# VACCINATION OF NON-DOMESTIC ANIMALS AGAINST EMERGING VIRUS INFECTIONS

VACCINATIE VAN NIET-GEDOMESTICEERDE DIEREN TEGEN OPKOMENDE VIRUSINFECTIES

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"He is able who thinks he is able."

*-Siddharta Gautama, "Buddha"*

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## ABBREVIATIONS

| | |
|---|---|
| ADV | Aleutian disease virus |
| AI | avian influenza |
| AIV | avian influenza virus |
| AZA | association of zoos and aquariums |
| AAZV | American association of zoo veterinarians |
| CAV | canine adeno virus |
| CCoV | canine corona virus |
| CDV | canine distemper virus |
| CI | confidence interval |
| CN | central nervous |
| CPIV | canine para influenza virus |
| CPV | canine parvo virus |
| DIVA | differentiation of infected from vaccinated animals |
| DMV | dolphin morbilli virus |
| dpi | days post-inoculation |
| DRV | dolphin rhabdo virus |
| EAZA | European association of zoos and aquariums |
| EAZWV | European association of zoo and wildlife veterinarians |
| EDTA | ethylene-diamine-tetraacetic acid |
| ELISA | enzyme linked immunosorbent assay |
| EU | European Union |
| F | fusion |
| FCoV | feline corona virus |
| FCS | foetal calf serum |
| FeLV | feline leukaemia virus |
| FHV | feline herpes virus |
| FPV | feline panleukopenia virus |
| GI | gastro-intestinal |
| GMT | geometric mean titre |
| H | haemagglutinin |
| HAO | precursor haemagglutinin |
| HI | haemagglutination inhibition |
| HPAI | highly pathogenic avian influenza |
| Ig | immunoglobulin |
| i.m. | intra-muscular |
| ISCOM | immuno-stimulating complex |
| IUCN | World conservation union |
| KV | killed vaccine |
| LPAI | low pathogenic avian influenza |
| M | matrix |
| MLV | modified live virus |
| MS | member states |
| NP | nucleoprotein |

| | |
|---|---|
| PBMC | peripheral blood mononuclear cells |
| PDV | phocine distemper virus |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| RV | rabies virus |
| SARS | severe acquired respiratory syndrome |
| s.c. | sub-cutaneous |
| SD | standard deviation |
| SRRC | seal rehabilitation and research centre |
| TCID | tissue culture infectious dose |
| TMB | tetra-methyl benzidine |
| VN | virus neutralisation |
| WNV | West Nile virus |

# 1.

# General Introduction

In part adapted from:

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and
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Since the 1980's, emerging and re-emerging infectious diseases have made an enormous impact on public and animal health, food supply, economies, and the environment. An estimated 75% of emerging infectious diseases in humans are zoonotic (pathogens of non-human vertebrate animals that may be transmitted to humans under natural conditions), mainly of viral origin and often vector-borne [1]. Since 1980, more than 35 new infectious agents have emerged in humans [2], including Severe Acquired Respiratory Syndrome (SARS coronavirus) [3,4], West Nile Virus (WNV) [5], Ebola virus [6] and Avian Influenza A Virus (AIV) [7,8]. Although the recognition of emerging or re-emerging infectious diseases can in part be attributed to increased interest or attention and to improved diagnostic methods, the foremost cause should be sought in human behaviour: (1) demographic changes: exponential growth of the human population, world-wide urbanisation, encroachment on wildlife habitat; (2) generalised social changes: globalisation of trade, increased and accelerated legal and illegal transport of animals, wildlife trade (live animal and bushmeat markets); (3) ecosystem disturbance: deforestation, eutrophication of waterways; and (4) climatic changes: global warming [9-12]. Livestock has been severely affected by direct mortality and depopulation policies, for example: to date, highly pathogenic avian influenza (H5N1) has resulted in culling of at least 220 million birds in 41 countries. The impact of emerging infections is of special concern for endangered wild animal populations, which can be pushed to the brink of extinction by such events [13-15].

Early detection of outbreaks, by improved surveillance in animals for (zoonotic) pathogens is critical for managing these infections [16]. Linking comprehensive pathogen surveillance of wild and domestic animals with public health surveillance will make an important contribution to the detection and control of emerging zoonotic infections [10,11]. Current technological capabilities facilitate a rapid response to emerging infectious diseases in terms of rapid identification and diagnostic techniques and, to a lesser degree, the development and use of vaccines and therapeutic agents.

Vaccination is one of the tools that can be used to combat infections in individual animals, or large scale outbreaks. Vaccination is only one of the factors (e.g., nutrition, parasite control, hygiene) associated with preventive medicine, and vaccines can never be absolutely guaranteed to provide adequate protection against emerging diseases.

The principal objective of vaccination is to induce a protective immune response that mimics protection acquired after natural infection. The ideal vaccine would: (1) induce a strong virus neutralising serum antibody response with high titres, of long duration, and broadly reactive; (2) induce T-cell mediated immunity, with a strong T-helper cell response and induction of cytotoxic T-cell responses; (3) induce mucosal immunity; (4) induce a robust protection; (5) be needle free; and (6) be safe. Obviously, no vaccine used today fulfils all these criteria. This is certainly true for vaccines used in non-domestic species.

Historically there have always been two major types of viral vaccines for non-domestic animals classified according to the nature of the antigens used. (1) Modified live virus (MLV) vaccines use attenuated pathogens which replicate in the vaccinated animal, thereby closely mimicking a natural infection, and eliciting an immunologic response without causing severe clinical disease signs (a "controlled" infection); (2) vaccines based on antigens that are non-living or inactivated – often termed killed vaccines (KV), for which the immunologic response they illicit is directed towards the inactivated antigen. Vaccines are generally not approved for non-domestic species and thus their use is always extra-label [17], and there is always a potential liability to such use[18].

(1) MLV vaccines infect host cells, cause viral replication, and the infected cells will process endogenous antigen. In this way immune responses of both T-cells (CD8+ and CD4+) and B-cells are triggered. MLV vaccines have been designed to be minimally virulent, while retaining maximal immunogenicity in the domestic species for which they have been developed. When used in other species or delivered by another route, residual virulence may cause clinical disease or death [19] which has occurred in numerous non-domestic species after vaccination with MLV vaccines registered for domestic animals (Table 1). Several viruses induce a suppression of the immune system, and it is known that some attenuated virus strains may still be able to cause immuno-suppression, like MLV canine distemper virus (CDV) and canine parvo virus (CPV) vaccines [20]. Sometimes the individual vaccine strains are not detectably immunosuppressive, but when used in a combination vaccine they may induce e.g. suppression of blood lymphocyte counts [21]. Immune-suppression caused by combination vaccines that contain live canine adenovirus type 1 (CAV-1) and canine corona virus (CCoV) may lead to clinical disease signs attributed to the attenuated CDV component of the vaccine, and may lead to CDV-encephalitis [22-24].

(2) Inactivated vaccines are preferred in case of safety concerns of the MLV vaccines, as they do not contain infectious virus and are therefore incapable of causing an infection. However, inactivated vaccines act as exogenous antigens, triggering an immune response dominated by CD4+ and often Th2 cells, which may not always be the most effective response to the pathogen vaccinated against. Additionally, the process of inactivation may dramatically reduce immunogenicity, usually resulting in an immune response that is shorter in duration, narrower in antigenic spectrum, weaker in cell-mediated and mucosal immune responses, and less effective in preventing viral replication [25].

| Virus | Species | Latin name | Reference |
|-------|---------|------------|-----------|
| CAV | Domestic dog | *Canis lupus familiaris* | [201] |
| | Maned wolf | *Chrysocyon brachyurus* | [127] |
| CDV | African wild dog | *Lycaon pictus* | [65,202,203] |
| | Black-footed ferret | *Mustela nigripes* | [121,125] |
| | Bush dog | *Speothos venaticus* | [123] |
| | Domestic ferret | *Mustela putorius furo* | [143] |
| | European mink | *Mustela lutreola* | [126] |
| | Fennec fox | *Fennecus zerda* | [128] |
| | Gray fox | *Urocyon cinereoargentus* | [58] |
| | Kinkajou | *Potos flavus* | [122] |
| | Maned wolf | *Chrysocyon brachyurus* | [127] |
| | Red panda | *Ailurus fulgens* | [120,204] |
| RV | Domestic cat | *Felis catus* | [205] |
| | Domestic dog | *Canis lupus familiaris* | [206] |
| | Striped skunk | *Mephitis mephitis* | [207] |
| FeLV | Cheetah | *Acinonyx jubatus* | [52] |
| FHV | Domestic cat | *Felis catus familiaris* | [208] |
| | Pallas's cat | *Felis manul* | [209] |
| FPV | Cheetah | *Acinonyx jubatus* | [210] |

Table 1. Examples of vaccine-induced disease.

To maximise the efficacy of vaccines, especially inactivated vaccines, adjuvants are usually added. These adjuvants can greatly enhance specific immunological responses to vaccination by several mechanisms, including: protecting the antigen from degradation, promoting efficient delivery of antigens to antigen presenting cells, and enhancing cytokine production. However, the use of adjuvants can also cause severe inflammation and systemic toxicity, have an impact on growth of the animal or reproductive rate, and repeated or high doses of antigen can induce hypersensitivity reactions [26]. In both domestic and non-domestic species, it is not unusual to observe side-effects such as elevated temperature, swelling, and irritation at the site of injection, or anaphylactic reactions like hyperaemia, hyper-salivation, or vomiting [27] and these side-effects may in some cases be severe [28]. Anaphylactic reactions may occur after the use of any vaccine, but are particularly thought to occur following the use of multivalent, adjuvanted vaccines containing large amounts of foreign proteins [29]. Adjuvanted inactivated vaccines are used more widely in domestic cats than in dogs, as several MLV vaccines have shown a significant association with upper respiratory tract infections in cats. However, a significant association between the use of these usually aluminium adjuvanted vaccines and local reactions (granulomas and sarcomas) has also been found [30]. This has led to recommendations such as alternating predisposed vaccination sites, avoiding aluminium adjuvanted and polyvalent vaccines, and avoiding over-vaccination [31-33].

Recent advances in immunology, molecular biology and biochemistry have allowed the construction of subunit vaccines based on recombinant viruses and bacteria, peptides, or bacterial and viral vectors, which may lead to safer, and more efficacious vaccines that can also be used in non-domestic species.

Effective vaccination should not only trigger cell-mediated responses, but also elicit a high titre of neutralising antibodies (humoral immune response) of the appropriate class: Immunoglobulin M (IgM), immunoglobulin G (IgG) and/or immunoglobulin A (IgA), directed against the relevant epitopes on the virion. The immunity induced by vaccination has mainly been evaluated and quantified in domestic animals by measuring the levels of serum antibodies. For certain infections (e.g. CDV, CAV, CPV, feline panleukopenia virus (FPV) or *Borrelia burghdorferi*) the level of the humoral response, although not the only mechanism involved, tends to correlate with level of protection from clinical disease, and therefore is a useful indicator of the immune status. Other agents (e.g. CCoV, feline enteric coronavirus (FCoV), canine para-influenza virus (CPIV), *Bordetella bronchiseptica*, and *Chlamydophila psittaci*) all replicate and cause damage on mucosal surfaces, and might require a mucosal immune response for protection. As a consequence, serum antibody titres do not necessarily correlate with (adequate) protection against these pathogens.

Effective vaccination induces not only a humoral and a cell-mediated response but also memory T and B cells, which will remain for years after the effector mechanisms have declined. These memory cells rapidly differentiate during a subsequent infection into effector cells that can eliminate an infection before clinical signs appear [34]. Additionally, "memory effector B-cells" can produce antibodies for years without overt antigenic stimulation [35,36]. In order to obtain a complete view of the immunologic status of an animal one therefore needs to look (ideally) at the humoral, cell-mediated, and local (mucosal) responses. One can only know if the measured level of immunity is protective by challenging the vaccinated animal with the pathogen. However, for non-domestic endangered or otherwise irreplaceable species (IUCN Red list, http://www.iucnredlist.org), challenge infection studies should not be conducted from a conservation point of view, and humoral and/or cellular immune responses have to be used as correlates of protection.

The recent debate concerning vaccine safety, efficacy and duration of immunity in domestic cats (*Felis catus familiaris*) and dogs (*Canis lupus familiaris*) [37-45] resulted in the need for more objective and scientific data. Vaccinated domestic dogs and cats have shown a range of protective antibody titres after challenge infections with the viruses used in standard vaccines. Additionally, these protective antibody titres may vary per virus due to the variety of techniques and standards used in different laboratories. There is little or no standardisation of serological assay methods, and this non-standardisation of serologic tests can make comparisons between laboratories of questionable use [46].

Currently, several long-term vaccination/infection studies have shown that protective immunity against certain viruses upon vaccination may last for several years, and annual re-vaccination of domestic dogs and cats may not always be required [36]. Recommendations based on these recent studies are currently different for "core" vaccines (which are considered essential: CDV, CPV, CAV, rabies virus (RV), FPV, FCV, FHV), and "non-core" vaccines (all other vaccines, which should be given in high risk situations, but are not considered essential). The vaccination schedule for carnivores should start at the age of 6-8 weeks, with 3 week intervals so that the last dose is given at the age of 12-14 weeks, then revaccination at 1 year, and then every 3 years for "core" vaccines. Antibodies induced against "non-core" vaccines are detectable for a shorter period of time after vaccination, and should be repeated yearly or more frequently [36].

In non-domestic animals controlled vaccination studies are limited, and their evaluation is largely restricted to evaluation of humoral responses extrapolated to known challenge infection data from domestic animals [47-73]. However, analytical tests are not standardised or validated for the different non-domestic species, thereby hindering evaluation and comparison of vaccine-induced immunity in the many different non-domestic species.

Recommendations for use in non-domestic mammals are generally based on tradition, anecdotal/personal experiences or taken from more precise, published data. This has led to a plethora of differing opinions and therefore the use of many different vaccination protocols (Table 2 for CDV vaccination). However, there are some general rules for using extra-label vaccines in non-domestic animals. Monovalent inactivated viral or bacterial vaccines are preferred, and the use of polyvalent vaccines containing unnecessary antigen should be avoided whenever possible. Animals with active clinical illness should preferably not be vaccinated. In the event of a viral disease outbreak in an animal collection, all susceptible species should ideally be vaccinated immediately and boostered 10-14 days later, regardless of age and last time of immunisation [74]. The use of some drugs, such as tetracycline, chloramphenicol, dapsone, clindamycin, griseofulvin, nalidixic acid and sulphamethoxypyradizine have been associated with an inadequate response to vaccination [40]. Vaccination should also be avoided in animals undergoing glucocorticoid therapy, although challenge studies have been performed which show that "immuno-suppressive" doses given at the time of vaccination do not significantly affect the level of post-vaccinal immunity to CDV or RV [75]. When using remote delivery systems one must be sure that a full dose is delivered, as syringe darts may rebound quickly on impact and fail to deliver the dose required to elicit a satisfactory immune response [76]. The type, serial number, and source of product should be recorded in the veterinary records [77]. Any vaccination programme should also take the current local infection risk by the pathogen into account, upon which the decision can be made if vaccination is warranted.

| Species | Vaccine | Regime | Booster | Reference |
|---|---|---|---|---|
| All susceptible | MLV [§] | Initial at 3-6 weeks, at 9 weeks combination vaccine. Single vaccination after 12 weeks | Semii-annually | [133] |
| Mustelidae | MLV [§] | 10 weeks | * | [133] |
| All susceptible | KV or MLV[‡] | Initial at 5-6 weeks repeat every 2 weeks until 15 weeks | Annually | [209] |
| Mustelidae | KV for black-footed ferret & initial, then MLV | Initial at 8 weeks, repeat after 2-3 weeks | * | [209] |
| Procyonidae | KV | Initial at 6-8 weeks, repeat every 2-3 weeks until 14 weeks | Annually | [209] |
| All susceptible | KV (unavailable) | Initial distemper/measles at 6-8 weeks, at 12-14 weeks combination vaccine. | Annually | [116] |
| Procyonidae | MLV [‡] | Initial at 8 weeks, repeat at 12 and 16 or 18 weeks of age | * | [66] |
| All susceptible | KV preferred (unavailable) thus MLV (avian origin)[‡] | Single dose after weaning im, monthly booster up to 4 months | Annually | [76] |
| All susceptible | MLV (avian origin)[‡] | Initial at 6-8 weeks, repeat every 2-3 weeks with a total of 3 vaccinations, in special cases (ie early weaning, ill juveniles, high probability of exposure to disease) extended to 4 or 5. | Annually | [74,211] |
| All susceptible | ISCOM | 8, 11, 14 weeks of age | Annually | Rotterdam Zoo |
| All susceptible | Canarypox-vectored recombinant | 3 doses with 3 weeks interval | Annually | AAZV |

Table 2. CDV vaccination regimes recommended for use in non-domestic species. Vaccination with modified live virus (MLV) vaccines were recommended (§) until the beginning of the 1980s, when a growing number of species were reported with vaccine-induced CDV infections. Hereafter, inactivated (or 'killed') virus (KV) were recommended, or MLV vaccines of avian origin - usually due to KV being unavailable (‡). Currently, the use of MLV vaccines in non-domestic carnivores is not recommended, although safe and effective CDV vaccines for non-domestic species are not commercially available or legal to use in many countries.

Vaccination of non-domestic species is especially useful in captive collections, where the population density may be higher than in wild populations, with an increased infection risk. Infection/exposure risks can

be minimised by strict hygiene and quarantine protocols, proper fencing to reduce contact with unvaccinated domestic and non-domestic species, and vaccination. Vaccination of endangered species in conservation projects can contribute to the survival of these species. African wild dog [13] and black-footed ferret [15] conservation projects have been severely affected by CDV outbreaks, and a safe and effective vaccine is much needed [65]. Following the phocine distemper virus (PDV) epidemic of 1988 [78-80] in harbour seals (*Phoca vitulina*) and the development of an experimental CDV-ISCOM vaccine [81], all rehabilitated seals from the Seal Rehabilitation and Research Centre (SRRC) in Pieterburen, the Netherlands, are vaccinated upon arrival. The duration of protective immunity following this vaccination in seals is unknown, but is intended to last for at least the duration of stay in the rehabilitation centre.

The question is often raised whether free-ranging non-domestic animals should be vaccinated. It is an interference with natural selection, and therefore a topic under discussion. Re-introduced or translocated animals may not have been challenged under natural conditions with the local pathogens when young (and maternal immunity is still present), but can be vaccinated while in captivity (prior to release) to obtain a level of immunity against these pathogens. The World Conservation Union (IUCN) guidelines for the reintroduction of captive animals into the wild include the description of preventive vaccination strategies [82]. A problem faced is the difficulty to boost under field conditions, so that the level of immunity may not be sufficient for prolonged periods of time. The vaccination regime should therefore be completed before release when possible, to allow sufficient time to develop the required immunity and detect possible adverse effects before release of the animals.

Vaccination of a wild population of Mediterranean monk seals (*Monachus monachus*) was considered during a morbillivirus epizootic on the west coast of Africa in 1992 [83], and this discussion flared up during the recent PDV epidemic in North European harbour seals in 2002, but it was generally not considered a viable option [84,85]. Vaccination of seals with a MLV vaccine should be considered contraindicated [86], as for all non-domestic animals. When vaccinating free-ranging wildlife it is of utmost importance to consider the fact that MLV vaccines may not be sufficiently attenuated for exotic species, and that vaccine induced disease or shedding of virus may occur, potentially infecting free-living populations.

The only examples of effective vaccination campaigns of free-ranging carnivores are those against rabies. The zoonotic and economic aspects of RV infection have resulted in prophylactic vaccination of free-ranging vector species, which are much more difficult to vaccinate than captive specimens. The development of oral MLV vaccines [87] proved its value when an advancing epidemic was stopped by the vaccination zone [88]. This vaccine has since been replaced by a poxvirus based recombinant vaccine, which has proven to be efficacious and safe for the target species (the European fox, *Vulpes vulpes*), as well as for numerous non-target species [89-91].

This thesis focuses on the vaccination of non-domestic species against two groups of viruses which have recently caused large outbreaks with high mortality: morbilliviruses and avian influenza viruses.

# MORBILLIVIRUSES

Morbilliviruses are relatively large (150-600 nm) negative sense single stranded RNA viruses which belong to the family of Paramyxoviridae. The viral RNA is associated with the nucleoprotein (NP), forming a helical nucleocapsid. The matrix (M) protein forms a linkage between the glycoproteins in the membrane and the nucleocapsid, thus stabilising the virus structure. Two membrane proteins, the haemagglutinin (H) and fusion (F) proteins form projections on the viral membrane, and are involved in attachment of the virion to receptors on target cells (H), and fusion of viral and target cell membranes or between host cells (F). Both the H and F proteins are the major immunogens for the induction of antibodies that play an important role in the prevention of and the recovery from infection.

All families of the taxonomic order Carnivora are in principle susceptible to CDV infection, which is among the most significant infections of domestic dogs and many non-domestic species in terms of mortality. Infections with CDV and the closely related PDV have caused major outbreaks in naive populations of terrestrial carnivores and marine mammals [13,15,80,92-99]. Transmission is mainly via droplet infection or direct and indirect contacts, and the highly contagious virus may be excreted for up to 90 days by domestic dogs. Morbidity and mortality varies per species, but case-fatality rates can be as high as 100% in naive animals.

Pathogenesis of CDV infection has been best described in domestic dogs, in which the clinical signs are dependent on the virus strain, environmental conditions, host susceptibility and immune status. Acute infection is clinically predominantly associated with catarrhal and respiratory infection (conjunctivitis, pneumonia, diarrhoea, anorexia, and dehydration). Neurologic manifestation of CDV infection often follows 1-3 weeks after recovery from acute generalised infection, and is most commonly seen in dogs with a poor immune response. Neurologic signs may manifest themselves as a chronic progressive disease, even if the infected dog has not shown systemic signs previously. In non-domestic species, clinical signs may vary between species, but respiratory, gastro-intestinal (GI) tract, integumentary and central nervous (CN) systems are most commonly affected.

