health of rabbit populations in order to optimise disease prevention and management strategies.

All the reviewed contemporary scientific experimental studies have produced a consensus that intradermal inoculation is esteemed to be the most effective administration method with live attenuated homologous or recombinant vaccines to protect against and alleviate severe symptoms of myxomatosis in all rabbits.

To effectively evaluate the efficacies of old, present and new vaccine strains, it would be imperative to standardise an extensively reviewed diagnostic tool and protocol that provides the highest sensitivity and specificity in determining the level of protection a vaccine strain endows on its recipients, given the current lacking department of serological assays in detecting cell-mediated immunity protection which has a superior role in defending against myxomatosis in hosts to neutralising antibodies.

With the complete revelation of the genome sequence of the myxoma virus, it has opened up a whole new era of endless possibilities in the development of different novel and previously untrialled attenuated and/or recombinant vaccine strains to prevent against myxomatosis. Despite a wide range of commercially available vaccines against myxomatosis on the market, this can be useful in developing an effective vaccine and/or establishing an effective dose of any of the current vaccine strains for use in wild hares. The criticality of this field is highlighted in the deficiency the currently commercially available vaccines used in rabbits in delivering cross-protection to wild hares. This would be an important control point in preventing vaccine breaks in wild rabbits population, as although wild hares are more resistant to MV, they can nonetheless contract and transmit the disease to the less resistant and more ecologically fragile species of wild rabbits. A mist vaccine can be a novel approach in vaccinating wild animals without the need for stressful

procedures including capturing, individual handling and parenteral injections, which could further lower the health status of the handled wild animals.

Myxomatosis is also an area of interest for human medical advancement due to its stenotic host range (rendering it harmless to other mammals non-lagomorphic) and clever tactics in evading and manipulating the host's immunity. Promising prospects have been demonstrated in researches that exploited these properties of MV in oncolytic therapy and curing disorders of an anti-inflammatory origin.

Depending on the rabbit's use, different vaccination protocols follow. Wildlife vaccination for the purpose of conservation has produced different experimental results and divided scientists on its useful place in field conditions. With the ongoing controversial blind vaccination approaches to wild rabbit populations, more research is needed on the topic in order to decipher the full extent – both harms and benefits examined – of the efficacy of the blind vaccination programmes fitted to the purpose of population abundance recovery currently in use in the field, in order to improve disease prevention and management strategies. An engaging discussion would ensue on exploring a balance between the practical, ethical and economical aspects of preliminary evaluation of vaccine candidates in the target species of wild rabbits, given the foreseeable negative effects of captivity and trapping on wild rabbits.

Reiterating the dual origins of the SG33 strain highly likely to be a by-product of mixing different vaccine strains with a wild viral strain in the same animals, more research is need on clarifying the hazards of combining the use of different vaccine strains that are essentially live attenuated viral strains, especially in the agricultural sector. In a more applicable and controllable context, such as in farm settings, some scientists have ventured out in an alternative direction to explore other ways to complement the arguably ineffective measures, such as the addition of oral supplementations of immunostimulants into the diet of farmed animals, which may have the capacity to elicit a positive effect if enough concrete evidence can be produced and replicated.

On the front role of veterinarians in a clinical scenario, owner's education on the need to regularly vaccinate pet rabbits against myxomatosis sets the foundational building block for success in protecting vulnerable patients against the deadly disease. The 2 novel recombinant vaccines, for instance, provide a convenient opportunity in offering sufficient

double immunity against 2 very deadly rabbits-specific infectious diseases, RHDV and MV.

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