Vaccination of susceptible species in zoos has been recommended since 1963 [100]. In general, CDV vaccination has always been recommended in all members of the taxonomic families Canidae, Procyonidae and Mustelidae. Vaccination of large cats is recommended in high risk situations [76,82,101-103], after several outbreaks occurred among captive and free-ranging large felids[93,99,104-

[106]. Although clinical disease as a result of CDV infection is rare in ursids, serologic surveys have shown the presence of CDV specific antibodies[107-111]. Clinical disease and presence of CDV specific antibodies have been documented in spotted hyaenas (*Crocuta crocuta*) [112-114] and a palm civet (*Paguma larvata*) [115], therefore vaccination is recommended in these species by some authors [74,82,101,116-118] However, the susceptibility, and therefore need for vaccination of members of the Ursidae, Hyaenidae and Viverridae to CDV is disputed by some [76,103,119].

All members of the taxonomic order Carnivora are potentially susceptible, and vaccination of non-domestic carnivores with a safe and efficacious vaccine is therefore recommended if local exposure is high, and contact with infected animals can not be prevented.

A problem faced in the prophylaxis of CDV in non-domestic carnivores is the variation between and within species in their reaction to MLV vaccines, and many species have been documented with vaccine-induced canine distemper with possible lethal consequences [58,120-127]. Currently the majority of commercially available morbillivirus vaccines are MLV vaccines. Between the different commercially available MLV vaccines there is a clear difference in vaccine efficacy [128-130] and adverse effects. Chicken embryo-attenuated MLV CDV vaccines specifically attenuated for domestic ferrets (*Mustela putorius furo*)[131] appear to be safe and efficacious in maned wolves (*Chrysocyon brachyurus*), bush dogs (*Speothos venaticus*) and fennec foxes (*Vulpes zerda*) [65], but have caused disease in several species of minks, ferrets, grey foxes (*Urocyon cinereoargenteus*) and red pandas (*Ailurus fulgens*) [121,124,126,132]. Vaccine virus attenuated by passages in canine kidney cells has been responsible for vaccine-induced distemper in a much larger number of species (Table 1). Until 1983 the use of MLV was mentioned without warning of the adverse effects [133]. After 1985 inactivated virus vaccines were recommended for use in non-domestic species [76,117,119,134,135] even though the efficacy of inactivated vaccines against CDV infection has been questioned [136,137]. Currently there are no monovalent inactivated CDV vaccines commercially available, due to their limited efficacy in domestic dogs compared to MLV vaccines, and the absence of a commercially interesting market for non-domestic animals [138].

The large range of (highly susceptible) host species in zoos for which vaccination is recommended underpins the need for the production of a safe and efficacious vaccine for use in non-domestic carnivore species. Several alternatives to MLV vaccines have been tried in non-domestic species.

(1) Non-domestic canine pups have previously (early 1980's) been vaccinated with a MLV measles vaccine [133,139]. Measles virus and CDV are antigenically closely related, but the measles virus is not neutralised by the maternal antibodies in 6-week-old puppies of domestic dogs [136], therefore a level of immunity is induced. However, the induced protection is not complete [136], and vaccination requires a booster vaccination with a modified live CDV vaccine, and is currently not recommended anymore.

(2) An experimental saponin-adjuvanted inactivated CDV vaccine has been used in red pandas and giant pandas (*Ailuropoda melanoleuca*) in several zoos. The vaccine appeared to be safe and efficacious, but produced low titres with inadequate durability, requiring booster vaccinations two to three times annually [124]. This vaccine is no longer produced. In Germany a small amount of aluminium hydroxide adjuvanted inactivated vaccine was previously produced for use in zoos (K. Frölich, pers. comm), although no published data exists of its efficacy in different non-domestic species.

(3) An experimental subunit vaccine incorporating the F and H surface proteins of CDV into immuno stimulating complexes (ISCOM) has been developed and tested in domestic dogs and harbour seals, producing humoral and cellular immunity [81,140]. ISCOMS are stable complexes containing cholesterol, phospholipids, saponin, and antigen, and can be used as an adjuvant. Micelles can be constructed using protein antigens and a matrix of a saponin mixture called Quil A. They are highly effective in targeting antigens to the antigen processing cells, while the saponin activates these cells, promoting cytokine production and the expression of co-stimulatory molecules. Depending on the antigen and adjuvant composition, Th1 or Th2 responses can be stimulated. Although the immunity achieved is not sterile (infection of the upper respiratory tract occurs), CDV-ISCOM vaccinated seals were protected from a potentially lethal challenge with the closely related PDV[81]. The ISCOM vaccine has since been used experimentally in several European zoos (W. Schaftenaar, pers. comm), although no data on its efficacy in different non-domestic species have been published.

(4) In 1997 a recombinant canarypox-vectored vaccine expressing the H and F surface antigens of CDV was introduced[141] and tested in domestic dogs for its safety and efficacy [142]. Recently a similar monovalent canarypox-vectored vaccine has become commercially available in the US (Purevax®, Merial, Duluth, Minnesota, USA). It is registered for use in domestic ferrets in which its efficacy and safety have been demonstrated[143]. In black-footed ferrets (*Mustela nigripes*) x Siberian polecat (*Mustela eversmanni*) hybrids the use of this vaccine has produced a good immune response[144], and it has since been used and evaluated in a large number of exotic species (R. Montali, pers. comm.). Its extra-label use in all susceptible species in zoos is recommended by the American Association for Zoo Veterinarians (AAZV) and the IUCN[82], although only limited published data on its efficacy in these non-domestic species exist [145]. In the EU its use is not permitted, as it is a non-registered genetically modified organism (although currently several other recombinant vaccines have been registered for domestic species[146]).

The main advantage of recombinant canarypox-vectored vaccines is their safety in mammals. Members of the Avipox genus (e.g. fowlpox and canarypox) are non-pathogenic and replication-deficient in mammals due to their natural host range restriction to avian species. However, they still have the ability to enter mammalian cells, reach an early stage of morphogenesis, and (importantly) express exogenous genes [147,148]. Protective cellular and

humoral immunity is induced in the absence of the complete virus, therefore eliminating the possibility of infection with CDV. Canarypox virus generally appears to be superior to fowlpox virus in the induction of immune responses in mammals [149]. Because the vector is replication deficient in mammalian cells, the potential for dissemination of the vector within the vaccinate is eliminated and therefore there is no spread of the vector to non-vaccinated contacts or the environment [150]. Currently there is no safe, commercially available CDV vaccine that can be used in non-domestic carnivores in the EU.

# AVIAN INFLUENZA VIRUSES

Avian influenza viruses (AIV) are type A influenza viruses and belong to the Orthomyxoviridae family, which also contains the influenza virus B and C types. The influenza A virion is a particle of approximately 120 nm in diameter, and its genome consists of eight segments of negative sense single stranded RNA. It can be classified according to the antigenicity of its surface proteins haemagglutinin (H) and neuraminidase (N). Currently 16 H (H1-16) and 9 N (N1-9) subtypes have been described in avian species [151]. Individual subtypes can theoretically be composed of any combination of one of the H and one of the N proteins. Furthermore the subtypes can be classified on the basis of their pathogenicity in chickens after intravenous inoculation.

Highly pathogenic avian influenza (HPAI, formerly termed fowl plague), an acute generalised disease in which mortality in chickens may be as high as 100%, is restricted to subtypes H5 and H7, although most viruses of these subtypes have low pathogenicity, and do not cause HPAI. All other AIV strains are low pathogenic avian influenza (LPAI) virus strains which cause more variable morbidity and mortality (ranging from sub-clinical to fatal) but are generally associated in poultry with mild, primarily respiratory disease with loss of egg production [152], or mild enteric disease in wild birds. In certain cases (in poultry flocks) the LPAI virus phenotype (of subtype H5 or H7) may mutate into the HPAI virus phenotype by the introduction of basic amino acid residues (arginine or lysine) at the cleavage site of the precursor haemagglutinin (HA0)[153], which facilitates systemic virus replication. H5 and H7 subtypes with an amino acid sequence at the HA0 cleavage site comparable to those that have been observed in virulent AI viruses are considered HPAI viruses, even when mortality in chickens is low [154]. However, the two forms of avian influenza (HPAI and LPAI) are distinctly different and should be regarded as such.

Avian influenza viruses have a worldwide distribution and are in principle infectious to all avian species (domestic and wild), with variable morbidity per virus isolate and species. Aquatic avian species, mainly those of the taxonomic orders Anseriformes (Anatidae: ducks, geese and swans; Anhimidae: screamers; and Anseranatidae: magpie goose) and Charadriiformes (Scolopaci: snipe-like waders; Thinocori: aberrant charadriforms; Larii: gulls

and allies; Turnici: buttonquails; Chionidi: thick-knees and allies; Charadrii: plover-like waders) are considered the main natural reservoir of all avian influenza viruses, including the LPAI ancestral viruses of HPAI strains [155,156]. Replication of LPAI viruses occurs mainly in the intestinal tract, with excretion of high virus loads for up to 45 days [157] and AI virus remains infectious in faeces for 30 to 35 days [158]. In lake water AI virus remains infectious for 4 days at 22°C to more than 30 days at 0°C [159], and the relatively high prevalence of AIV infection in birds living in aquatic environments may be due in part to efficient transmission via the faecal-oral route via surface waters [159,160]. Migrating waterfowl are thought to carry LPAI viruses over long distances, and can initiate outbreaks of HPAI by the introduction into poultry flocks of these LPAI viruses, which subsequently can change into HPAI viruses [160]. Recent HPAI H5N1 viruses have been predominantly associated with oropharyngeal shedding [161,162]: the impact of this on environmental contamination, persistence and transmission is yet unknown.

Terrestrial poultry species (e.g., chickens, turkeys, quail and ostriches) are generally highly susceptible to infection with HPAI virus, but waterfowl were considered resistant until 2002. However, in 2002 an outbreak of HPAI H5N1 virus occurred in wild migratory avian species and resident waterfowl, and the high pathogenicity in ducks was confirmed in laboratory infections [163]. Since 2002, this particular HPAI virus subtype has made an unprecedented spread from South East Asia throughout Asia and into the Middle East, Europe and Africa, with morbidity and mortality not only in poultry, but in a large number of other avian species. To date HPAI virus infection with the H5N1 subtype has been confirmed in at least 105 species (spp.) from 14 orders: Anseriformes (33 spp.), Charadriiformes (5), Ciconiiformes (6), Columbiformes (3), Falconiformes (11), Galliformes (10), Gruiformes (4), Passeriformes (22), Pelecaniformes (2), Phoenicopteriformes (1), Strigiformes (4), Struthioniformes (1), Psittaciformes (1), and Podicipediformes (2) [164]. Outbreaks along the recognised flyways from South East Asia into Europe have suggested that this HPAI virus subtype may be distributed directly by migrating waterfowl, and HPAI virus infections have been detected in several migratory species [165-169]. However, domestic waterfowl [162,170,171], specific farming practices, agro-ecological environments, and transportation of domestic avian species or their products with trade at local markets may all have played a key role in the amplification and spread of HPAI H5N1 virus in Asia [167,172].

Several mammalian species (including ferrets, horses, pigs [170], seals and humans [7,173]) had been reported with infections with the H5 and H7 subtypes of AIV up to 1997. The recent HPAI H5N1 virus subtype has caused mortality in a large number of mammalian species (Table 3), and has caused 313 human cases with 191 deaths to date (30th of June 2007) [174]. Noteworthy are the fatal HPAI H5N1 virus infections with severe pneumonia of domestic cats, tigers and leopards that fed on infected poultry carcasses, as felids had previously been considered to be resistant to disease upon AIV infection [175]. Horizontal spread of infection was suspected [176], and has been demonstrated

experimentally in domestic cats [177], with excretion from both the respiratory and intestinal tracts [178].

| Taxonomic family | Common name | Latin name | Reference |
|---|---|---|---|
| Canidae | Domestic dog | *Canis lupus familiaris* | 212 |
| Felidae | Domestic cat | *Felis catus* | 177,178,213,214 |
| Felidae | Clouded leopard | *Neofelis nebulosa* | 215 |
| Felidae | Tiger | *Panthera tigris* | 176,216 |
| Felidae | Leopard | *Panthera pardus* | 216 |
| Mustelidae | American mink | *Mustela vison* | 164 |
| Mustelidae | Stone marten | *Martes foina* | 164 |
| Viverridae | Owston's banded palm civet | *Chrotogale owstoni* | 218 |

Table 3. Mammalian species documented with natural infections with highly pathogenic avian influenza virus (H5N1).

Documented outbreaks of Asian lineage H5N1 HPAI virus in zoo birds have been limited to 5 cases: Penfold Park, Hong Kong, (People's Republic of China, 2002) and Kowloon Park, Hong Kong (People's Republic of China, 2002), Ragunan Zoo, Jakarta (Indonesia, 2005), Dresden Zoo (Germany, 2006) and Islamabad Zoo (Pakistan, 2007). Large felids with H5N1 infection have been reported in Suphanburi Zoo (Thailand, 2003), and Sri Racha Tiger Zoo (Thailand, 2004). To curtail these outbreaks, a combination of increased bio-security measures (isolation and quarantine of infected animals, disinfection of the area), feeding of cooked poultry only, treatment of infected animals in quarantine areas, selective culling, extensive surveillance of migratory and captive birds and vaccination were used.

Vaccination is a useful means of reducing the horizontal spread of AIV in poultry [179,180]. An inactivated vaccine (Nobilis Influenza H5, Intervet International, Boxmeer, the Netherlands), using an H5N2 strain (A/Chicken/Mexico/232-CPA/94) proved efficacious in chickens in Hong Kong under field conditions and after high dose laboratory challenge with HPAI H5N1 viruses [181]. Furthermore, other inactivated vaccines, H5N1 reverse genetics based vaccines, and fowlpox recombinant vaccines with H5 inserts have been shown to be protective in chickens [182,183], domestic ducks [184,185], and domestic geese [186] against diverse HPAI H5N1 virus strains. Vaccination protects against disease and mortality, but does not always prevent infection and virus spread. However, the dose required for infection is much higher, and vaccinated birds shed far less field virus after infection than unvaccinated birds [187,188].

Protective antibodies produced in response to infection or vaccination

are directed against the H and N surface proteins. Vaccine-induced protection is species-, dose-, and vaccine strain-dependent. The degree of homology of the H protein will largely affect the level of cross-protection and therefore efficacy of the vaccine [189]. A so-called Differentiation of Infected from Vaccinated Animal (DIVA) strategy, with a heterologous vaccine (using the same H subtype as the field virus, but a different N subtype), is recommended to differentiate between vaccinated and field-virus infected animals [190]. However, in housing systems where birds are not housed permanently indoors (e.g., in zoos), contact with free-ranging birds can result in LPAI virus infections that go by unnoticed, but which may interfere with the DIVA principle.

Antibody responses upon AIV vaccination may vary between avian species, being higher in chickens than in other poultry species [191]. Published minimum serum antibody titres measured by HI test in vaccinated chickens that correlate with protection after challenge with HPAI are 1:10 [183] or 1:16[181,186]. However, domestic ducks with very low or undetectable antibody titres post vaccination have been shown to be protected from HPAI virus challenge [184,185]. Duration of protection from HPAI virus challenge may vary between species: chickens for up to 40 weeks after one dose of vaccine, domestic ducks for more than 52 weeks after 2 doses, while domestic geese which received 3 doses were protected for 34 weeks [186].

In the EU there is currently a non-vaccination policy with regard to routine vaccination of poultry against AIV due to interference with stamping-out policies and international trade agreements. Instead, eradication measures during an outbreak in poultry include (long-term) confinement, large-scale culling and safe disposal of carcasses of all poultry on the infected farm, and - depending on the poultry density in the area and the epidemiological situation - pre-emptive culling of poultry on neighbouring farms (since 2003, more than 220 million birds have been culled world-wide to eradicate H5N1 avian influenza outbreaks), and emergency vaccinations (Directive 92/94/EEC).

The standard eradication measures used to prevent and eradicate HPAI virus outbreaks in poultry would be detrimental to the welfare and breeding programmes of avian species in zoos. Large scale culling in zoological collections that include endangered species would be highly undesirable from a conservation point of view. Directive 2005/94/EC foresees a derogation from killing of birds provided the birds can be brought inside and are subjected to virus detection tests that give negative results (after the last death/positive finding, 2 tests at an interval of 21 days have to be performed according to the diagnostic manual Decision 2006/437/EC). However, most zoos will not have the facilities to suitably confine their entire bird collections for prolonged periods of time, and many species will not be able to adjust to confinement and increased stress with subsequent welfare problems and increased exposure to pathogens resulting in disease (e.g. aspergillosis, bumblefoot) [192-194].

Vaccination of zoo birds as an additional preventive measure against HPAI virus infection (while reducing confinement measures) in Belgian, Dutch and German zoos was first allowed during an outbreak of HPAI H7N7 virus

in poultry in 2003 (Decision 2003/291/EC). In 2005, Decision 2005/744/
EC allowed vaccination in European zoos against the encroaching H5N1
subtype. Targeted preventive vaccination campaigns in poultry have since
been authorized in the Netherlands: voluntary vaccination of hobby poultry
and free-range laying hens as an alternative to the requirement that these
birds be kept indoors (Decision 2006/147/EC), and France: domestic ducks
and geese which cannot easily be kept indoors and be separated from wild
birds (Decision 2006/148/EC). These campaigns were subject to rigorous
surveillance and control requirements.

Surveillance of wild birds can provide early warning signs for the
introduction of HPAI virus [156,195]. Several countries have initiated surveillance
campaigns of free-ranging wild birds. Wild bird populations that experience
high mortality rates should be submitted to national or regional reference
laboratories for testing (for a European listing see the EAZWV handbook of
infectious diseases [196]). Birds showing clinical signs can be captured, isolated
and selectively culled when testing is positive to HPAI virus. There is no
scientific basis for large scale culling of free-ranging wild bird populations to
control outbreaks or their spread, and it would be highly undesirable from a
conservation perspective [197]. Instead, measures should be taken to prevent
contact between non-vaccinated captive and wild bird populations.

Increased bio-security remains the first line of defence during
outbreaks of HPAI, and can be complemented by vaccination. Accreditation of
zoos (e.g., by AZA, EAZA or other (inter-)national organisations) has resulted
in standardised high levels of bio-security, decreasing the risk of introduction
and increasing the likelihood of containment of infectious diseases. However,
in the face of an outbreak of HPAI, levels of bio-security should always be
raised immediately with hygienic measures implemented accordingly to
prevent entrance or spread of the virus. Attention should be paid to both
exclusion (identification and elimination of possible routes of entrance [e.g.,
by live birds, cages, equipment, clothing]), and containment (reduction of the
risk of infection for neighbouring cages) of the virus, as described in guidelines
for zoos [198,199]. Derogations to bio-security measures (such as an alleviation
of confinement measures) can be made in zoos, when birds are vaccinated
(Decision 2005/94/EC), provided that such derogations do not interfere with
disease control.

# OUTLINE OF THIS THESIS

The last 20 years a growing number of (re-) emerging infectious diseases have had an enormous impact on public and animal health. Early detection of outbreaks, by improved surveillance in animals for pathogens is critical for managing these infections. Preventive vaccination can be used in individual animals to prevent morbidity and mortality from infectious agents, but it is also one of the tools that may be used to combat outbreaks of emerging infections in captive populations of non-domestic species.

The first part of this thesis (chapter 2) focuses on morbillivirus infections of non-domestic carnivores and marine mammals. Morbilliviruses have caused several large outbreaks in these animals, with high morbidity and mortality. Clinical signs observed in juvenile harbour seals during an outbreak of PDV are documented in chapter 2.1. To evaluate the prevalence of morbilliviruses and other viral pathogens in free-ranging terrestrial carnivores and marine mammals, hundreds of serum samples from Canada and France were tested for antibodies to these pathogens (chapters 2.2.1 and 2.2.2), providing important management implications for re-introduction of certain species like the European mink. To protect susceptible species from CDV infection, they can be vaccinated. However, commercial vaccines against CDV have caused fatal infections in numerous non-domestic carnivore species, and currently there is no safe, commercially available vaccine for use in non-domestic species in the EU. An experimental CDV-ISCOM vaccine developed for seals during a PDV epidemic was evaluated and compared to a canarypox-based recombinant vaccine (which is authorised for use in ferrets in the USA) in conservation projects of highly endangered European mink and African wild dogs (chapter 2.3.1 and 2.3.2).

The second part of this thesis focuses on avian influenza A viruses (chapter 3). In the past decade, HPAI outbreaks have occurred frequently, and the current outbreaks of the HPAI H5N1 subtype are unprecedented in their duration and spread. Standard eradication measures used in poultry in the EU (e.g., long-term confinement and large scale culling) would be detrimental to the welfare and conservation of the often endangered species kept in zoological collections. Two separate EC Decisions allowed vaccination in zoological collections to alleviate confinement measures and prevent large scale culling. However, detailed information about the safety and efficacy of vaccines for poultry against HPAI viruses in the large variety of bird species in zoos was not available at the time of vaccination. During an outbreak of HPAI H7N7 virus in poultry in the Netherlands, and due to the encroaching threat of HPAI H5N1 virus, birds in zoos were vaccinated and the safety and efficacy of these vaccination campaigns were evaluated (chapter 3.1 and 3.2). The longevity of serum antibodies after vaccination, and the effect of one vaccination one year after the initial two vaccinations are discussed in chapter 3.3. The susceptibility of pigeons and other species in the taxonomic order Columbiformes to HPAI virus and the efficacy of vaccination has been

questioned Therefore the effect of vaccination towards challenge with two strains of HPAI H5N1 virus from different antigenic clades in pigeons was studied (chapter 3.4.).

The findings presented in chapters 2 and 3 are evaluated and discussed in chapter 4 in the light of additional data on CDV vaccination in Rotterdam Zoo, and additional data on AI vaccination in other European zoos. The latter was presented in a recent EFSA report [200].
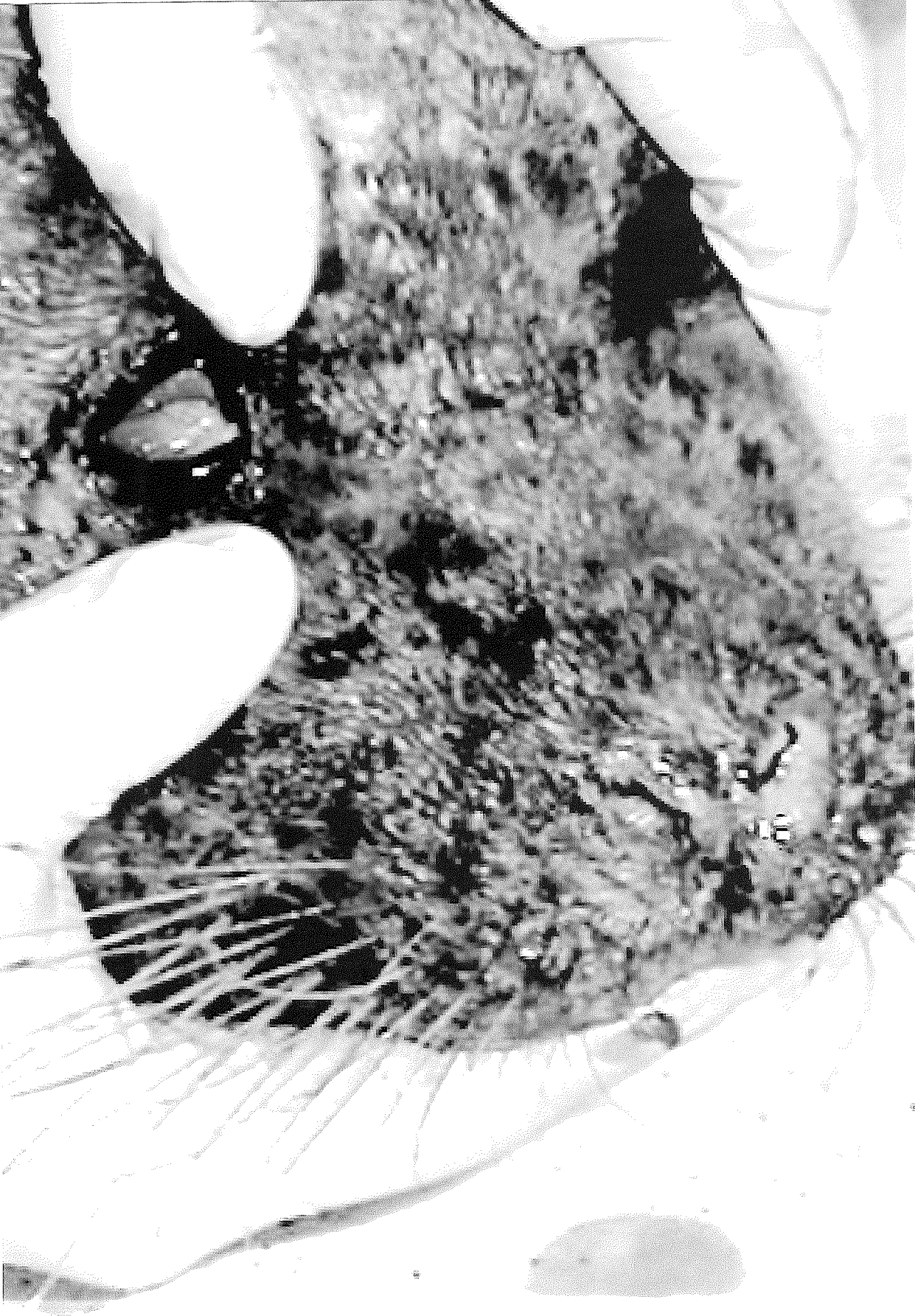
# 2.

# Morbillivirus infections

# 2.1.

# Neurological signs in the majority of juvenile harbour seals (*Phoca vitulina*) with fatal phocine distemper during a recent outbreak

*Submitted*

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The Northern European harbour seal (*Phoca vitulina*) population experienced a phocine distemper virus (PDV) epidemic with high mortality (22.000) during the summer of 2002. Clinical signs were recorded for 20 harbour seal pups that were admitted to the Seal Rehabilitation and Research Centre (SRRC) with clinical disease and were diagnosed PDV infection positive by RT-PCR at necropsy, confirming that indeed they had died from PDV infection. The most prominent clinical signs were respiratory signs of varying extent in 100%, conjunctivitis in 70%, and neurological signs developed in 50% of the infected seals. Severe neurological signs were one of the euthanasia criteria during the epidemic, and a large number of juvenile seals that were euthanised on humane grounds and not admitted to the SRRC are not included in this study, due to lack of complete data sets. Consequently, neurological signs were among the most prevalent signs of fatal PDV infection in harbour seal pups. Reported lymphoid depletion in dead seals collected during the epidemic was not reflected in the total mononuclear leukocyte count of seals upon admittance. Haematological tests further showed absolute granulocytosis, thrombocytosis, anaemia, and an increase in total white blood cell count. At time of admittance, 55 % had a positive serum immunoglobulin G (IgG) titre, and IgM titres were positive in 20%. High levels of PDV-specific IgG serum antibodies at admittance were not correlated to absence of clinical signs or to longer survival.

## INTRODUCTION

Phocine distemper virus (PDV), a single stranded RNA virus belonging to the genus Morbillivirus, is a highly contagious pathogen that has caused two rapidly progressing epidemics with high mortality in naive seal populations. The Northern European harbour seal (*Phoca vitulina*) population was struck by a PDV epidemic in 1988 [97], resulting in the death of 18 000 seals. During the summer of 2002 this population was struck by another PDV epidemic, and 22 000 seals died [219]. A nucleotide sequence analysis of this virus showed a close match (>97% homology) to the virus from 1988 [94]. Canine distemper virus (CDV), the closest relative of PDV [220], has a wide host range including all terrestrial carnivores, and has also caused epidemics among pinnipeds: Baikal seals (*Phoca sibirica*) [221,222] and Caspian seals (*Phoca caspica*) [95,223].

Clinical signs of PDV infected seals are usually compared to those seen in dogs with CDV infection, focussing on the respiratory (coughing, dyspnoea) and catarrhal aspects (oculo-nasal mucopurulent discharge, conjunctivitis), but also fever, diarrhoea, abortion and with a small number of infected seals exhibiting neurological signs [78,224]. CNS involvement in CDV infected dogs is dependent on the host's immune response [128]. Lymphocytic depletion has been reported in PDV infected seals, and the subsequent immune suppression increases the susceptibility to secondary infections [225,226]. These secondary infections (viral, bacterial and parasitic) contributed in part to the large variety

of clinical signs seen in 1988. Main necropsy findings in PDV infected seals in 1988 were pulmonary congestion and emphysema, atrophy of lymphoid tissue resulting in secondary bacterial infection, and degenerative changes in the mucosa of the airways, while only 1/29 seals showed evidence of encephalitis [227]. Clinical signs of CDV infected seals have included debilitation, ocular and nasal exudation, muscle spasms and tremors; and main necropsy findings were severe pneumonia, lymphoid depletion, and microscopic lesions of non-suppurative demyelinating encephalitis [95,228].

The clinical signs alone are not sufficiently characteristic to make the diagnosis of PDV or CDV infection of marine mammals, and therefore laboratory examinations are essential [86]. Paired sera demonstrating a rise in PDV-specific immunoglobulin G (IgG) titre, or a single high immunoglobulin M (IgM) titre are used to diagnose infection [224]. Seals are routinely vaccinated against morbilliviruses upon admittance to the SRRC [81], therefore testing of paired sera cannot be used for diagnostic purposes in this setting. A fast diagnosis of PDV infection is essential in seal rehabilitation centres to ensure that the necessary precautions are taken to minimise the dramatic effect of this highly contagious pathogen.

The objective of this study was to obtain an improved picture of clinical signs of harbour seals with natural PDV infection, and to assess diagnostic methods which may assist in future diagnosis of PDV infected seals admitted to rehabilitation centres.

## MATERIALS AND METHODS

We used data collected from 20 juvenile harbour seals that were admitted with clinical disease signs suggestive for morbillivirus infection to the Seal Rehabilitation and Research Centre (SRRC) in Pieterburen, the Netherlands during the PDV epidemic in 2002, and later diagnosed with fatal PDV infection by RT-PCR analysis of tissues collected at necropsy. Importantly, we did not include seals that were euthanised on humane grounds outside the rehabilitation centre. Severe neurological signs were one of the euthanasia criteria used during the outbreak, and a large majority of these euthanised seals had severe respiratory and neurological signs. However, no detailed descriptions of clinical signs or haematology and serum biochemistry data of these animals were available.

On arrival at the SRRC, a clinical examination was performed, and clinical signs were monitored daily, together with body temperature measurements twice daily. The seals were manually restrained, and blood was collected from the epidural vertebral vein using a 20 gauge x 38mm needle and vacutainer, into ethylene diamine tetra-acetic acid (EDTA) tubes and serum separator tubes.

Haematological parameters (platelet count, total leukocyte count, granulocyte count (neutrophils, eosinophils and basophils), mononuclear leukocyte count (lymphocytes and monocytes), percentage granulocytes,

and percentage mononuclear leukocytes) were determined in duplicate using a Quantative Buffy Coat analyser (QBC®, Becton Dickinson, USA), then averaged. No further differentiation was done. The haematocrit (HCT) was determined by centrifugation of blood in a microhaematocrit tube. A Reflotron® (Hoffmann- La Roche, Switzerland) was used to analyse serum chemistry: serum glutamate pyruvate transaminase / alanine aminotransferase (SGPT or ALT), serum glutamic oxaloacetic transaminase / aspartate aminotransferase (SGOT or AST), glucose, creatinin and urea levels were determined.

Two-sample t-tests were used to compare mean blood values of the confirmed PDV infected seals with those of juvenile harbour seals which had all tested negative to PDV infection when admitted to the SRRC during the same time of year in 2001. A significance level of P≤0.05 was used for all statistical tests.

An indirect enzyme-linked immunosorbent assay (ELISA) was used to determine IgG titres, and IgM titres were determined using an antibody capture ELISA, as previously described [223]. These tests were done on serum taken at admittance only, as CDV-ISCOM vaccination would interfere with further diagnostic tests for PDV.

Necropsies were performed following a standard protocol, and tissues (brain, lung, kidney and urinary bladder) were collected for detection of nucleic acid by reverse transcriptase-polymerase chain reaction (RT-PCR) [223], with a set of universal morbillivirus primers, P1 (5'-ATGTTTATGATCACAGCGGT-3') and P2 (5'-ATTGGGTTGCACCACTTGTC-3'), that are based on conserved sequences in the phosphoprotein gene, for viral antigen by means of immunohistochemistry (IHC) with rabbit-α-Measles as primary antibodies [228], and bacteriologic testing.

## Treatment

All seal pups (bodyweight 8-10kg) were tube-fed 300-400 ml oral re-hydrating solution (ORS) before transport to the SRRC. On arrival, the seal pups were vaccinated with a CDV-ISCOM vaccine [81], given ORS, and housed in single quarantine units. All seals were treated with a bronchodilator, Clenbuterol (Ventipulmin® syrup, Boehringer Ingelhiem Vetmedica Inc.), a mucolytic, N- Acetylcysteine (Fluimicil®, Zambon Group), and broad-spectrum antibiotics (enrofloxacin [Baytril®, Bayer] and/or amoxicillin-clavulanic acid [Synulox®, Pfizer]). Seizures were controlled symptomatically with diazepam (0,1-0,2 mg/kg i.m.) or phenobarbitone (0,03-0,04 mg/kg i.m.). Anti-parasitic medication was given following standard protocols, and additional fluid therapy was given as needed.

## RESULTS

Respiratory signs of varying severity were seen and heard in 100% (20/20) of the pups examined, conjunctivitis in 70% (14/20), and a combination of neurological signs in 50% (10/20) of the pups. Further specifications of the clinical signs are given in table 1. Occasional peaks in body temperature above 38°C were seen in 35% (7/20), and in general the animals became hypothermic a few days prior to death (to as low as 33.4°C). Eighty percent (16/20) of the seal pups died or had to be euthanised on humane grounds within 14 days after admittance to the SRRC. On the day of admittance to the SRRC, 45% (9/20) had detectable IgG antibody titres, while 20% (4/20) had IgM antibody titres.

| Clinical signs | n | % |
|---|---|---|
| Conjunctivitis | 14 | 70 |
| Myoclonus (flipper or facial muscles) | 10 | 50 |
| Productive cough | 8 | 40 |
| Depression | 8 | 40 |
| Nasal mucopurulent discharge | 7 | 35 |
| Laboured breathing | 7 | 35 |
| Dyspnoea | 6 | 30 |
| Oculo-nasal mucopurulent discharge | 6 | 30 |
| Cranial nerve deficits: uni- or bilateral blindness | 6 | 30 |
| Head tremors | 5 | 25 |
| Seizures | 5 | 25 |
| Tachypnoea | 5 | 25 |
| Palpable subcutaneous emphysema | 3 | 15 |
| Nystagmus | 2 | 10 |
| Paresis and ataxia | 1 | 5 |
| Skin lesions | 1 | 5 |
| **Auscultation** | | |
| Ronchi | 10 | 50 |
| Wheezing | 10 | 45 |
| Crackling | 7 | 35 |
| Increased respiratory sounds | 5 | 25 |
| Decreased respiratory sounds | 2 | 10 |

Table 1. Clinical signs recorded in 20 PDV infected juvenile harbour seals during their stay at the SRRC.

## Haematology and serum chemistry

Of the haematological parameters, total granulocyte count, percentage granulocytes, and thrombocyte count were statistically higher than mean values of PDV-negative pups admitted the previous year (Table 2.). The total WBC was higher compared to wild pups, but not statistically higher than that of PDV-negative pups from the previous year. Serum chemistry values were statistically lower for AST/GOT and ALT/GPT compared to PDV-negative pups from the previous year.

## Bacteriology

*Bordetella bronchiseptica* was cultured from the lungs of 35% (7/20) of the seals at necropsy. *Escherichia coli* was cultured from the lungs of 25% (5/20) at necropsy.

## Parasitology

*Otostrongylus circumlitis* infections were found in 25% (5/20) of the lungs at necropsy. Sporadic infections with *Parafilaroides gymnurus* (5% - 1/20) and *Dipetalonema spirocauda* (5% - 1/20) were also seen.

## RT-PCR and IHC

All animals were positive in at least one organ tested by means of RT-PCR, and 80% (16/20) by means of IHC. Lung tissue was positive in 50% (10/20) and 55% (11/20) of seals with respiratory signs by RT-PCR and IHC respectively. Brain tissue was positive in 40% (8/20) and 10% (2/20) of animals with-, but also 40% (8/20) and 20% (4/20) of animals without neurological signs by RT-PCR and IHC respectively.

| | Admit pups, PDV infected, 2002 | | | Admit pups, PDV negative, 2001 | | | | Wild pups, PDV negative | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Range | n | Mean ± SD | Range | n | P | Mean ± SD | Range |
| HCT (%) | 47.4 ± 11.8 | 22.8– 69.8 | 20 | 51.5 ± 7.9 | 31.4 - 70.85 | 80 | 0.066 | 61.0 ± 6.7 § | 42.4– 78.8 § |
| Thrombocyte count ($10^9$/l) | 445.1± 10.8 | 284.5- 631 | 20 | 368.3 ±136.2 | 44- 805 | 80 | **0.012** | ND | 373– 1164 ‡ |
| WBC ($10^9$/l) | 18.7 ± 9.1 | 3– 34.5 | 20 | 14.2 ± 7.9 | 5.4- 43.8 | 80 | 0.055 | 8.7 ± 3.2 § | 4.6– 20.8 § |
| Granulocytes ($10^9$/l) | 15.2 ± 8.2 | 2.15– 28.2 | 20 | 10.3 ± 7.4 | 3.4– 41.2 | 80 | **0.022** | ND | ND |
| Mononuclear leukocytes ($10^9$/l) | 3.8 ± 2.3 | 0.85– 11.1 | 20 | 4.0 ± 1.7 | 0.8– 11.5 | 80 | 0.682 | ND | ND |
| % Granulocytes | 76.9 ± 11.1 | 50.5– 95.5 | 20 | 68.6 ± 12.0 | 44.5– 95.5 | 80 | **0.006** | ND | ND |
| %Mononuclear leukocytes | 24.4 ± 14.1 | 4.5- 64 | 20 | 31.9 ± 12.1 | 4.5– 55.5 | 80 | 0.072 | ND | ND |
| AST/GOT (U/l) | 96.8 ± 29.2 | 56.4- 164 | 19 | 139.0 ± 83.7 | 13.8- 471 | 80 | **0.034** | 133 ± 45 ‡ | 70– 241 ‡ |
| ALT/GPT (U/l) | 32.4 ± 11.3 | 13.6– 61.3 | 19 | 91.3 ± 76.7 | 13.4- 421 | 80 | **0.000** | 21.6 ± 7.7 § | 12.0– 43.0 § |
| Glucose (mmol/l) | 7.9 ± 2.6 | 3– 13.6 | 16 | 7.8 ± 3.25 | 0.78– 26.6 | 78 | 0.538 | 5.55 ± 10.23 ‡ | 2.28– 9.02 ‡ |
| Urea (µmol/l) | 13.2 ± 5.7 | 5.6- 28 | 19 | 11.4 ± 4.9 | 3.3– 28.5 | 78 | 0.232 | 15.5 ± 3.4 § | 8.7– 22.7 § |
| Creatinin (mmol/l) | 52.9 ± 7.8 | 44.3– 67.3 | 7 | 50.4 ± 7.1 | 26.6– 66.1 | 31 | 0.598 | 61.6 ± 17.6 ‡ | 35.2– 123.2 ‡ |

Table 2 (left). Mean and standard deviation (SD), range, and sample size (n) for juvenile harbour seal pups on day of admittance to the SRRC (Admit), in 2002: with PDV infection, and at the same in 2001, without PDV infection. In bold are given significant differences between the groups (P< 0.05). In the last column, published values for wild harbour seals are given (§ = [257]; ‡ = [260]).
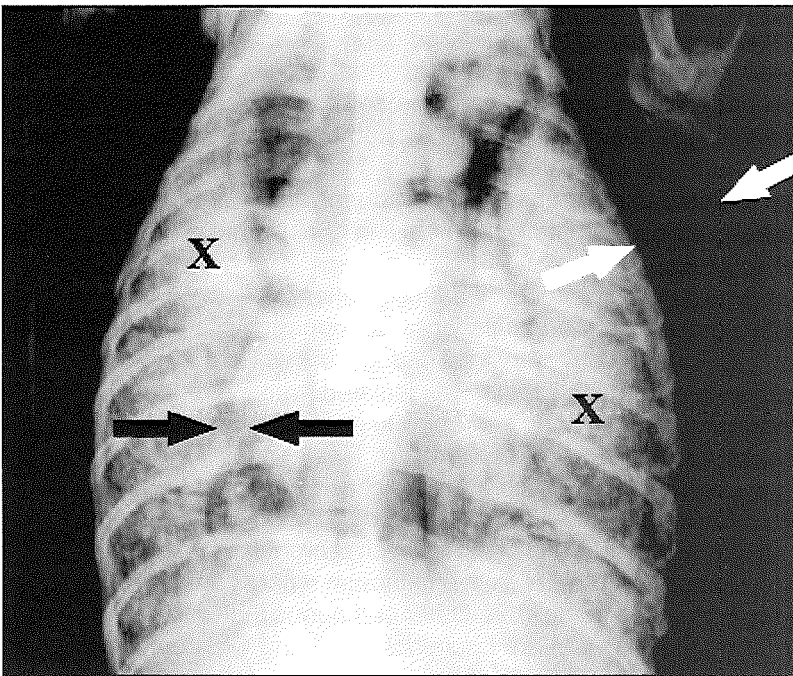


Figure 1. Dorso-ventral thoracic radiograph of a juvenile harbour seal (*Phoca vitulina*) exhibiting diffuse interstitial pneumonia (X), and mediastinal, pericardial (black arrows) and subcutaneous emphysema (white arrows).

## DISCUSSION

In the present paper we have shown that although there are many similarities, there are certain differences in the clinical signs observed during the PDV epidemic of 2002 compared to those previously described for the 1988 outbreak. The respiratory signs (100% of the seals: 20/20) and catarrhal aspects (conjunctivitis in 14/20 or 70%, and mucopurulent oculonasal discharge in 7/20 or 35%) were prominent as in 1988. Pulmonary emphysema was heard as crackles on auscultation in 7 animals (35%), and in severe cases (3/20) it tracked via the mediastinum to subcutaneous cervical and thoracic regions (figure1.), where it could be palpated, causing a crackling sensation. Subcutaneous emphysema can have patho-physiological consequences for seals by affecting their diving ability, and it is thought to be extremely uncomfortable for the animal (in analogy with humans), therefore attempts were made to remove air with a large gauge needle, but a relapse was seen soon afterwards. A more effective method in humans is by subcutaneous catheter [229], and this may be useful in seals. Seals with laboured breathing had an abnormal posture - with an arched back, and front-flippers stretched out at right angles to facilitate breathing. The prominent respiratory signs are in accordance with necropsy findings during the epidemic of 2002, where approximately 80% of stranded seals in the Netherlands had pulmonary consolidation [219].

An important secondary infection after CDV infection in dogs [230] and seals [95,231] is *Bordetella bronchiseptica*, an opportunistic pathogen commonly carried in the upper respiratory tract of dogs, but which may cause suppurative pneumonia [232]. *B.bronchiseptica* was isolated from the lungs of 35% (7/20) of the seals in this study during necropsy. In another study *B.bronchiseptica* was isolated from 60% of dead seals in the Netherlands during the 2002 PDV epidemic (Wagenaar, pers.comm), making it the most common secondary bacterial infection, as in 1988 [233]. It should be noted that the seals used in this study were treated with antibiotics, to which *B.bronchiseptica* strains previously isolated had proven to be sensitive, although most of these seals died before the therapy could be completed. Other pathogens that will have contributed to the respiratory signs seen are *Escherichia coli* and *Otostrongylus circumlitus,* which were both found in 25% of these seals at necropsy.

Lethargy/depression was seen in 40% of the seals, possibly as a result of exhaustion and malnourishment, or with a neurological cause. Neurological signs were observed in 50% (10/20) of the pups, more often than was expected from publications and experiences from the 1988 outbreak, where "a small number of seals exhibited nervous signs" [78]. The percentage of pups with neurological signs reported in the current study would have been higher if animals that were euthanised outside the rehabilitation centre were included, but no exact data of these animals were available. Morbilliviruses are known to be highly neurotropic and capable of causing chronic persistent infections of the CNS [234], and experimental PDV and CDV infections of harbour seals

have shown neurological signs in 2/2 and 1/9 seals respectively [235,236].

Neurological signs we observed in PDV infected seals correlate with those seen in dogs with acute CDV encephalitis (myoclonus, seizures, ataxia) and chronic / multifocal CDV encephalitis (uni- or bi-lateral menace deficits, head tilt, head tremors, nystagmus, weakness / paralysis of pelvic limbs). A myoclonus, or muscle spasm of the flippers or facial muscles was seen in 50% (10/20) of seal pups, and is also the most common sign of acute distemper encephalitis in dogs [237]. Grand mal seizures were recorded for 24% of the seals. As in dogs with CDV infection [237], neurological signs of PDV-infected seals became more frequent and severe over time.

Neurological signs were varied and suggest a spread of the virus throughout the CNS. PDV could be detected by RT-PCR in the brains of only 40% (8/20) of animals with neurological signs, but also in 40% (8/20) of animals without neurological signs. Although IHC is recognised as a sensitive and specific method, PDV was detected in the brain by IHC in only 10% (2/20) of animals with, and 20% (4/20) of animals without neurological signs. Although the neurological signs should reflect the distribution of the virus and lesions in the central nervous system, a clinico-pathological correlation is often lacking in dogs with CDV infection [238], and variation with virus strains in the extent of encephalomyelitis, and therefore the clinical signs has been shown [239]. Also, IHC was only performed on one section of cerebrum and one section of cerebellum, therefore PDV antigen expression in a large part of the CNS was not detected. Whereas PDV infected seals exhibited a combination of neurological and catarrhal signs, in dogs with CDV infection neurological signs typically follow catarrhal signs after about 2-3 weeks.

Differentially, neurological signs in pinnipeds have been associated with electrolyte imbalances associated with renal disease and / or nutritional deficiencies [240] and domoic acid intoxication [241]. Encephalitis has been attributed to infections with herpes viruses [242], West Nile virus [243], bacteria (*Enterococcus* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Salmonella* spp. [244]), fungi [245], and protozoa (*Toxoplasma gondii* [246] *Sarcocystis neurona* [247] and *Eimeria phocae* [248].

Haematological parameters showed an absolute granulocytosis, anaemia, thrombocytosis compared to PDV-negative seals from the previous year, and increased total WBC count (compared to healthy wild seals, not significant with pups at admittance the previous year). Granulocytosis or neutrophilia (the most abundant granulocytes) is most commonly associated with bacterial infection. In the later stages of canine distemper with secondary bacterial infection, the total WBC count may be normal or increased with neutrophilia, lymphopenia, and sometimes increased band neutrophils [249]. Physiological neutrophilia may occur with epinephrine and corticosteroid release during exercise, excitement, or stressful situations [250], but levels were significantly higher than those of seal pups undergoing similar stressful situations without PDV infection. A decrease of the haematocrit was seen instead of the expected haemoconcentration, although a possible effect of

ORS administered prior to transport to the SRRC can not be excluded. Virus-induced immune mediated haemolytic / haemophagocytic anaemia (resulting in a neutrophilia, anaemia and thrombocytopenia) has been described for CDV-infected dogs, and measles infected humans [251-254]. The thrombocytopenia is thought to reflect megakaryocytic damage by the virus, or be the result of immunologic components on the thrombocytes (eg phagocytosis and peripheral depletion). However, thrombocyte count of PDV infected seals was significantly higher than PDV-negative seals in the previous year. Physiologic mobilisation from splenic and non-splenic platelet pools occurs following epinephrine release, but this stress-related thrombocytosis is expected be found in the control seals from 2001 as well, and the thrombocytosis is therefore more likely to have been secondary to virus-induced endothelial or inflammatory changes [255].

Extended periods of lymphopenia and haemoconcentration were previously reported in harbour seals with phocine or canine distemper [97,235,256]). The described lymphopenia ($\leq 10^9$/l) after experimental CDV infection of seals starts 5 days post-infection (dpi), and lasts for about 3 weeks [235]. Marked lymphoid depletion was found in dead PDV-infected seals during the 2002 epidemic in Germany [226]. However, this previously described lymphopenia was not reflected in the total mononuclear leukocyte counts of PDV-infected seals in 2002.

ALT/GPT and AST/GOT levels were significantly lower than in PDV-negative pups admitted the previous year, and comparable (in the case of ALT/GPT) to values of healthy wild pups. The serum levels of these liver enzymes have been documented to be significantly lower in wild pups compared to pups admitted for rehabilitation [257].

A PDV-specific IgG titre $\geq 30$ was found in 55% (11/20) of the seals on the day of admittance. In a naive population, as the northern European harbour seals had been for at least 10 years [94] a single IgG titre can be of diagnostic value, although these may be of maternal origin in juvenile seals. There was no correlation between IgG titre and severity of clinical signs, or duration of survival. In (experimentally) infected seals neutralising antibodies appear 10-20 dpi. However, Duignan et al reported the absence of IgG titres in harbour seals with natural morbillivirus infection, especially in those with respiratory infection [258]. Dogs that succumb to infection between 2-4 weeks post infection have little or no circulating antibodies and the antibody response is inversely correlated with the severity of disease [259].

IgM titres have proven to be more useful for diagnosis in the past, as these are produced sooner after infection (7 dpi) (Harder 1992) but false negative results may also occur in this assay. In the present study on the day of admittance, only 20% of the seals in this study had a positive IgM antibody titre, making it a less useful method of detection of PDV infection in juvenile harbour seals admitted to a rehabilitation centre than IgG titre. Caspian seals with CDV infection had a lower percentage IgM positive animals at necropsy (61%), compared to IgG antibody titres (92%) [223].

Pyrexia was occasionally recorded in only 33% of the pups during their stay at the SRRC. Although the time of infection and duration of clinical disease of the seals before admittance to the SRRC is unknown, the onset of clinical signs usually coincides with the second peak of pyrexia (for CDV infection of harbour seals 7-9 dpi [235], suggesting that 66% of the seals had been infected more than 7 days prior to being taken to the SRRC.

In conclusion, it can be said that the clinical signs observed were similar to those seen in 1988, however with some striking differences. In 2002 the virus caused clinical signs of respiratory and catarrhal infection in the majority of seals, with a higher prevalence of neurological signs than in 1988. Fifty-five percent (11/20) of the seals had serologic responses on admittance to the SRRC, and the percentage of seals with a positive IgG titre was higher than for IgM. No correlation between level of IgG titres and presentation of clinical signs were seen. The most prominent changes in haematological parameters were total granulocytosis, anaemia, thrombocytosis and an increase of total WBC count. Previously described lymphocytic depletion was not reflected in total mononuclear leukocyte counts.

## ACKNOWLEDGEMENTS

# 2.2.

# Serologic surveys in free-ranging animals

# 2.2.1.

# Survey for antibodies to selected pathogens in free-ranging terrestrial carnivores and marine mammals in Canada

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We determined antibody titres to selected pathogens [canine adenovirus (CAV-2), feline herpesvirus (FHV), phocine herpesvirus (PHV-1), canine distemper virus (CDV), dolphin morbillivirus (DMV), phocine distemper virus (PDV), canine para-influenzavirus (CPIV), rabiesvirus (RV), dolphin rhabdovirus (DRV), canine coronavirus (CCoV), feline coronavirus (FeCoV), feline leukaemiavirus (FeLV), *Borrelia burghdorferi*, and *Toxoplasma gondii*] in whole blood or serum samples collected from selected free-ranging terrestrial carnivores and marine mammals, including cougars (*Felis concolor*), lynxes (*Felis lynx*), American badgers (*Taxidea taxus*), fishers (*Martes pennanti*), wolverines (*Gulo gulo*), wolves (*Canis lupus*), black bears (*Ursus americanus*), grizzly bears (*Ursus arctos*), polar bears (*Ursus maritimus*), walruses (*Odobenus rosmarus*), and belugas (*Delphinapterus leucas*). These samples had been collected at several locations in Canada between 1984 and 2000. The results show, among other findings, the presence of antibodies against a number of viruses in species in which these infections have not been reported before, e.g. antibodies to CAV-2 in walruses, to PDV in black bears, grizzly bears, polar bears, lynxes, and wolves, to DMV in grizzly bears, polar bears, walruses and wolves, to CPIV in black bears and fishers, and to DRV in belugas and walruses.

## INTRODUCTION

Mass mortalities due to virus infections may occur in previously unaffected, and therefore susceptible populations of free-ranging terrestrial and aquatic carnivores. Epizootics resulting in mass mortalities caused by infections with morbilliviruses [80,99,105], herpes viruses [242] and influenza viruses [261] have raised considerable public interest. Besides such high-profile epizootic infections, several other pathogens may influence population dynamics with less dramatic effects or infect species that act as intermediate reservoirs, e.g. infections with canine adenovirus [108], canine coronavirus [262], dolphin rhabdovirus [263], *Borrelia burghdorferi* [264] and *Toxoplasma gondii* [265].

Introduction of "new" pathogens in populations may result from ecological changes in relation to the host, pathogen or both. A shrinking or fragmented wildlife habitat has changed population numbers and in some areas has caused increased population densities. These population changes can result in increased inter- and intra-species contacts, and exposure to new pathogens. The ability of a pathogen to infect multiple hosts, including hosts in other taxonomic orders, poses a direct threat of the "spill-over" of infectious agents from reservoir animal populations. By means of reverse spill-over, or "spill-back", these pathogens may represent a threat to sympatric populations of domestic animals. Domestic carnivores like dogs and cats may be maintenance hosts and sources of virulent pathogens to free-ranging carnivores [266]. The risk of interspecies transmission is likely to depend on both the intensity of inter-species contact rates and the possible modes of pathogen

transmission. The translocation of wildlife for conservation, agriculture, or hunting brings an additional inherent risk of exposure of wildlife species to exotic infectious agents. This form of emergence is of particular concern to conservation programmes that bring allopatric species into close proximity or that alter host-infectious agent variables such as population density [267]. Examples of this are certain rehabilitation centres where the risk of spill-over and spill-back of infections includes transmission of possible zoonotic infections present in wildlife populations.

This study aims to determine the prevalence of antibodies to selected pathogens, and to estimate the possible biological importance of intra- and interspecies transmission of infections (including zoonotic infections) on population dynamics and health status in different species of free-ranging Canadian carnivores and marine mammals.

## MATERIALS AND METHODS

### Samples

The survey was conducted on serum and whole blood samples collected at several locations where they were banked in -20$^0$C freezers. The terrestrial carnivore blood and/or serum samples were taken from animals that had been involved in biological research projects during which they were caught and manually restrained, or were chemically immobilised prior to blood collection, after which they were released. Between 1994 and 2001, 23 black bears (*Ursus americanus*), 11 grizzly bears (*Ursus arctos*), 8 wolves (*Canis lupus*) and 8 cougars (*Felis concolor*) were sampled in Banff National Park, Alberta. Between 1996 and 2001, 15 badgers (*Taxidea taxus*), 15 black bears, 25 grizzly bears, 15 cougars, 28 fishers (*Martes pennanti*), 1 wolf and 20 wolverines (*Gulo gulo*) were sampled in various locations in British Columbia. In 1997, 60 polar bears (*Ursus maritimus*) were sampled in Resolute Bay, Nunavut. The 5 lynxes (*Felis lynx*) were hospitalised at the veterinary faculty on Prince Edward Island after they were taken from Cape Breton Island and Nova Scotia mainland in 2000.

The marine mammal samples: 54 belugas (*Delphinapterus leucas*) and 102 walruses (*Odobenus rosmarus*), were obtained from hunter-killed animals from Nunavut and the Northwest Territory between 1986 and 1993.

None of the animals had a known history of vaccination.

### Serology

Serum samples were centrifuged for 5 minutes at 10 000 x G, heat inactivated at 56$^0$C for 30 minutes and screened for antibodies against selected viral and protozoan pathogens using an indirect enzyme-linked immunosorbent assay (ELISA), as previously described [268]. In short, anti-dog immunoglobulins were used for species belonging to the *Canidae*, anti-cat immunoglobulins were used for the species belonging to the *Felidae*, and

horseradish-peroxidase conjugated Protein A was used for all other species to detect the pathogen-specific immunoglobulins bound to the antigen coated wells (European Veterinary Laboratory, Woerden, the Netherlands). After the addition of a tetramethylbenzidine solution and the development of the colour reaction, the reaction was stopped by the addition of a sulphuric acid solution (2M), and the resulting optical density was read at 450 nm. Dilutions of whole blood or serum (1:50 and 1:100) were made using a buffer consisting of phosphate buffered saline solution + 0.2% bovine serum albumin + 0.1% milk powder + 5% NaCl. Control sera of known positive and negative animals were included in the test. An optical density of three times the background optical density in both dilutions was considered positive.

A confirmatory screening was done by a virus neutralisation (VN) test using a serum dilution of 1:40, and 50-100 $TCID_{50}$ of the respective viruses, essentially as previously described [269]. Specific cell lines were used for the different viruses: Vero cells for the dolphin rhabdovirus (DRV, [263]) and morbilliviruses [canine distemper virus (CDV Brussel strain), dolphin morbillivirus (DMV-16a, [270]), phocine distemper virus (PDV-1 /88/NL)], Madin-Darby canine kidney cells (MDCK, ATCC CCL-34) for canine adenovirus (CAV-2 Manhattan strain), and Crandell feline kidney cells for the herpesviruses (feline herpesvirus (FHV): F1134, phocine herpesvirus (PhHV-1): PB-84). The serum antibody titres of samples considered positive (equal to or larger than 40) were subsequently determined by means of a VN test using 2-log dilution series of the pre-diluted samples (1:10-1:1280). The end point titre of each serum was expressed as the reciprocal of the highest dilution that completely inhibited cytopathic effect (CPE) after 5 days incubation. The VN assay proved unsuitable for the whole blood samples due to cytotoxicity of the samples.

Serum samples of animals belonging to the Canidae, Mustelidae, or Ursidae families were analysed for antibodies against CAV-2, canine coronavirus (CCoV), morbilliviruses (CDV, DMV and PDV), canine parainfluenza virus type 3 (CPIV), rabiesvirus (RV) and *Toxoplasma gondii*.

Serum samples of members of the Felidae family were analysed for antibodies against *Borrelia burghdorferi*, morbilliviruses, feline coronavirus (FCoV), FHV, feline leukemiavirus (FeLV), and *T. gondii*.

Serum samples of the waluses and belugas were analysed for antibodies against CAV-2, morbilliviruses, PhHV-1, CPIV and DRV.

## RESULTS

### Canine adenovirus

The total prevalence of CAV-2 specific antibodies in certain species is comprised of the prevalences found in different areas: 8% in black bears (9% from Banff National Park, 7% from British Columbia), 17 % in walruses (15% from Igloolik, 2% from Hall Beach) and 89% in wolves (88% from Banff, 1/1 from BC). Six of the polar bears with antibodies to CAV-2 also had antibodies

to at least one of the respective morbilliviruses. One of the black bears also had antibodies to CPIV.

## Herpes viruses

In cougars, the prevalence of antibodies to FHV ranged from 38% in BC to 57% in Banff, making the total prevalence 47%.

| | n | B. burgh | CAV | CCoV | FeCoV | CDV | DMV | PDV | FHV | PhHV | FeLV | CPIV | RV | DRV | T. gondii |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| badger | 15 | x | 0 | 0 | x | 7 | 0 | 0 | x | x | x | 0 | 0 | x | 0 |
| beluga | 54 | x | 0 | x | x | 0 | 0 | 0 | x | 2 | x | 0 | x | 7 | x |
| black bear | 38 | x | 8 | 0 | x | 3 | 0 | 3 | x | x | x | 5 | 0 | x | 13 |
| cougar | 15 | 0 | x | x | 0 | 0 | 0 | 0 | 47 | x | 0 | x | x | x | 11 |
| fisher | 28 | x | 4 | 4 | x | 0 | 0 | 0 | x | x | x | 4 | 4 | x | 18 |
| grizzly bear | 36 | x | 0 | 0 | x | 28 | 33 | 47 | x | x | x | 0 | 0 | x | 0 |
| lynx | 5 | 0 | x | x | 0 | 20 | 0 | 20 | 60 | x | 0 | x | x | x | 20 |
| polar bear | 60 | x | 17 | 0 | x | 39 | 13 | 35 | x | x | x | 0 | 0 | 2 | 0 |
| walrus | 102 | x | 17 | x | x | 14 | 13 | 6 | x | 0 | x | 0 | x | 15 | x |
| wolf | 9 | x | 89 | 0 | x | 67 | 22 | 33 | x | x | x | 0 | 0 | x | 0 |
| wolverine | 20 | x | 0 | 0 | x | 0 | 0 | 0 | x | x | x | 0 | 0 | x | 0 |

Table 1. Overall prevalences of antibodies to the different pathogens tested for, samples taken from one species from several locations are depicted as one total percentage. For the percentages per location see text. X: Not tested. 0: All samples negative

## Morbilliviruses

The total prevalence of antibodies to at least one of the three morbilliviruses tested for were comprised of the prevalences found in different areas: 3% of the black bears (Banff 0%, BC 13%), 64% of the grizzly bears (Banff 55%, BC 68%), 53% of the polar bears, 18% of the walruses (18% Igloolik; 15% Hall Beach), and 77% of the wolves (Banff 75%, BC 1/1).

## Para-influenza virus

None of the black bears from BC, compared to 9% of the black bears from Banff, showed antibodies to CPIV, making the total prevalence 2%. One of the black bears also had a positive titre to CAV.
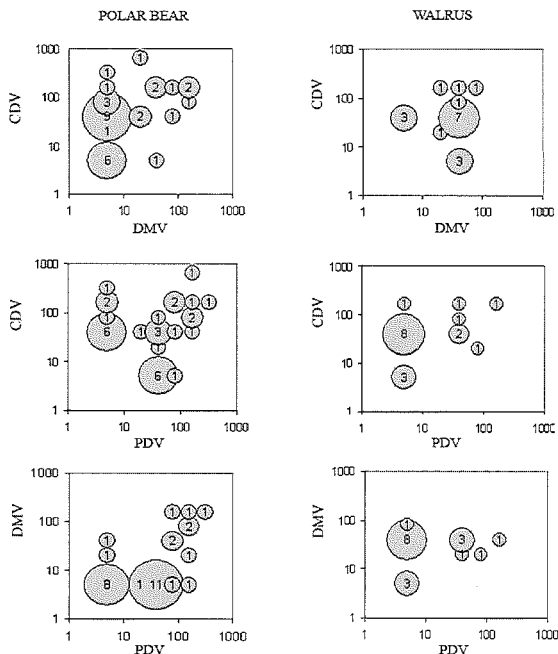
Figure 1 and 2. Antibody titres to the different morbilliviruses in polar bear (figure 1, left hand panels) and walruses (figure 2, right hand panels). The sizes of the bubbles are related to the number of samples. Only samples which tested positive to at least one of the three morbilliviruses tested for are plotted on the graphs, samples depicted in a graph as having titres of <10 on both axes are therefore positive to the third morbillivirus tested for.

### *Toxoplasma gondii*

None of the samples taken from Banff showed antibodies to *T. gondii*. In BC 33% of the black bears tested were positive (including one Kermodie bear, *Ursus americanus kermodie*). The only cougar with antibodies to *T. gondii* was 1 of 8 cougars (13%) tested in BC, making the total prevalence 7%. One of the 2 *T. gondii* positive lynx samples (40%) came from the Nova Scotia mainland, the other from Cape Breton Island.
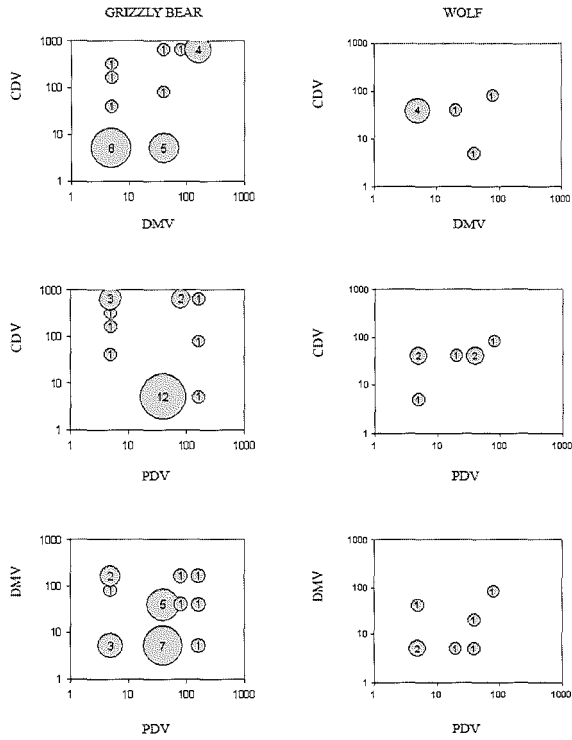
Figures 3 and 4. Serum antibody titres to the different morbilliviruses in grizzly bears (3, left hand panels) and wolves (4, right hand panels). The sizes of the bubbles are related to the number of samples.Only samples which tested positive to at least one of the three morbilliviruses tested for are plotted on the graphs, samples depicted in a graph as having titres of <10 on both axes are therefore positive to the third morbillivirus tested for.

## DISCUSSION

In the present paper we have shown, among other findings, the presence of antibodies against a number of viruses in species in which the infections have not been reported before. This includes the presence of antibodies to CAV-2 in walruses; antibodies to DMV in grizzly bears, polar bears, walruses and wolves; antibodies to PDV in black bears, grizzly bears, lynxes, polar bears and wolves; antibodies to CPIV in black bears and fishers, and antibodies to DRV in belugas and walruses. These infections, or infections with closely antigenically related viruses, may either be endemic in these species or result from interspecies transmission.

The humoral immune response to virus infections (e.g. CDV, CAV, FPV), although not the only mechanism involved, tends to correlate with the level of protection from disease and sometimes infection. it may be an indicator of immune status: a high concentration of virus neutralising antibodies usually implies that an animal is protected from infection or disease, but protective antibody titres may differ between viruses and animal species, and cannot generally be extrapolated from those known to correlate with these parameters in domestic animal species.

Antibodies to morbillivirus were found in sera from all locations, suggesting a wide-spread presence among Canadian free-ranging carnivores. There have been no mass-mortalities related to CDV infection in free-ranging animals in Canada. CDV specific antibodies have been documented in wolves[271] and lynxes [272], and clinical infections of river otters, raccoons, and coyotes [273] have been reported. The only two animals in this survey reported with clinical symptoms correlating with a distemper infection (emaciation, neurological signs and abnormally tame) were the two lynxes with a positive morbillivirus titre (Daoust pers. comm.).

In several species the antibody response was directed at more than one of the morbilliviruses tested for. Figure 1 suggest that production of antibodies in polar bears is probably caused by two different viruses: CDV and PDV. Figure 2 suggest that the antibody response in walruses is caused mainly by infection with CDV and DMV. In grizzly bears (Figure 3) titres to CDV are generally higher, with incomplete cross-reactivity to DMV and PDV. Most of the positive samples had a titre to PDV, often in combination with a titre to DMV. Antibody titres suggest that morbillivirus infection in wolves is mainly by CDV with cross reactivity to PDV. One of the wolves only had antibodies to DMV. The only positive badger had a much higher titre to CDV than to the other morbilliviruses. The antibody reaction in black bears was to CDV in one bear, and PDV in the other. In the lynx samples there was a high level of cross-reactivity between CDV and PDV. The presence of antibodies to PDV and DMV in terrestrial species may be explained as a result of infection by a closely antigenically related virus, or interspecies transmission.

A possible explanation for the presence of antibodies to PDV and DMV in polar bears, and to PDV in walruses may be predation, which provides

a means of direct contact with subsequent production of antibodies. Polar bears are the top predator in the Arctic marine ecosystem and rely largely on seal blubber as their main energy source. They are also known to predate on larger cetaceans at breathing holes when food is scarce. Walruses feed almost entirely on bottom dwelling, or benthic shellfish, supplemented by an assortment of other invertebrate species. On rare occasions certain adult males display a shift in food preference and start feeding on pinnipeds. They feed mainly on carrion, but have reportedly attacked and killed young pinnipeds [274]. However, these antibodies were also found in species in which contact with phocids and cetaceans is unlikely or virtually impossible (black bear, grizzly bear, lynx and wolf). All the morbilliviruses cross-react serologically, but titres are highest against the homologous virus [269], therefore suggesting infection by a closely antigenically related (PDV- or DMV- like) virus, as well as CDV.

The results for morbillivirus specific antibodies in walruses, which suggest infection mainly by CDV, but also DMV, are not consistent with those reported in walruses in arctic Canada over the time period 1984-1993 [275], where 50% had PDV neutralizing antibodies, versus 16% CDV. In their study, however, a titre of 16 was considered positive, and specific antibodies to DMV were not tested.

The low prevalence of CAV-2 specific antibodies found in black bears is in agreement with those found in black bears in Florida [108]. Previously reported serological surveys in grizzly bears in Alaska showed a prevalence of 12% [276] and 14% [277], but grizzly bear sera collected from both British Columbia and Alberta in this survey did not have CAV-2 specific antibodies. Previous surveys among wolves from Canada have shown a prevalence of less than 40% [271]. More recent studies conducted in the USA have shown prevalences of 81% [278] and 94.7% [279], which are more in agreement with those found in our survey.

One adult black bear from Banff National Park tested positive to both CAV-2 and CPIV, a combination that causes infectious tracheo-bronchitis (kennel cough) in domestic dogs. The only other species that tested positive to CPIV was the fisher. The fisher was also the only species to have antibodies to CCoV, indicating its susceptibility to a large number of virus infections found in domestic carnivores.

The prevalence of antibodies to FHV in cougars (46%) and lynxes (60%) is higher than those from previous studies, which have shown 19% prevalence among free-ranging cougars in California [280], and 0,5% among free-ranging lynxes in Canada [272]. Our results show that FHV is widely present in the free-ranging Canadian feline species, on the east and west coast.

The polar bear with an antibody titre against DRV did not have a titre above the threshold for RV neutralizing antibodies, suggesting that the polar bear has been exposed to a rhabdovirus that is antigenically distinct from RV. These titres might result from an interspecies infection with DRV, or an infection by a virus that is closely antigenically related to the DRV. There has

been one documented case of RV infection in polar bears [281], but arctic foxes, a species which shares the polar bear's habitat and relies on food left over by polar bears, are the primary reservoir species in the Arctic [282]. The walrus and beluga samples were not tested for rabies specific antibodies.

The results obtained from this survey show that *T. gondii* infections occur among free-ranging felids in the Western and Eastern regions of Canada. None of the samples collected in Banff had *T. gondii* specific antibody titres. The infection is being sustained in BC, as it is seen in its primary host (*felidae*), as well as various secondary hosts. A previous survey conducted by Aramini and others [283] on Vancouver Island, BC, showed a much higher prevalence (92%) among cougars than this survey (13%). The cougar population density on Vancouver island is much higher than that of the BC mainland, a possible explanation for the higher prevalence. In the United States studies have shown prevalences ranging from 9-58% [280,284]. A previous study among lynxes in Alaska showed a prevalence of 15% [285]. In our survey all grizzly bears were negative, and only 13% of the black bears from BC had antibodies to *T. gondii*. Previous serosurveys of antibodies to *T. gondii* among bears in the United States have shown titres ranging from 8-84% in black bears [277,286-288], and between 9-37% in grizzly bears [277,289]. The only other species with an antibody titre was the fisher, with a prevalence of 18%.

Regional differences in the prevalences of the different pathogens may be related to differences in population densities, and the distribution of other species (including domestic species) that may act as sources of infection. For example, wolverines were sampled in very remote areas, where a lower exposure to sources of infection may be a possible explanation for the absence of antibodies to any of the pathogens tested for.

In conclusion, our data provide information on the prevalence of a number of infectious diseases among different species of free-ranging wildlife throughout Canada. The morbidity or mortality of these infections is often not known in these species. Therefore serologic surveys such as these may be useful in directing further studies on the impact of infectious diseases on free-ranging populations.

# 2.2.2.

**Serologic survey for selected viral pathogens in European mink (*Mustela lutreola*) and other free-ranging mustelids in south-western France**

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In order to investigate the possible role of selected pathogens in the decline of-, and the threat posed to the survival of the endangered European mink (*Mustela lutreola*), a serologic survey was conducted using serum samples collected from March 1996 to March 2003 in eight departments of south-western France. In total, 481 free-ranging individuals of five mustelid species (including the European mink) were tested, as sympatric mustelids can serve as 'sentinels' to determine the presence of antibodies to viruses in the study area and could potentially be a source of infection. Antibodies to canine distemper virus (CDV) were detected in all species, i.e., in 9% of 127 European mink, 20% of 210 polecats (*Mustela putorius*), 5% of 112 American mink (*Mustela vison*), 33% of 21 stone marten (*Martes foina*) and 5% of 20 pine marten (*Martes martes*). Seroprevalence was significantly higher in stone marten and polecats, possibly because their ranges overlap more closely with that of domestic species than that of the other species tested. Antibodies to canine adenovirus were detected in all species but the pine marten, with seroprevalence ranging from 2 to 10%. Seroprevalence of canine para-influenza virus was 1% in European mink, 1% in American mink and 5% in polecats, and was not detected in Martes species. Antibodies to rabies virus (RV) detected in three animals may be due to inter-species transmission of bat lyssaviruses as the sampling area is considered to be free of RV, or to a lack of specificity of the test, as titres measured were borderline. Higher prevalence of the potentially lethal CDV than that of the other viruses suggests that this pathogen could have significant effects on the free-ranging populations, and has implications for the conservation efforts for the endangered European mink.

## INTRODUCTION

The European mink (*Mustela lutreola*), a small semi-aquatic mustelid, has retracted dramatically from its former territory during the last century [290-293] and is currently listed as endangered (i.e., facing a very high risk of extinction in the wild in the near future) by the International Union for the Conservation of Nature and Natural Resources [294]. Presently, the remaining population is spread out over two distinct areas: a relatively large Eastern population (in the Russia, Belarus, Kazakhstan, and Romania) and a very small Western population located in south-western France and northern Spain [294]. In France, there are probably no more than a few hundred individuals, and population density seems to be low. Possible reasons for the decline include excessive trapping, change or loss of habitat and competition with the larger introduced American mink (*Mustela vison*), and infectious diseases [290,295]. Recent studies in the western population of European mink have shown the presence of Aleutian disease virus (ADV), that could contribute to the decline because of its persistent nature and its potential negative effects [296,297]. So far, the incidence of other infectious diseases has not been investigated in free-ranging

European mink. A number of other viruses have been reported in captive or free-ranging mustelids, which could potentially damage free-ranging European mink populations: canine distemper virus (CDV) [15,128,298-300], rabies virus (RV) [301], canine adenovirus (CAV) [110,302-304], canine parainfluenza virus (CPIV) [110,305-307], parvoviruses, including feline panleukopenia, mink enteritis and canine parvoviruses [308,309], corona virus associated epizootic catarrhal enteritis [310], SARS corona virus [311], feline leukaemia virus, rotavirus, Powassan virus, and herpes viruses including Aujeszky's disease virus, and a alpha herpes virus (herpes necrotizing encephalitis) [312].

In order to investigate the potential threat of viruses to the western range of European mink, a serologic survey was conducted in several mustelid species, including European mink, feral American mink, polecat (*Mustela putorius*), stone marten (*Martes foina*) and pine marten (*Martes martes*). All of these mustelids have much larger ranges than the European mink, and co-inhabit certain habitats with European mink, therefore providing opportunities for interspecies transmission of infections. American mink were introduced in Europe for the fur trade, but subsequent escapes from fur farms and successful colonisation of habitats have led to the establishment of populations in large parts of Europe [313]. Polecats are found throughout most of Europe, pine martens are found throughout central/northern Europe and as far East as Siberia, while stone martens are found throughout central and southern Europe [314]. There are no current studies on population sizes and densities of these four mustelid species in the sample area.

Serological surveys can be used to determine prevalence of antibodies to different pathogens, and to gain knowledge on whether these pathogens are endemic in the region tested, if repeated infections occur from an external source, or if an epidemic has occurred. Differences in prevalences should furthermore be attributed to differences in population density, or differences in host-virus interaction. However, prevalence of antibodies should be interpreted with caution, as it does not necessary equate to the prevalence of exposure. Exposed animals that died from the infection, that have not yet seroconverted, or that no longer have detectable antibody titres will not be detected in such ad hoc surveys. Furthermore, serum antibody tests are usually produced for use in domestic species, and have not been validated for use in non-domestic species.

To our knowledge, free-ranging mustelids in Europe have previously only been tested for the presence of antibodies to RV [315], ADV [296,316,317] and CDV [299,300,318-320]. Apart from recent data on prevalence of ADV [296], there is no knowledge of the infection status of the mustelid populations in south-western France.

We tested for antibodies against four viruses which are common in domestic animals (CDV, CAV, CPIV, and RV) and for which serological tests are readily available. Although ADV is seen as a potential threat to the European mink, this virus was not included in this survey, as the data on ADV prevalence in this population have been published recently [296].

Mustelids are known to be very susceptible to CDV infection [321,322]. In the highly endangered black-footed ferret (*Mustela nigripes*) of Wyoming, CDV has contributed to the decline of free-ranging and captive populations [15]. Effects of CDV on European mink is poorly documented, but fatal vaccine-induced distemper has been published [126,323]. The endangered European mink is therefore expected to be very susceptible to infection with virulent CDV. Members of the Canidae, Ursidae and Mustelidae (including striped skunks (*Mephitis mephitis*), American mink, and ferrets) are susceptible to CAV-1 infection, and transmission among domestic and wildlife species is well documented [324], but reports of clinical disease in free-ranging species associated with natural infection are limited [304]. Experimental intranasal infections with CPIV in ferrets usually cause mild respiratory symptoms [306], but its prevalence and significance in free-ranging mustelids is largely unknown. The zoonotic potential of RV has initiated effective vaccination programmes of domestic dogs and free-ranging vector species, which have eradicated it in many areas, including our study area.

The objectives of this study were (1) to determine the prevalence of antibodies to CDV, CAV, CPIV and RV in free-ranging European mink from south-western France as a measure of exposure to these major pathogens; and (2) to determine antibodies in sympatric mustelids which co-inhabit home ranges of the European mink and which can serve as sentinels to determine the presence of these four viruses in the study area or could potentially pass virus to them.

## MATERIALS AND METHODS

Serum samples were collected from 127 European mink, 112 American mink, 201 polecats, 20 pine martens and 21 stone martens trapped during several studies [296] in eight departments of south-western France (42°47 to 46°22'N and 0°54' to 4°7'W) between March 1996 and March 2003 (Figure 1). Most animals (n=327) were caught in live traps, between September and April to avoid birth and nursing periods. Some animals (n=154) were also accidentally captured in live traps during pest control campaigns. Individuals were sometimes caught several times.

Animals were anaesthetised with an intramuscular injection of 150 µg/kg medetomidine (Domitor® 1 mg/ml, Pfizer Sante Animale, Paris, France) and 7.5 mg/kg ketamine (Ketamine UVA 500® 50 mg/ml, Laboratories UVA, Ivry-sur-Seine, France) and a detailed clinical exam was performed [296]. All animals were marked by a cut on the ear and received a subcutaneous transponder (Injectable Trovan®, Eid Aalten B.V., Aalten, The Netherlands) between the shoulders. Blood was taken from the jugular vein using a disposable syringe with a 0.6 x 25mm disposable needle (Terumo®, Terumo Europe N.V., Leuven, Belgium), and transferred into a plain silicone coated glass tube (Venoject, Terumo). When the procedures were completed, anaesthesia was reversed

with 750 µg/kg Atipamezole (Antisedan®, 1 mg/ml, Pfizer Santé Animale), and the animal was placed back in the trap to recover, and released at the capture site 2-3 hr after recovery. Blood was centrifuged at 3000 g for 5 minutes on the same or the next day and serum was stored at –20°C.
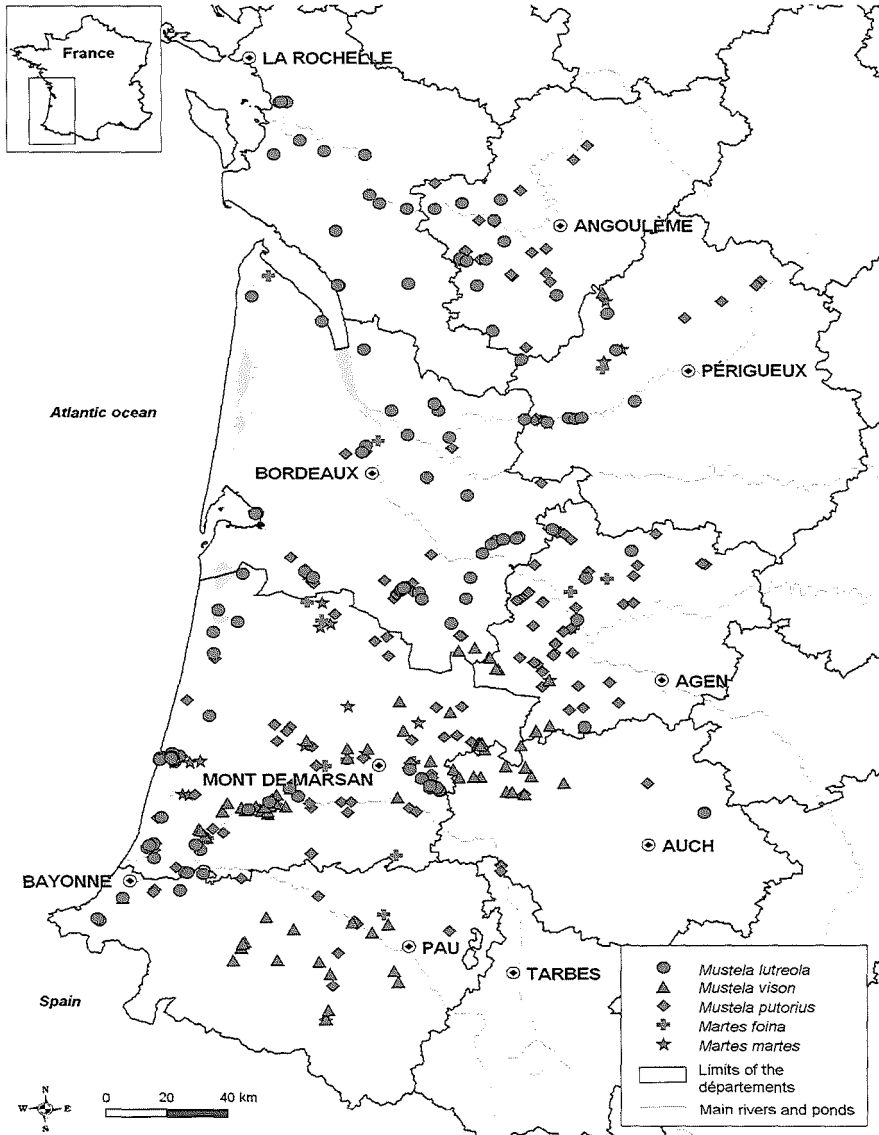


Figure 1 : Geographic distribution of 480 free-ranging mustelids tested for antibodies to canine distemper virus, canine adenovirus, parainfluenza A virus and rabies virus in southwestern France.

Serum was centrifuged for 5 minutes at 10 000 g, heat-inactivated at 56°C for 30 minutes, and screened for antibodies against CDV, CAV, CPIV and RV using an indirect ELISA, as described [268]. In short, horseradish-peroxidase conjugated protein A was used to detect the pathogen-specific immunoglobulins bound to the antigen coated wells (European Veterinary Laboratory, Woerden, the Netherlands). An optical density (OD) read at 450nm of three times the background OD was considered positive. Dilutions of serum were made in a buffer consisting of phosphate buffered saline solution, 0.2% bovine serum albumin, 0.1% milk powder and 5% sodium chloride. Positive and negative control sera were included in the tests. Positive samples in the screening dilution of 1:50 were then retested using 2-log dilution series (1:10 – 1:1280) to determine the titre. CAV-1 and CAV-2 are closely related viruses (CAV-2 causes milder, predominantly respiratory disease in domestic species) and antibodies against these viruses can not be distinguished with the methodology used. Results are therefore given for CAV (without specification of the subtype).

The CDV-specific serum antibody titres of samples considered positive by ELISA were subsequently determined by means of a virus neutralisation (VN) test as previously described [269] using 2-log dilution series of the pre-diluted samples (1:10-1:1280). The end point titre of each serum was expressed as the reciprocal of the highest dilution that completely inhibited cytopathic effect (CPE) in Vero cells after 5 days incubation.

Twenty European mink, four polecats and two American mink were sampled repeatedly, two to four times (one European mink three times and one four times), with a mean interval of 48 weeks (6 to 123 weeks). For determination of antibody prevalence and for all statistical tests, re-sampled animals were represented once (the first sample that tested positive). Cytotoxic sera in the VN test (n=11) were excluded from calculations of prevalence.

For each virus, we used the chi-square test to compare the prevalence of antibodies between sex within species, or a Fisher exact test when the contingency table contained an expected frequency of less than 1.0 in any cell [325]. For CDV, the same tests were used to compare, within species, the difference between prevalences measured by ELISA and VN test. Difference of prevalence of antibodies between species was tested using a Chi-square test followed by a multiple comparisons test [325,326]. For all statistical tests $P \leq 0.05$ was considered significant.

# RESULTS

## Clinical Examination
None of the animals sampled showed clinical signs of disease upon capture and sampling.

## Canine distemper virus

Antibody titres to CDV were detected in all species (Table 1 and Figure 2), without significant differences in prevalence between sexes, tested per species. For each species, the difference of prevalence between the ELISA and the VN test was not significant. Prevalence tested by ELISA was significantly different between species ($x^2$=26.8, p<0.005) and the multiple comparison test (with $\alpha'$= 0,0051) revealed that for both polecat and stone marten, prevalence was significantly higher than in European mink ($x^2$=8.0 and $x^2$=10.3, respectively), and than in American mink ($x^2$=14.6, and $x^2$=17.9, respectively). Prevalence tested by VN was also significantly different between species ($x^2$=18.8, p<0.005) and multiple comparisons test only revealed significantly higher prevalence for both polecat and stone marten versus American mink ($x^2$=10.2 and $x^2$=12.1, respectively).

| | CDV by ELISA | | CDV by Virus neutralisation | |
| --- | --- | --- | --- | --- |
| | Positive /tested | Prevalence (95% confidence interval) | Positive /tested | Prevalence (95% confidence interval) |
| *Mustela lutreola* | 11/127 | 8.7% a, b | (3.4-14.0) | 8/126 | 6.3% f, g | (1.7-11.0) |
| *Mustela putorius* | 41/201 | 20.4% a, c | (14.6-26.2) | 30/192 | 15.6% f, h | (10.2-21.0) |
| *Mustela vison* | 5/112 | 4.5% c, d | (0.2-8.8) | 4/111 | 3.6% h, i | (0.0-7.5) |
| *Martes foina* | 7/21 | 33.3% b, d, e | (14.6-57.0) | 5/20 | 25.0% g, i | (8.7-49.1) |
| *Martes martes* | 1/20 | 5.0% e | (0.1-24.9) | 1/20 | 5.0% | (0.1-24.9) |

Table 1: Antibody prevalence to canine distemper virus (CDV) in free-ranging small mustelids from south-western France using indirect ELISA and virus neutralisation tests. [a, b, c, d, e, f, g, h, i] : Mean values with the same superscript are significantly different between species (P ≤ 0.05).

VN titres ranged from 40 to 640 in European mink and polecat, 20 to 160 in American mink, 80 to 160 in stone martens and was 320 in the positive pine marten.

No seroconversion was observed in 21 negative re-sampled individuals. One European mink had an increased titre when recaptured 48 weeks later (320 – 640). One other, positive in ELISA test only, was negative in both tests 13 months later. Three polecats with an antibody titre of 80 were

negative (≤ 20) 12, 42 and 44 weeks later, respectively.

| | CAV (ELISA) | | CPIV (ELISA) | | RV (ELISA) | |
|---|---|---|---|---|---|---|
| | Positive /tested | Prevalence (95% CI) | Positive /tested | Prevalence (95% CI) | Positive /tested | Prevalence (95% CI) |
| *Mustela lutreola* | 3/126 | 2.4% (0.0-5.5) | 1/126 | 0.8% (0.0-2.7) | 1/126 | 0.8% (0.0-2.7) |
| *Mustela putorius* | 5/201 | 2.5% (0.1-4.9) | 9/201 | 4.5% (1.4-7.6) | 2/201 | 1.0% (0.0-2.6) |
| *Mustela vison* | 6/112 | 5.4% (0.7-10.0) | 1/112 | 0.9% (0.0-3.1) | 0/112 | 0.0% (0.0-0.4) |
| *Martes foina* | 2/21 | 9.5% (1.2-30.4) | 0/21 | 0.0% (0.0-16.1) | 0/21 | 0.0% (0.0-16.1) |
| *Martes martes* | 0/20 | 0.0% (0.0-16.8) | 0/20 | 0.0% (0.0-16.8) | 0/20 | 0.0% (0.0-16.8) |

Table 2: Antibody prevalence to canine adenovirus (CAV), parainfluenza virus (CPIV) and rabies virus (RV) in free-ranging small mustelids from southwestern France using an indirect ELISA test.

## Canine adenovirus

Antibody titres to CAV were detected in all species except pine marten (Table 2 and Figure 2), without any significant difference in prevalence between sexes. No significant difference was observed between species ($\chi^2=5.4$). All re-sampled individuals were negative without any serologic conversion.

## Canine parainfluenza virus

Antibody titres to CPIV were only detected in one European mink, 9 polecats and two American mink, without any significant difference between these three species ($\chi^2=5.9$) (Table 2 and Figure 2). One negative polecat was positive 12 weeks later. All other re-sampled animals were negative without any serologic conversion.

## Rabies virus

Low "borderline" antibody titres to RV (≤ 50) were only detected in one European mink, and two polecats (Table 2). All re-sampled individuals were negative without any serologic conversion.

## Multiple exposures

One stone marten and two polecats were positive to both CDV and CAV, three polecats were positive to both CDV and CPIV, and one European mink was positive to both CDV and with a low, possibly non-specific titre to RV.

Figure 2 : Geographic distribution of the free-ranging mustelids seropositive for antibodies to canine distemper virus (CDV), canine adenovirus (CAV), canine parainfluenza A virus (CPIV) in south-western France.

**CDV** — **CAV** — **CPIV**

La Rochelle, Angoulême, Périgueux, Bordeaux, Agen, Auch, Mont de Marsan, Bayonne, Pau, Tarbes

⬤ *Mustela lutreola* ▲ *Mustela vison* ◆ *Mustela putorius* ✤ *Martes foina* ★ *Martes martes*

▭ Limits of the départements    Main rivers and ponds
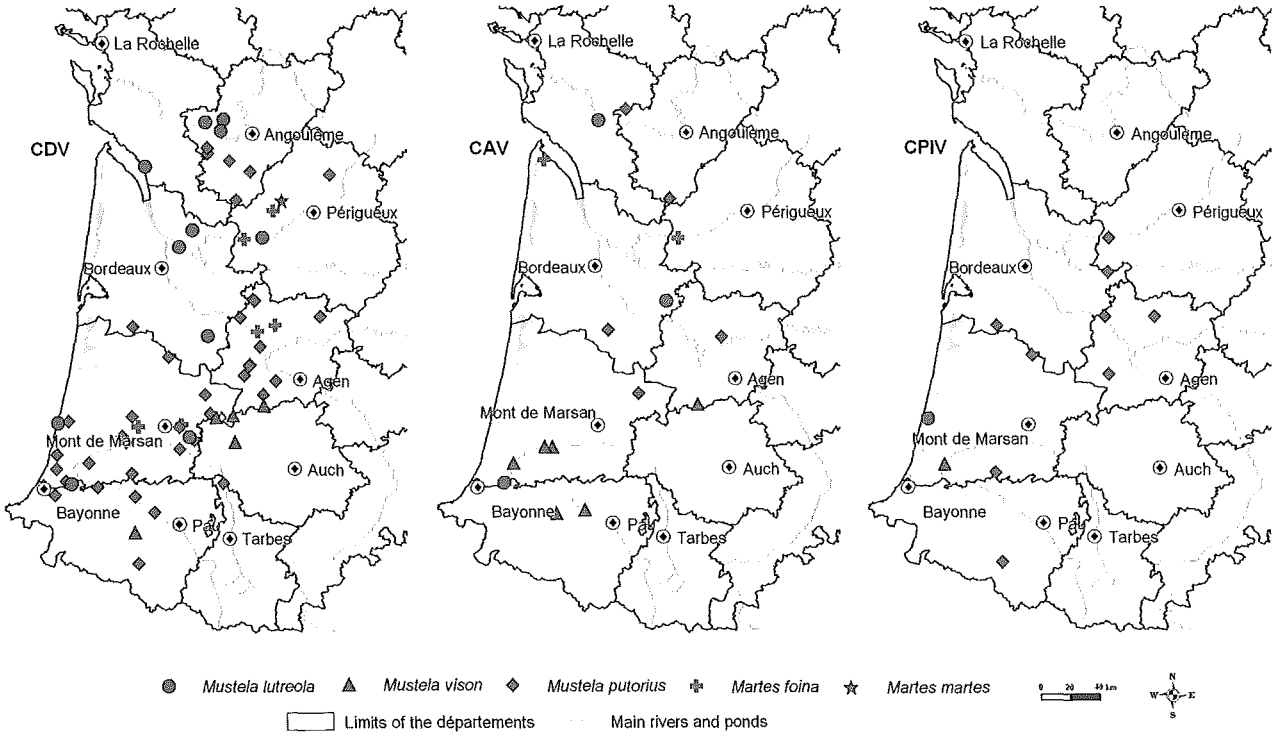
## DISCUSSION

In the present study we showed the presence of antibodies to CDV in all species investigated, to CAV in all species but the pine marten, and to CPIV and possibly RV in all *Mustela* sp. Serological evidence of exposure to CDV occurred in all five mustelid species tested and throughout the sample area. The significantly higher prevalence observed in polecats and stone martens (33% and 20%, respectively, versus 9 and 5 % in European mink and American mink, respectively) correlates with previous prevalence found in stone martens from Germany [259,299]. The high prevalence of CDV is possibly related to the natural habitat of these species. They live in close proximity to humans [327,328], making it more likely that they have come into direct or indirect contact with CDV-infected domestic dogs, which can act as an external source of virus for free-ranging populations. Studies have shown that CDV strains in dogs and free-ranging carnivores in Germany are identical, suggesting transmission of the virus between these populations [259,299]. In the study area, hunting with hounds is widespread in rural regions, and CDV infection probably occurs regularly in these hounds. Although European mink is strongly specialised in aquatic habitats, generally far from humans, they have very large home ranges [329], occasionally resting near rural human habitation [330]. American mink are known to cause damage to hen houses and poultry farms. Therefore interspecies contact with domestic species is also likely to occur, but less frequently than the polecat or stone marten. High prevalence was observed in free-ranging polecats, while CDV has a very high mortality rate in naïve ferrets [321]. A CDV epidemic could not be demonstrated, as the numbers of animals sampled were too small to perform statistical tests between years in the study period to determine a peak in prevalence. This therefore indicates that CDV may be endemic in this species or these species collectively, or that repeated infections occur from an external source. Whatever the source of infection of polecats, this species is known to have close contact with European mink, as hybrids have been found in the wild [331], and the high prevalence in this species suggests that CDV poses a serious threat to the European mink.

Although specific data on longevity of antibody titres are unknown for these species, three polecats which were CDV-positive at the time of their first capture, were negative when recaptured, illustrating that serologic studies like this document the prevalence at particular points in time. The neutralisation titres of 20 to 640 are higher than those previously reported [299], although differences in methodology impedes direct comparisons.

Antibodies to CAV were detected in all species, except pine marten, and throughout the sample area. In our study, prevalence ranged from 2 to 10%. Previous serological surveys of Canadian mustelids have shown prevalences of 4% in 28 fischers (*Martes pennanti*), and 0% in 15 American badgers (*Taxidea taxus*) [110], but 62% prevalence was observed in striped skunks, and two cases of fatal disease have been described in this species [302]. Disease caused by CAV infection is generally not severe in carnivores and our results

probably reflect a relatively low exposure to the virus.

Antibodies to CPIV were found mainly in polecats (5% of 201 individuals). Of all other species, only one positive American mink and one positive European mink were detected. Our results probably reflect a low exposure to the virus, particularly in minks, and suggest that CPIV infection is a lesser threat for free-ranging mustelids.

The detection of antibodies against RV is surprising, as the sampling areas are considered rabies-free. There are two possible explanations. First, it may be attributed to a lack of specificity of the test method used, as the titres detected were low ($\leq 50$). Usually a higher cut-off (=100) is used for positivity in this ELISA. Second, the antibodies detected may be directed against European bat-lyssa virus type-2 (EBLV-2), since there is a high level of cross-reaction between the closely related rabies and bat-lyssa viruses, and it is difficult to distinguish the antibodies to either of these viruses by the serologic method used. Spill-over of bat-origin lyssa virus type-1 has been documented in stone martens in Germany [332], although these spill-over infections do not occur frequently, are supposed to be fatal, and mustelids are dead-end hosts, so the infection is self-limiting [333]. It is interesting to note that experimental infection of ferrets with EBLV-2 has induced high neutralising titres, and all ferrets survived [334], although no natural infections have been documented. A VN test could confirm the specificity of the borderline titre against RV. However, insufficient volumes of serum of these animals would not allow this confirmation.

We have shown that free-ranging mustelids of South-western France are exposed to all the viruses investigated (possibly except RV). The high prevalence of antibodies against the potentially lethal CDV suggests that this pathogen could have significant effects on the free-ranging populations and its contribution to the decline of the weakened population of endangered European mink cannot be excluded. This has several implications for the conservation of the species. Strict sanitary protocols should be implemented during (pest-) trapping programs - European mink are occasionally accidentally captured in live-traps used for pest control -, to exclude live-traps as sources of infection. Recently a breeding program has been set up in Spain, with the intention to eventually release European mink, and a similar program may also be set up soon in France. Virus burdens in the release areas may be reduced by vaccination campaigns of domestic dogs in the region, as is done to protect endangered free-ranging carnivores in Africa [335,336], or by restrictive dog-hunting measures. Vaccination of immunologically naive European mink (especially against CDV) before release into endemic or epidemic areas is recommended (and the vaccine should be administered early enough to allow for the development of protective immunity prior to release) in order for these programmes to be maximally successful. Vaccines against the viruses reported in this study are commercially available for domestic dogs, but unfortunately contain a modified-live CDV component, among other modified-live viruses. Only inactivated vaccines (or other vaccines that have proven to be safe and

effective in the targeted species) should be used in non-domestic animals, as fatal vaccine-induced diseases have occurred in several non-domestic species, including vaccine-induced CDV infections in European mink. Currently there is no safe and effective CDV vaccine commercially available for non-domestic species in the European Union, and the safety and efficacy of (the extra-label use of) vaccines against other pathogens have not been described in European mink.

In conclusion, this study has shown that free-ranging mustelids in south-western France have been exposed to CDV, CAV and CPIV. Future studies should focus on isolation and identification of these viruses in order to improve our understanding of their epidemiology and impact in these species, and on the development and evaluation of preventive measures like vaccination with safe and effective vaccines.

## ACKNOWLEDGEMENTS

# 2.3.

# Canine distemper vaccination of non-domestic terrestrial carnivores

# 2.3.1.

# ISCOM vaccine against canine distemper induces stronger humoral immune response in European mink (*Mustela lutreola*) than a canarypox-vectored recombinant vaccine

*Submitted*

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The endangered European mink (*Mustela lutreola*) is highly susceptible to infection with canine distemper virus (CDV), and live vaccine-induced CDV infections have been reported in this species. Currently there is no safe and efficacious commercially available CDV vaccine for use in highly susceptible non-domestic species like the European mink. We evaluated the safety and efficacy of an experimental CDV immuno-stimulating complex (ISCOM) and a canarypox-vectored recombinant vaccine which is commercially available in the USA, but which is not authorised for use within the European Union. Both vaccines were inoculated in six European mink each, and proved to be safe and induced antibodies to CDV. However, compared to the recombinant group, CDV-specific antibody titres in the ISCOM group appeared sooner after first vaccination, peaked at higher levels, and remained higher one year after the third vaccination. These results indicate that while both CDV vaccines are effective, using virus neutralising serum antibody titres as correlate of induced protection, the CDV-ISCOM vaccine is more immunogenic than the recombinant vaccine in European mink.

## INTRODUCTION

All families of the order Carnivora are susceptible to infection with canine distemper virus (CDV), a ubiquitous and potentially fatal pathogen which has caused outbreaks in several species. Mustelids (black-footed ferret [*Mustela nigripes*], Siberian polecat [*Mustela eversmanni*], and the domestic ferret [*Mustela putorius furo*]) are extremely susceptible and exhibit almost 100% morbidity and mortality after experimental and natural infections.

Domestic dogs and domestic ferrets can be protected against canine distemper by vaccination with a modified live virus (MLV) vaccine. However, other carnivores show a large variation (between and within species) in reaction to MLV vaccines. Vaccines approved for domestic ferrets and dogs which use MLV attenuated via passage in avian- or primate cell lines, are generally safer for use than those of canine kidney cell origin [337]. However, MLV vaccine-induced disease and mortality have been reported in several non-domestic carnivore species, including highly endangered mustelid species like the European mink (*Mustela lutreola*) [126,323] and black-footed ferret [121,125].

Safer alternatives are inactivated virus vaccines, subunit vaccines, or recombinant vaccines. Monovalent inactivated virus vaccines do not induce disease after vaccination, although the process of inactivation may affect their immunogenicity, usually resulting in an immune response that is shorter in duration, narrower in antigenic spectrum, weaker in cell-mediated and mucosal immune responses [25]. Recombinant poxvirus-based CDV vaccines have proven to be safe and efficacious in Siberian polecats, a species closely related to the European mink, and in other carnivore species [73]. In the USA, a canarypox-vectored recombinant CDV vaccine is commercially available for use in domestic ferrets (Purevax™, Merial, Duluth, GA, USA), and is

recommended for use in exotic species by the American Association of Zoo Veterinarians (AAZV). However, the use of this recombinant CDV vaccine is currently forbidden in the European Union, and monovalent inactivated vaccines are no longer commercially available. An experimental CDV immuno-stimulating complex (ISCOM) vaccine was developed and tested in harbour seals (*Phoca vitulina*) during an outbreak of the closely related phocine distemper virus [338]. This vaccine protects seals and domestic dogs against challenge infection [140,236], and has since then been used in several non-domestic carnivore species in zoos, although little published data exist of its use in these species. Currently there are no safe and effective CDV vaccines registered and available for use in highly susceptible non-domestic species within the EU.

The European mink, a small semi-aquatic mustelid, has withdrawn dramatically from its former territory during the last century [290-293] and is currently listed as endangered by the International Union for the Conservation of Nature and Natural Resources (IUCN, 2006). Presently, the population is spread out over two distinct areas: a relatively large eastern population (in Russia, Belarus, Romania, Kazakhstan and the Ukraine) and a very small western population located in South-western France and northern Spain. Reasons implicated for the decline include excessive trapping, change or loss of habitat and interspecies competition by the larger American mink (*Mustela vison*), but the role of diseases has been also suggested [290,295]. An EU LIFE project (2001-2004) was set up together with Foundation Lutreola in Estonia for the recovery of the species and the preservation of biological diversity, both *in situ* and *ex situ*. The *ex situ* project includes a breeding facility at the Tallinn zoo with a capacity of 100 animals as the core of the European endangered species breeding programme (EEP) and to serve as a source of animals to eventually re-establish a population on two Estonian islands. A similar breeding programme has recently been started in Spain.

The impact of CDV infections in captive breeding and re-introduction programmes has been devastating for endangered species like the black-footed ferret [15] and African wild dogs [13]. A safe and effective vaccination campaign is therefore essential for the protection (and therefore survival) of valuable and endangered susceptible species in breeding centres and re-introduction projects. The endangered status of the European mink precludes challenge infections, but serum antibody titres measured by virus neutralisation (VN) test, which is regarded as the gold standard method for determination of immunity to morbilliviruses [339] can be used as correlate of protection. Survival rates after challenge infection with CDV increases with vaccine-induced VN titre [73] in polecats - closely related mustelids, which may be regarded as sympatric sister species of the European mink [340]. The goal of the present study was to evaluate and compare the humoral immunogenicity and safety of a commercial recombinant CDV vaccine and an experimental CDV-ISCOM vaccine in European mink.

## MATERIALS AND METHODS

Previously unvaccinated captive European mink housed in a breeding centre in Tallinn Zoo, Estonia were used for this study, which was conducted in the summer of 2003. Six mink were vaccinated with one ml of a commercial canarypox-vectored recombinant CDV vaccine (Purevax®, Merial, Duluth, GA, USA) and six mink were vaccinated with one ml of an experimental immuno-stimulating complex (CDV-ISCOM, Erasmus MC, Rotterdam, the Netherlands) with an antigen content of 10 µg/ml, produced as previously described [140]. As a negative control group, five mink were injected with a phosphate buffered saline solution. The mink were vaccinated three times intramuscularly at three week intervals, monitored daily for the development of adverse reactions or CDV-like disease, and clinically examined at times of vaccination.

Blood was collected from the jugular vein prior to each vaccination, and at 3 weeks and 1 year after the third vaccination. To collect blood, the European mink were manually restrained in a cloth bag, and anaesthetised with an intramuscular injection of 150 µg/kg medetomidine (Domitor® 1 mg/ml, Pfizer Animal Health, Capelle a/d Ijssel, the Netherlands) and 7.5 mg/kg ketamine (Ketamine 10%, Alfasan, Woerden, the Netherlands). Anaesthesia was reversed using 750 µg/kg atipamezole (Antisedan®, 1mg/ml, Pfizer Animal Health, Capelle a/d Ijssel). Initial dosage was based on estimated weight, and subsequently on weight measured during previous anaesthesia.

### Study design

The VN test is regarded as the gold standard test for determination of immunity against morbilliviruses. The enzyme-linked immunosorbent assay (ELISA), is easy and rapid to perform, conducted in most veterinary diagnostic labs, and commercially available as kits. Future evaluation of vaccine-induced antibody titres would be more practical if the ELISA test can be used. Therefore serum antibody titres were determined by both methods and compared.

Blood was centrifuged for five minutes at 10 000 $g$, serum was separated and stored at -20°C. Serum was then heat-inactivated at 56°C for 30 minutes and tested for CDV-specific antibodies by means of a VN test using 2-log dilution series (1:10 to 1:1280), and 100 median tissue culture-infectious doses ($TCID_{50}$) of CDV in Vero cell culture, essentially as previously described [269]. The end-point titre of each serum was expressed as the reciprocal of the highest dilution that completely inhibited a cytopathic effect after five days incubation. We considered a high serum antibody titre ($\geq 80$) protective, based on published protective antibody titres to CDV infection in polecats which varied between $\geq 3$ and $\geq 152$, and showed higher survival rates with increasing VN serum antibody titres [73].

Serum antibody titres were then determined by an indirect ELISA [268], using 2-log. dilution series. An optical density of three times the background optical density in both dilutions, read at 450 nm was considered positive. An ELISA using uninfected Vero cell lysate was used as a negative control

antigen for each sample tested, providing the background optical density using 2-log dilution series, and control sera from known positive and negative animals were included in the tests. Undetectable titres were regarded as 5 for calculation of geometric mean titres (GMT).

For comparison, three Asiatic small-clawed otters (*Aonyx cinerea*) and two European otters (*Lutra lutra*) that had been vaccinated three times with three week intervals with the same batch of CDV-ISCOM in Rotterdam Zoo were also tested for the development of VN serum antibody titres.

## RESULTS

None of the vaccinated animals showed clinical signs of CDV infection, and no local or systemic side effects that could be attributed to vaccination were noticed. Control animals did not produce serum antibody titres throughout the study.
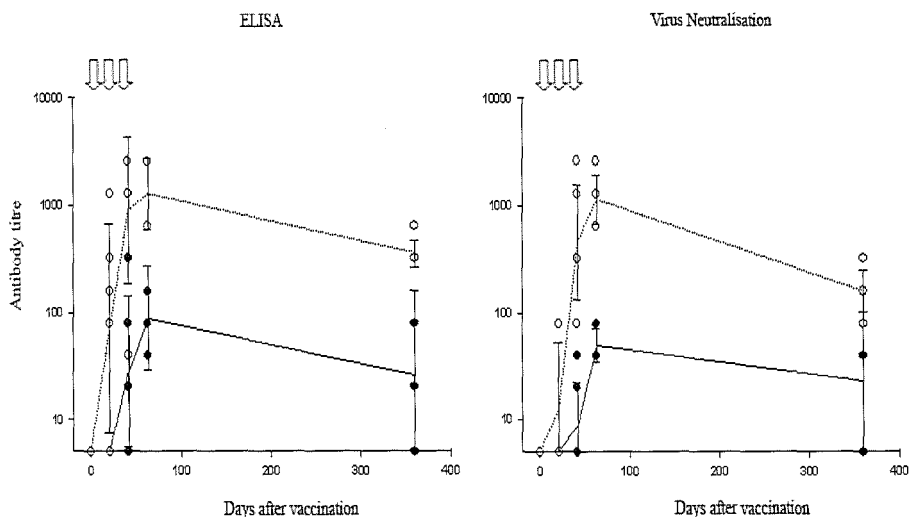


Figure 1. Antibody titres induced in European mink by CDV-ISCOM and a canarypox-vectored recombinant CDV vaccine, measured by ELISA and virus neutralisation. Mink were vaccinated three times with three weeks interval, and blood was collected on days of vaccination, three weeks after the third vaccination, and one year later. White circles depict CDV-ISCOM vaccinated animals, black circles depict canarypox-vectored vaccinated animals. Dotted line connects geometric mean titres of CDV-ISCOM vaccinated animals (n=6), black line connects GMT of canarypox-vectored recombinant vaccinated animals (n=6). Arrows depict time of vaccination.

## CDV-ISCOM vaccinated animals

Low GMTs (by VN and ELISA test) were induced by one dose of the ISCOM vaccine (figure 1). Virus neutralising serum antibody titres were high (≥ 80) in 33% of vaccinated animals after one vaccination (67% measured by ELISA). After 2 doses, high GMTs were induced by the ISCOM vaccine, and 100% had high serum antibody titres (measured by both VN and ELISA). A third dose boosted antibody titres, with VN titres ≥ 640 in all animals, and VN GMT 1140. The GMTs measured by ELISA followed the same trend, but were higher (Figure 1). One year after the last vaccination, VN titres had declined (GMT: 160) but 100% had high (≥ 80) serum antibody titres (measured by both VN and ELISA).

In comparison, two other mustelid species: Asiatic small-clawed otters (*Aonyx cinerea*) and European otters (*Lutra lutra*) that had been vaccinated three times at three week intervals with the same batch of CDV-ISCOM in Rotterdam Zoo showed high VN GMT after 2 vaccinations. The GMT in these species remained high one year after the first three vaccinations, and one booster-vaccination at this time resulted in high GMT one year later in the Asiatic small-clawed otters (Figure 2)

## Recombinant vaccine vaccinated animals

After 2 doses low GMTs were induced in the European mink by the recombinant vaccine (Figure 1), and 0% had high (≥ 80) VN serum antibody titres (33% measured by ELISA). A third dose boosted antibody titres, and 33% had high VN serum antibody titres (50% measured by ELISA). GMT declined but remained for at least one year. In all animals, both VN and ELISA serum antibodies were induced after three vaccinations, with titres ≥ 40 in all animals, and VN GMT 50. The GMTs measured by ELISA followed the same trend, but were higher (Figure 1). One year after the initial three vaccinations, VN titres had declined, but were still detectable (GMT: 22) although only 33% had high VN and ELISA titres, and 33% did not have a detectable titre (by both VN and ELISA) at that time
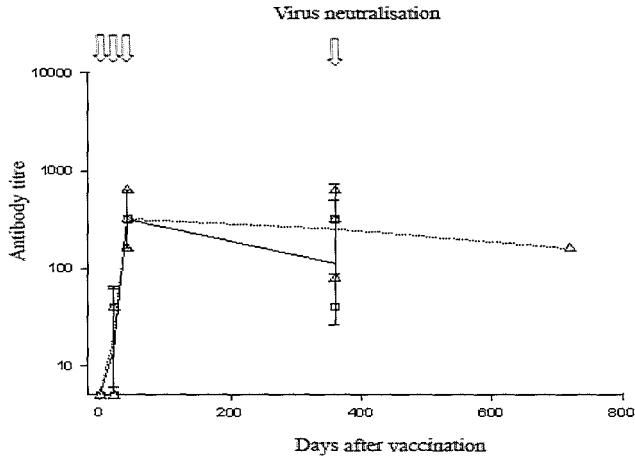
Figure 2. Virus neutralising antibody titres in two other mustelid species: European otter (n=2) and Asiatic small-clawed otter (n=3) induced by the same batch of CDV-ISCOM as used in the European mink. The otters were vaccinated three times with three weeks interval, and blood was collected on days of vaccination, three weeks after the third vaccination, and one year later. One year after the initial three vaccinations, the Asiatic small-clawed otters were re-vaccinated with one vaccine dose, and blood was collected one year later. Triangles depict Asiatic small-clawed otters, squares depict European otters, dotted line connect geometric mean titres (GMT) of Asiatic small-clawed otters, black line connects GMT of European otters

## DISCUSSION

In the present study we have shown that the CDV-ISCOM and canarypox-based recombinant CDV vaccines do not cause adverse effects or canine distemper-like clinical signs and induce humoral immune responses in European mink as determined by VN and ELISA tests. Using CDV neutralising serum antibody titres as correlate of protection, these results indicate that the CDV-ISCOM vaccine is more efficacious in European mink, whilst both seem to be safe. Furthermore, vaccination with this CDV-ISCOM proved also efficacious in terms of VN serum antibody titres in two other mustelid species.

Serum antibody titres measured by ELISA followed a similar trend as those measured by VN test, but were generally higher. The ELISA, which is based on Vero cell culture-grown detergent-treated virus antigens, detects antibodies directed at a large range of epitopes on the haemagglutinin (H) and fusion (F) surface proteins of CDV, but it also detects antibodies against the nucleoprotein (NP) antigen and cell components. The VN antibodies are exclusively directed to the H and F surface proteins of the virus, and mostly recognise conformational epitopes [341]. The ISCOM vaccine uses virus grown on Vero cells as used in the ELISA. It is produced in a manner that

incorporates the H and F proteins into the ISCOMs, and then purified, leaving little or no NP or cell components in the vaccine. However, the presence of small amounts of NP or cell components in the vaccine may induce additional antibodies against these additional epitopes, which are measured by ELISA. The inclusion of an ELISA coated with an uninfected Vero cell lysate used as a control for these sera excludes serum antibodies directed against the Vero cell epitopes. Antibodies induced by the ISCOM and recombinant vaccine are thus directed predominantly against the H and F surface proteins. Nevertheless, a difference in serum antibody titre is seen between the two test methods, which may be explained by the fact that the ELISA detects antibodies not exclusively against VN inducing epitopes on the H and F, like the VN test does.

Vaccination with a canarypox-vectored recombinant CDV vaccine has induced protection in dogs without eliciting a pronounced serologic response, and it has been suggested that the vaccine-induced cell-mediated responses can result in adequate protection in the absence of high serum antibody titres [141,142]. Efforts to document T-cell responses after vaccination of ferrets with canarypox-vectored CDV vaccines were not successful [342]. Morbillivirus ISCOMs have been shown to effectively induce specific T-cell responses in macaques [343]. Therefore it is likely that both the CDV-ISCOM and the recombinant vaccine used in this study will have induced virus specific T-cell responses in this species, thus adding to the protective efficacy of both vaccines.

For breeding and re-introduction programmes to be maximally effective, it is important to know the status of infectious agents in the designated area, so that animals can be vaccinated and thus obtain suitable levels of vaccine-induced protection before release. Equally important is a screening of the animals before release to ensure that they do not introduce a new pathogen into the designated release area.

In conclusion, these results suggest that while both vaccines induce a humoral response, the CDV-ISCOM vaccine may be expected to protect European mink better than the recombinant vaccine against CDV infection for at least one year after 3 vaccinations, based on the induction of higher virus neutralising antibody titres in this species. However, the contribution of vaccine-induced specific T-cell responses by each of the vaccines tested may contribute to the protective efficacy. Since no specific T-cell responses were measured, and no challenge infections can be carried out in this species, the relative protective efficacy between both vaccines for the European mink cannot be estimated.

## ACKNOWLEDGEMENTS

# 2.3.2.

## ISCOM-based and recombinant vaccines induce poor immunity against canine distemper virus in African wild dogs (*Lycaon pictus*)

*Submitted*

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Modified live virus vaccines against canine distemper virus (CDV) for dogs have induced disease and mortality in several non-domestic carnivores, including the endangered African wild dog (*Lycaon pictus*). We evaluated safety and efficacy of alternative vaccines in African wild dogs: an experimental immunostimulating complex (ISCOM) vaccine and a commercially available canarypox-vectored recombinant vaccine. Two vaccine doses of both vaccines induced low virus neutralising (VN) antibody titres (GMT: 8 and 28 for the ISCOM and recombinant vaccinated groups respectively). One year after 3 vaccinations at three week intervals the VN GMT was 6 and 38 for the ISCOM and recombinant groups respectively. The percentage of animals with high VN serum antibody titres (≥80) at this time was 39% in the recombinant vaccine group, whereas no high VN serum antibody titres were found in the ISCOM group. Antibody titres measured by ELISA one year after the initial three vaccinations were higher than VN titres (GMTs: 84 and 80 for the ISCOM and recombinant vaccinated groups respectively, with 69% ≥ 80 for both vaccines). These results indicate that both vaccines were safe, but using vaccine-induced VN antibodies as a correlate of protection, neither vaccine proved to be adequately efficacious, for up to one year post-vaccination in this species. Interestingly, the same batch of CDV-ISCOM vaccine induced adequate VN serum antibody titres in several other non-domestic carnivore species, using the same vaccination regimens. This shows that the African wild dog is a low responder to CDV vaccination with these vaccines.

## INTRODUCTION

The African wild dog (*Lycaon pictus*) is one of the most endangered species of the taxonomic family Canidae. They once ranged widely throughout 39 sub-Saharan African countries, but currently their population is estimated at 3 000 - 5 500 in 15 countries, and have been listed as endangered by the International Union for the Conservation of Nature and Natural Resources since 1990 (IUCN, 2006). Their dramatic decline is largely due to human persecution (snaring, shooting, and road accidents), habitat fragmentation, reduced prey availability, competition with other carnivores, and disease. A shrinking or fragmented wildlife habitat has changed population numbers and may have resulted in increased inter-species contacts and exposure to new pathogens. Outbreaks of canine distemper virus (CDV) infection [92,344] and rabies virus (RV) infection [345,346] have had a devastating impact on African wild dog populations, and have been associated with the close proximity to unvaccinated domestic dogs [92].

Domestic dogs can be routinely vaccinated against CDV with modified-live virus (MLV) vaccines. A problem faced in the prophylaxis of CDV infection in exotic carnivores is the variation between and within species in

their reaction to vaccines. Vaccination with MLV CDV vaccines have resulted in clinical canine distemper (often resulting in death) in many non-domestic species, including the African wild dog [202,203,347]. Inactivated virus vaccines are safer because they do not cause infection, but have decreased efficacy, and have largely failed to induce sufficient immunological responses in domestic [136] and non-domestic carnivores, including the African wild dog [65,70]. There is one report in which an inactivated oil-adjuvanted vaccine has produced virus neutralising (VN) serum antibody titres > 20 in African wild dogs [348]. However, currently there are no monovalent inactivated CDV vaccines commercially available, due to their lower efficacy in domestic dogs compared to MLV vaccines, and since the market for non-domestic animals is too small [138].

One safe alternative would be the use of an immuno-stimulating complex (ISCOM) vaccine, which does not contain live virus. Such a vaccine has been developed and protects harbour seals (*Phoca vitulina*) and domestic dogs against phocine distemper virus infection [81,338], which is closely related to CDV. African wild dogs in a breeding programme in Mkomazi, Tanzania, were vaccinated with the CDV-ISCOM vaccine, which resulted in antibody titres to CDV in African wild dogs monitored at the beginning of this captive breeding program. However, despite recent vaccination there was a lack of VN antibody titres to CDV in sera of these African wild dogs just prior to an outbreak of CDV that subsequently killed 49 of the 52 animals [13].

Another safe alternative is a recombinant canarypox-vectored vaccine which is registered for use in ferrets in the USA, and recommended for use in non-domestic carnivore species by the American Association of Zoo Veterinarians. However, its use in the European Union is not authorised.

The endangered status of the African wild dog precludes challenge infections, but the presence of serum antibody titres measured by VN test, the gold standard method for determination of immunity to morbilliviruses [339] can be used as a correlate of protection. Published vaccine induced serum antibody titres that protect domestic dogs against CDV infection range from 20 [349,350] to 100 [65,351,352]. Survival rates after challenge infection with CDV correlates with the level of vaccine-induced VN serum antibody titres in dogs and polecats (*Mustela eversmanni*) [73,353]. The objective of the present study was to evaluate safety and humoral immune responses to two different CDV vaccines – CDV-ISCOM and canarypox-vectored CDV vaccine - in previously unvaccinated African wild dogs.

## MATERIAL AND METHODS

Previously unvaccinated and unexposed, young captive African wild dogs housed in a breeding centre in Mkomazi, Tanzania, or in Artis Amsterdam Zoo, The Netherlands, were used for this study. Thirteen African wild dogs in Mkomazi were vaccinated with one ml of a canarypox-vectored recombinant CDV vaccine (Purevax™, Merial, Duluth, GA, USA) and thirteen African

wild dogs in Artis were vaccinated with one ml of an experimental ISCOM vaccine, with an antigen concentration of 10 µg/ml (CDV-ISCOM, Erasmus MC, Rotterdam, the Netherlands). The African wild dogs were vaccinated three times intramuscularly at three week intervals, monitored daily for the development of adverse reactions including CDV-like disease, and clinically examined by a veterinarian at the times of vaccination.

Blood was collected from the jugular vein prior to each vaccination, and 1 year after the third vaccination. The number of serum samples available at time of second and third vaccination was 8 in the recombinant group, but one year post vaccination all 13 animals could be evaluated. In the ISCOM group blood was collected from all 13 animals prior to each vaccination. No blood was collected after the third vaccination to minimise the number of times the animals had to be anaesthetised. To collect blood, the African wild dogs were anaesthetised with an intramuscular injection by blowpipe of 0.05 mg/kg medetomidine (Domitor® 1 mg/ml, Pfizer Animal Health, Capelle a/d Ijssel, the Netherlands) and 5 mg/kg ketamine (Ketamine 10%, Alfasan, Woerden, the Netherlands). Dosage was based on estimated weights.

## Study design

The VN test is regarded as the gold standard test for determination of immunity against morbilliviruses. The enzyme-linked immunosorbent assay (ELISA), is easy and rapid to perform, conducted in most veterinary diagnostic labs, and commercially available as kits. Future evaluation of vaccine-induced antibody titres would be more practical if the ELISA test can be used. Therefore serum antibody titres were determined by both methods and compared.

Blood was centrifuged for five minutes at 10 000 $g$, serum was separated and stored at -20°C. Serum was then heat-inactivated at 56°C for 30 minutes and tested for CDV-specific antibodies by means of a VN test using 2-log dilution series (1:10 to 1:1280), and 100 median tissue culture-infectious doses ($TCID_{50}$) of CDV in Vero cell culture, essentially as previously described [269]. The end-point titre of each serum was expressed as the reciprocal of the highest dilution that completely inhibited a cytopathic effect after five days incubation. Serum antibody titres were then determined by an indirect ELISA [268], using 2-log. dilution series of these sera. An optical density of three times the background optical density in both dilutions, read at 450 nm was considered positive.

An ELISA using uninfected Vero cell lysate was used as a negative control antigen for each sample tested (providing the background optical density using 2-log dilution series), and control sera from known positive and negative animals were included in the tests. Undetectable titres were regarded as 5 for calculation of geometric mean titres (GMT).

For comparison, five red pandas (*Ailurus fulgens*), two maned wolves (*Chrysocyon brachyurus*) and five Malay civets (*Viverra tangalunga*) that had been vaccinated in Rotterdam Zoo three times with three week intervals with the same batch of CDV-ISCOM, were also tested for VN serum antibody titres.

For 16 red pandas that had previously been vaccinated with another batch of CDV-ISCOM (with an antigen concentration of 5 µg/ml), only serum antibody titres measured by ELISA were available.

The African wild dog is an endangered carnivore species, therefore challenge infections using a highly virulent CDV virus would not be acceptable from a conservation point of view. We considered a high VN titre (≥ 80) as a correlate of protection to measure vaccine efficacy, in analogy with published data in several other carnivore species, in which the protective titre ranges between 20 and 100 [65,73,143,349,352,354,355].

## RESULTS

None of the vaccinated animals showed local or systemic clinical signs or side effects that could be attributed to vaccination.
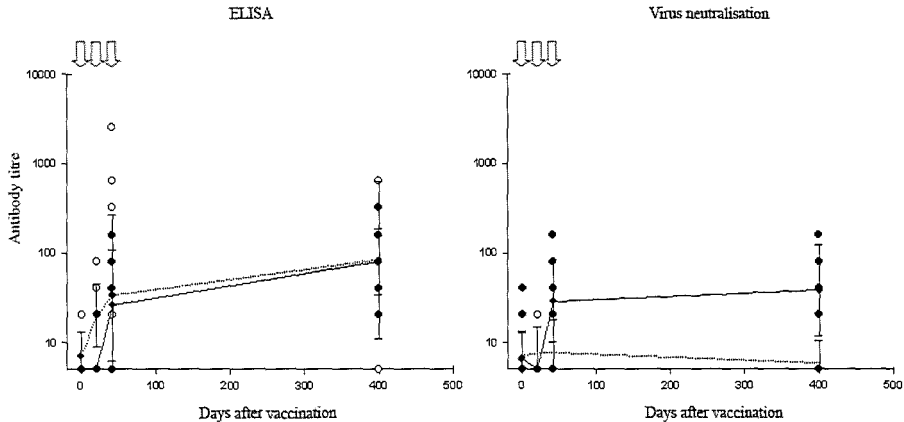


Figure 1. Serum antibody titres measured by ELISA and virus neutralisation, at times of vaccination with an experimental CDV-ISCOM vaccine (white circles) and a commercially available canarypox-vectored recombinant CDV vaccine (black circles) in 13 African wild dogs (*Lycaon pictus*). Dotted lines depict geometric mean titres (GMT) after CDV-ISCOM vaccination, straight lines depict GMT after recombinant vaccination. Arrows depict times of vaccination.

### CDV-ISCOM vaccinated animals

On day zero, 31% (4/13) and 15% (2/13) of the animals had low CDV-specific antibody titres (20 - 40) by ELISA and VN respectively. After one vaccination four animals had low VN titres (20), and 11 animals had low ELISA titres (20-40). High VN serum antibody titres (≥ 80) were first detected in 8% (1/13) of the animals after 2 vaccinations (GMT: 8) (Figure 1). Serum antibody titres measured by ELISA after 2 vaccinations showed higher percentages of animals with high titres: 31% (4/13), and a GMT of 38 (Figure 1).

One year after the initial three vaccinations none of the vaccinated

animals had high VN serum antibody titres. Furthermore, antibodies were undetectable by VN in 92% (12/13) at this time. Percentages of animals with high serum antibody titres measured by ELISA were higher one year after the initial three vaccinations: 69% (9/13) (GMT: 84).

In contrast, VN serum antibody titres in red pandas, maned wolves and Malay civets after CDV-ISCOM vaccination showed high GMT after two vaccinations, which had declined but remained adequate one year later (Figure 2). One yearly booster vaccination of Malay civets ensured that VN GMTs remained adequate. Vaccination of red pandas with the CDV-ISCOM vaccine with lower antigen concentration (5μg/ml) induced low serum antibody titres measured by ELISA.

## Recombinant vaccine vaccinated animals

On day zero, 15% (2/13) animals had low CDV-specific antibody titres (20), only detectable by VN. One animal in the recombinant group had a titre of 80 on day zero. All animals were negative by both ELISA and VN after one vaccination. High VN serum antibody titres (≥ 80) were first detected in 25% (2/8) of the animals after two vaccinations (GMT: 28) (Figure 1). Serum antibody titres measured by ELISA after 2 vaccinations showed higher percentages of animals with high titres: 38% (3/8), and a GMT of 26 (Figure 1).

One year after the initial three vaccinations 39% (5/13) had high VN titres (GMT: 38). Antibodies were undetectable by VN in 16% (2/13) of the animals at this time. Percentages of animals with high serum antibody titres measured by ELISA were higher one year after the initial three vaccinations: 69% (9/13) (GMT: 80).

## DISCUSSION

In the present study we have shown that the canarypox-vectored recombinant CDV vaccine and CDV-ISCOM vaccine do not cause adverse effects, but induce poor immunity based on VN serum antibody titres in African wild dogs. For both vaccines 2 doses were required to induce low levels of VN serum antibodies, although this was only seen in a minority of the animals tested. One year post-vaccination the VN titres were low for the recombinant vaccine: GMT 38 and 39% ≥ 80, and absent for the ISCOM vaccine.

The VN test is regarded as the gold standard method for determination of immunity to morbilliviruses [339] because it measures functional neutralising antibodies. These VN antibodies are exclusively directed to the haemagglutinin (H) and fusion (F) surface proteins of the virus, and mostly recognise conformational epitopes [341]. The H-specific antibodies are the main correlate of vaccination-induced measles virus neutralisation [356]. Antibodies to the nucleoprotein (NP) do not contribute directly to neutralisation and will therefore be missed in a VN test; however, antibodies against these structures are most abundantly produced in response to infection or vaccination with traditional vaccines [357].
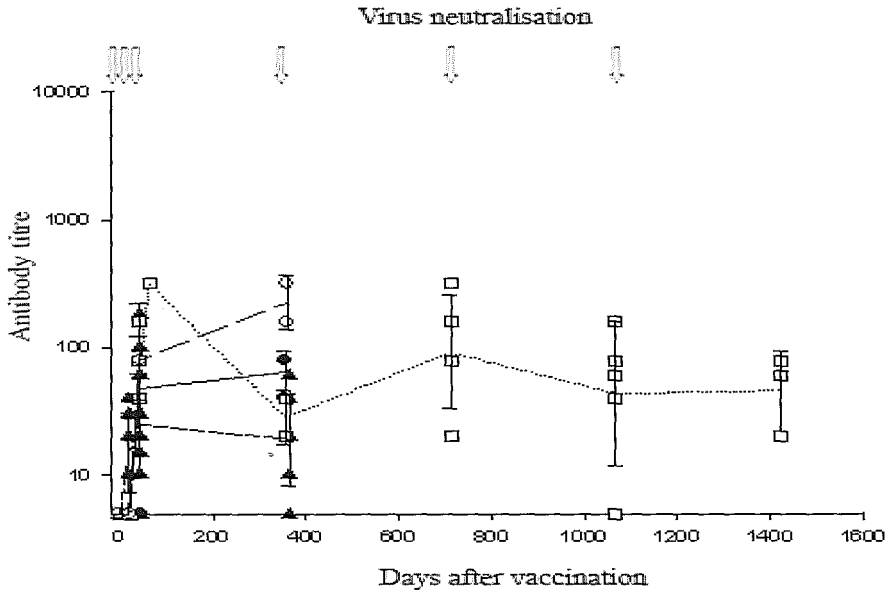
Figure 2. CDV-ISCOM induced virus neutralising antibody titres in 3 species of non-domestic carnivores. Black triangles depict red pandas vaccinated with 5um/ml (measured by ELISA), black circles depict red pandas, white squares depict Malay civets, white circles depict maned wolves. Short dashed line connects geometric mean titres (GMTs) of red pands vaccinated with 5ul/ml; straight line connects GMTs of red pands vaccinated with 10 ug/ml; dotted line connects GMTs of Malay civet cats; long dashed line connects GMTs of maned wolves.

We have previously vaccinated other species of non-domestic animals e.g. the European mink (*Mustela lutreola*) with the same vaccines (submitted), and the GMTs and percentages of African wild dogs with high titres after vaccination with the recombinant vaccine proved to be comparable to those seen in European mink. However, the vaccine-induced response of the African wild dog to CDV-ISCOM vaccination is much lower than the responses to the same batch of CDV-ISCOM in European mink, maned wolves, red pandas, and Malay civets (Figure 2).

Interruption of the cold chain required for preservation of the CDV-ISCOM vaccine as was previously suggested as one of the causes of CDV-ISCOM vaccine failure in African wild dogs [13] could be excluded this time. In red pandas we have seen a major improvement in antibody response by increasing the antigen concentration of the ISCOM vaccine from 5 to 10µg/ml (Figure 2). The increased dose may still not be sufficient to induce a humoral immune response in the African wild dogs. Alternatively, a loss of immunogenicity of the CDV antigen during ISCOM preparation, may have rendered it sub-optimal in terms of inducing a humoral response in the African

wild dog. Collectively, these data indicate that the reason for this relative vaccine failure should be sought in the intrinsic inability of the African wild dog to produce neutralising antibodies in response to CDV vaccination with these vaccines. It may e.g. be speculated that the population bottleneck created by the decimated and fragmented populations of African wild dogs may have resulted in a lack of genetic variability of this species, which subsequently can have had negative consequences for the immune system.

Surprisingly, vaccination with both vaccines did induce satisfactory antibody titres detected by ELISA one year after the initial three vaccinations, suggesting that CDV-ISCOM vaccination does induce CDV specific antibodies in African wild dogs, although they do not neutralise virus in the VN test. The ELISA, which is based on Vero cell culture-grown and detergent-treated virus antigens, detects antibodies directed at the H and F surface proteins of the virus, but also against the NP and cell components. The ISCOM vaccine uses virus grown on Vero cells as used in the ELISA. It is produced in a manner that incorporates the H and F proteins into the ISCOMs, and then purified, leaving little or no NP or cell components in the vaccine. The presence of small amounts of NP or cell components in the vaccine may induce additional antibodies against these epitopes, which can be measured by ELISA. However, the inclusion of an ELISA coated with an uninfected Vero cell lysate used as a control for these sera excludes serum antibodies directed against the Vero cell components.

Vaccine-induced antibodies tested in European mink were higher when tested by ELISA compared to VN (for both vaccines), but followed a similar trend, with a much smaller discrepancy than that observed in African wild dogs. Antibody titres measured by ELISA can be expected to be higher than by VN, because the ELISA detects antibodies against a large range of epitopes on the H and F, and not exclusively against virus neutralising epitopes which are conformational. This also explains the higher ELISA titres induced by the recombinant vaccine, which expresses only the H and F proteins.

Challenge infections have not been performed in the endangered African wild dogs, therefore protective titres are not known. CDV-specific antibody titres tend to correlate with level of protection against distemper. Therefore the recombinant vaccine may be expected to protect populations of African wild dogs better against infection with CDV, than the ISCOM vaccine. However, GMTs and the percentages of animals with high antibody titres are low, therefore a large percentage of the vaccinated animals is not expected to be protected against CDV infection. Models indicate that for measles virus (a related morbillivirus), elimination is achieved by maintaining the fraction of fully immunized individuals with protective VN serum antibody titres above a threshold value (estimated to be 90-95%), to interrupt virus transmission [358].

The presence of low antibody titres at day zero may be attributed to the presence of maternal antibodies, or alternatively to previous exposure to virulent CDV virus. This last scenario is unlikely for the African wild dogs housed in Artis Amsterdam zoo, but cannot be fully excluded for the animals

in Mkomazi game reserve.

Effective vaccination induces not only a humoral, but also a cellular immune response. Vaccination with a canarypox-vectored recombinant CDV vaccine has induced protection in dogs without eliciting a pronounced serologic response [142]. T-cell responses are likely to be of importance in providing immunity to infection with CDV, and it has been suggested that these vaccine-induced cell-mediated responses complement the humoral immunity, which can result in adequate protection in the absence of high serum antibody titres[141,142]. Morbillivirus ISCOMs have been shown to effectively induce specific T-cell responses in macaques [343]. Therefore it cannot be excluded that the CDV-ISCOM used in this study would have induced protective immunity in this species.

In conclusion, the results indicate that both vaccines are safe, but neither vaccine proved to be efficacious, using vaccine-induced VN antibodies as correlate of protection. The recombinant vaccine proved to be more effective in inducing VN antibody responses. This study may aid in eventually providing the necessary adequate protection against infectious diseases through vaccination of this, and other endangered species in conservation projects. We recommend further research on the genetic variability and functioning of the immune system of the African wild dog, on specific T-cell responses and B-cell responses induced by vaccination, to understand the lower vaccine-induced serum antibody responses compared to those seen in other species, and to study the reasons for the discrepancy between ELISA and VN tests.

## ACKNOWLEDGEMENTS

# 3.

# Avian Influenza

# 3.1.

**Highly pathogenic avian influenza (H7N7):
vaccination of zoo birds and transmission
to non-poultry species**

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In 2003 an outbreak of highly pathogenic avian influenza virus (H7N7) struck poultry in the Netherlands. A European Commission directive made vaccination of valuable species in zoo collections possible under strict conditions. We determined pre- and post-vaccination antibody titres in 211 birds by haemagglutination inhibition test as a measure of vaccine efficacy. After booster vaccination, 81.5% of vaccinated birds developed a titre of ≥ 40, while overall geometric mean titre (GMT) was 190 (95% CI: 144 to 251). Birds of the orders Anseriformes, Galliformes and Phoenicopteriformes showed higher GMT and larger percentages developed titres ≥ 40 than those of the other orders. Antibody response decreased with increasing mean body weight in birds ≥ 1.5 kg body weight. In the vicinity of the outbreak, H7N7 was detected by RT-PCR in wild species (mallards and mute swans) kept in captivity together with infected poultry, illustrating the potential threat of transmission from poultry into other avian species, and the importance of protecting valuable avian species by means of vaccination.

## INTRODUCTION

Avian influenza A virus (AIV) is a member of the *Orthomyxoviridae* family and can be classified according to the antigenicity of its surface proteins haemagglutinin (H) and neuraminidase (N), and on the basis of its pathogenicity in chickens. Highly pathogenic avian influenza (HPAI), an acute generalised disease in which mortality may be as high as 100%, is restricted to subtypes H5 and H7, although not all viruses of these subtypes necessarily cause HPAI. All other AIV strains are low pathogenic avian influenza (LPAI) virus strains and cause a much milder, primarily respiratory disease with loss of egg production [152].

The host range of AIV includes a large number of avian species [359-362], with a worldwide distribution and variable morbidity per virus isolate and avian species [361-367]. Free-ranging bird species of the order Anseriformes (ducks, geese, swans and screamers) and Charadriiformes (shorebirds), are considered natural reservoirs in which morbidity is low [361,160,368]. HPAI outbreaks have generally been restricted to poultry (turkeys and chickens), and farmed ostriches [369,370]. Exceptions, where HPAI outbreaks have been reported in free-ranging birds, have been in terns in 1961 [371], several species of wild migratory birds as well as captive Phoenicopteriformes (flamingos) in 2002 [163,372], and in wild migratory birds in 2005 [168].

The Netherlands did not have an outbreak since 1927, but in March 2003 the diagnosis HPAI virus (H7N7) infection was made by RT-PCR [7,373]. HPAI is categorised as an Office International des Epizooties (OIE) list A disease, for which the European Union (EU) has a non-vaccination policy. Eradication measures during an outbreak include confinement, stamping out of animals on the infected farm, pre-emptive culling of animals on neighbouring farms, and emergency vaccinations (EU Directive 92/40/EEC). The area

of the outbreak expanded [374], and threatened to encroach on Dutch zoos. The Dutch Zoo Federation (Nederlandse vereniging van dierentuinen, NVD) proposed vaccination to avoid not only pre-emptive culling of the valuable and sometimes endangered species kept in zoo collections, but also confinement (of unknown duration) of birds, which they considered to be detrimental to welfare. Commission Decision 2003/291/EC of 25 April 2003 replaced the implementation of pre-emptive culling and confinement in zoos with the preventive vaccination of zoo bird species listed as susceptible to avian influenza: Galliformes (fowl, quail, pheasants), Anseriformes, Struthioniformes (emus, nandus and ostriches) and rock doves (*Columba livia*) kept for consumption, provided that far-reaching requirements were met.

Inactivated monovalent and polyvalent AIV water-in-oil emulsion vaccines have been demonstrated to be effective in reducing mortality and/or preventing morbidity, as well as reducing viral shedding [188] after HPAI virus challenge in chickens and turkeys [187]. The use of a heterologous vaccination (in which the N protein differs from the field strain) makes it possible to distinguish vaccinated birds from infected birds, while maintaining acceptable efficacy [190].

The goals of this study were to determine whether transmission of HPAI H7N7 virus from poultry to free-ranging birds during an outbreak occurred, to evaluate the efficacy and safety of an inactivated H7 vaccine in a large number of exotic avian species, and whether vaccination would be a suitable alternative to pre-emptive culling and confinement during future outbreaks of H7 HPAI virus.

## MATERIALS AND METHODS

### Serology

An inactivated H7N1 vaccine, strain A/CK/Italy/473/99, inducing at least 4 $\log_2$ haemagglutination inhibition (HI) units according to potency test, with liquid paraffin adjuvant, (Nobilis influenza®, Intervet International, Boxmeer, the Netherlands) was used. The vaccine strain had a homology of 97.4% to the field strain on the basis of nucleotide sequence (1174 base pairs, excluding basic cleavage site), and 98.7% on the basis of amino acids. The vaccine was produced and assayed according to the requirements made in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter 2.7.12 (further details may be obtained from the manufacturer). The birds were vaccinated twice with 6 weeks interval. Mode of vaccination [subcutaneous (s.c.) or intramuscular (i.m.)] differed according to zoo. Birds with a body weight smaller than 1.5 kg (n = 74) were given 0.25 ml, those with a body weight of 1.5 kg or more (n = 137) were given 0.5 ml. Bodyweights were not assessed individually, but published mean weights of the species were used [375,376]. Blood was collected from 211 birds from 13 orders (Appendix 1), approximately 10% of the total number vaccinated in 10 participating zoos

from the Netherlands. Blood was collected using manual restraint from the right jugular vein or the ulnar vein (left or right) on the day of vaccination and between 30 - 60 days following the last vaccination. Blood collection on the second vaccination date was not mandatory, and was only done in 3 of 10 zoos.

To evaluate humoral immunologic response to vaccination, H7N1 (A/CK/Italy/473/99) specific antibody titres were determined by HI test, following standard procedures, using turkey erythrocytes [377,378]. Undetectable titres (<10) were regarded as 5 for calculation of geometric mean titres (GMT). Vaccine-induced HI titres of ≥ 40 were considered protective, as in humans [379]. GMT and 95% confidence intervals (CI) were then calculated per order. For orders Chariidiformes, Ciconiiformes (storks, herons, egrets), Columbiformes (doves, pigeons), Coraciiformes (hornbills), Gruiformes (cranes), Pelicaniformes (pelicans, cormorants), Psittaciiformes (parrots, cockatoos), Sphenisciformes (penguins), and Strigiformes (owls), it was not meaningful to calculate 95% CI of the GMT because of small numbers (<10). Birds in these orders were combined in two groups: "other orders <1.5 kg", and "other orders ≥ 1.5 kg".

To evaluate vaccine-induced immunity against the HPAI H7N7 strain, a virus neutralisation (VN) assay was performed on post-vaccination serum from 48 randomly selected birds with different HI titres. VN assays were performed essentially as described previously [343]. Briefly, serial dilutions of the respective serum samples, which had been heat-inactivated for 30 min at 56°C, were incubated 1 h at 37°C with 100TCID$_{50}$ of A/Chicken/Netherlands/1/03 (H7N7). The mixture was then transferred to MDCK monolayers in microtitre plates. After incubation for 1 h, the cells were washed with PBS and incubated in infection medium for 5 days. The supernatants of the cultures were then tested for HA activity.

## Virus detection

A cloacal swab was taken from 108 birds from one zoo on the first day of vaccination, and stored in 1 ml transport medium (Hank's balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 µg/ml gentamycin (ICN, Zoetermeer, the Netherlands) to detect active shedding of H7 AIV. RNA was isolated from 200 µl supernatant using the MagnaPure LC system (Roche Diagnostics, Almere, The Netherlands) and influenza A virus was detected by a Taqman assay, based on influenza A virus H7 gene sequences of A/Chicken/Netherlands/1/03, as described [7,380].

## Detection of AIV transmission from poultry to other species.

Free-ranging domestic and wild birds (n=109, Appendix 2.), mainly road casualties but also birds found dead or euthanized, or shot, were collected either directly in the vicinity of depopulated HPAI virus infected poultry farms, or arbitrarily throughout the infected area. Species, sex and age category were

determined and the location and date found were recorded. Cloacal swabs were taken from all birds, with additional tracheal swabs from dead birds, and stored in transport medium.

Full necropsies were performed on 4 mallards kept at a camping near an infected poultry farm, which had cloacal swabs positive for H7 AIV. Their carcasses were stored frozen prior to necropsy. Tissue samples of lung, brain, kidney, liver, spleen, duodenum, jejunum, ileum, caecum, colon, pancreas, heart, skin, and proventriculus were homogenized in 3 ml transport medium using a Polytron PT2100 (Kinematica, Lucerne, Switzerland) and subsequently centrifuged (1590 x g). Virus detection was performed as described above, and a sequence analysis was performed on H7 AIV positive specimens.

**Sequence analysis.**

RT-PCR specific for the non-coding regions of H7 AIV was performed on the original material as described [381]. PCR products were run on a 1% agarose gel / 1 × TBE and purified by using the QIAquick gel extraction kit (Qiagen, Leusden, The Netherlands) and sequenced directly. The entire H and N gene segments of the 7 H7 AIV positive birds were sequenced. Sequencing was performed using the Big Dye Terminator sequencing kit, version 3.1 (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands) and an ABI Prism 3100 genetic analyser (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands). Primer sequences are available on request. Nucleotide sequences were aligned using BIOEDIT 5.0.9.

## RESULTS

## Serology

For 116 birds, GMT after first and second (booster) vaccination could be assessed. Overall, GMT after second vaccination [166 (95% CI: 117 – 237)] and percentage of birds with titre $\geq$ 40 (80.2%) were much higher than after first vaccination [20 (95% CI: 14 - 30) and 36.2%], demonstrating the need for booster vaccination. All but three birds (a domestic chicken, an emu and an Egyptian goose) had a pre-vaccination GMT < 10. In the following text, titres after one vaccination are ignored, and only titres after two vaccinations of all 211 birds in this study are used. The post-vaccination GMT was 190 (95% CI 144 - 251) and 81.5% of all tested birds produced an antibody titre $\geq$ 40. GMT was significantly different between orders (Figure 1). No significant differences were found when Anseriformes, Galliformes, and Phoenicopteriformes were analysed for species differences within the orders.
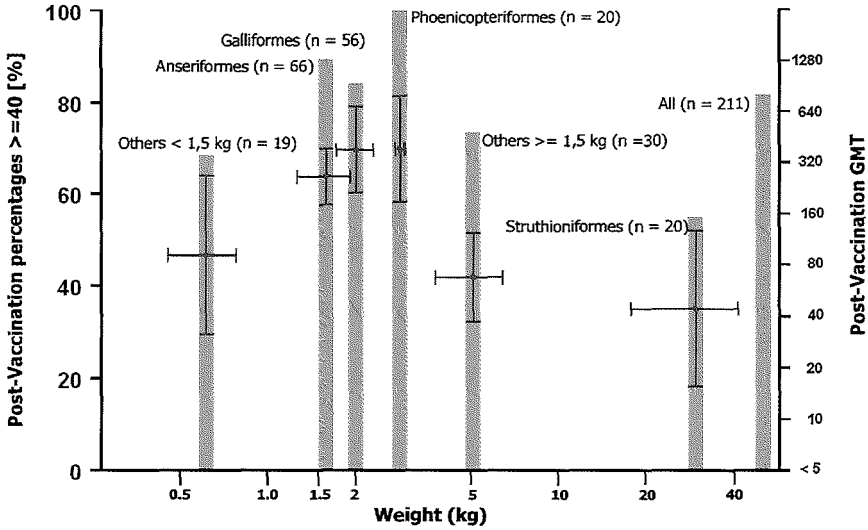
Figure 1. Post-vaccination percentages titre ≥ 40, and GMT versus mean body weight per order (lines: 95% CI's). The humoral response of different avian orders to vaccination against avian influenza (H7) using an inactivated vaccine administered twice with 6 weeks interval. Titres shown were measured 30 to 60 days following the second vaccination. The blue bars represent the percentage of birds with titre ≥ 40. The points represent the geometric mean titre (GMT) and mean body weight per order, with the 95% confidence intervals (CI) represented by vertical (GMT) and horizontal lines (mean body weight), respectively.
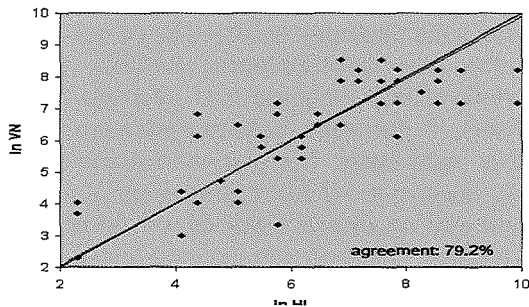


Figure 2. Post-vaccination natural logarithms of titres measured by virus neutralisation (ln VN) and by haemagglutination inhibition (ln HI). Natural logarithms of titres shown are 30 to 60 days following the second vaccination with an inactivated H7N1 vaccine in 48 birds chosen at random. Titres measured by haemagglutination inhibition are against the vaccine strain, those measured by virus neutralisation are against the highly pathogenic H7N7 strain. The red bar represents the regression line, the black bar represents the diagonal.

There was an inverse correlation between weight and antibody response (Figure 1) in birds ≥ 1.5 kg (regression coefficient: −1.543, P < 0.001), but not for those < 1.5 kg (regression coefficient: −0.076, P < 0.913). However, it should be noted that body weights used were published species averages, and not from the individual birds, therefore these results should be treated cautiously, and may not be regarded significant.

Generally, i.m. administration (n = 109) resulted in both a higher GMT [214 (95% CI: 149 - 306) vs 168 (95% CI 110 - 257)] and higher % of animals with a titre ≥ 40 (84.4 vs 78.4) than sc administration (n = 102). However, a statistical comparison between s.c. and i.m. administration with regard to antibody response was not possible (no random administration). Mode of administration was nonetheless not regarded as a factor influencing antibody response in the statistical analysis of the results, as no large differences were observed.

The degree of agreement (within a 4-fold margin) between the post-vaccination results of HI and VN test against the HPAI virus strain was 79.2% (fig. 2).

Necropsies of birds that died during this period did not reveal any HPAI virus- or vaccine-related cause of death.

## Virus Detection

H7 AIV could not be detected by RT-PCR carried out on the cloacal swabs collected in the zoo. H7 AIV virus was detected by RT-PCR from 7 captive birds (4 mallards and 3 mute swans, *Cygnus olor*). All 7 H and N gene segments were identical to the A/Chicken/Netherlands/1/03 H7N7 AIV (GenBank H AY338458, and N AY340077). All positive animals had been kept at a camp site situated about 100 metres from three of the first HPAI-positive poultry farms. These birds had been confined since the beginning of the HPAI outbreak, in the same shed as 8 domestic chickens (*Gallus gallus*), 1 emu (*Dromaius novaehollandiae*), 1 guinea fowl (*Numida meleagris*), 2 greylag geese (*Anser anser*), 2 domestic turkeys (*Meleagris gallopavo*), 1 other mallard, and 1 other mute swan. The guinea fowl and one chicken died and tested serologically positive to H7 AIV (pers comm. Koch, 2005). The rest of the birds, which showed no clinical signs of AIV infection, were euthanized the next day. The turkeys, chickens and the emu tested serologically positive. From the remaining birds, tracheal and cloacal swabs were tested for the presence of H7 AIV, but were negative – except from the 4 mallards and 3 mute swans described above. Free-ranging wild birds shot on the premises [1 greylag goose, 1 mallard and 1 pheasant (*Phasianus colchicus*)] also tested negative for H7 AIV.

Full necropsies on the 4 RT-PCR positive mallards showed the presence of H7N7 virus in the jejunum (3 birds), ileum (4 birds), caecum (3 birds), colon (1 bird), lung (1 bird), spleen (2 birds) and heart (1 bird).

## DISCUSSION

In the present paper we have shown that H7 AIV vaccination of exotic birds with Nobilis influenza® is safe and is expected to be efficacious. We consider it to be useful and necessary in case of an outbreak, as transmission of H7N7 from poultry into non-poultry species did occur. Overall 81.5% of the birds tested developed to a titre ≥ 40 30-60 days after the second vaccination (Figure 1). Antibodies produced against the surface protein H act as major determinants for protection against infection and disease in poultry [187,382,383]. Antibody titres to H7 were therefore used as a measure of immunogenicity, especially as efficacy could not be tested by challenge infections of these valuable or endangered species. Post-vaccination titres ≥ 32 in chickens have prevented shedding after challenge infection with the vaccine strain[384], and proven to be protective in chickens during an outbreak of HPAI virus (H5N1) [181]. On the basis of the antibody titres induced and the high degree of agreement (79.2%) between post-vaccination HI titres against the vaccine strain (H7N1) and VN titres against the HPAI virus strain (H7N7), vaccination may be expected to be efficacious during an outbreak. In analogy with vaccination of poultry, vaccination of exotic species may be expected to prevent morbidity and mortality, reduce environmental contamination with AI virus [179,182,385], and thus reduce or prevent subsequent bird-to-bird transmission. However, because clinically healthy birds can shed HPAI virus, enhanced bio-security measures and regular virologic monitoring of vaccinated and non-vaccinated birds in the infected area will remain necessary [152].

A booster vaccination was required to provide efficacy, using the criterion for efficacy as used by the Committee for Proprietary Medicinal Products (CPMP) for validation of human influenza vaccines (antibody titre ≥ 40 in ≥ 70% of the vaccinated population [386]); whether this criterion is valid for use in these avian species is unknown. In the present study only 36.2% of 116 birds developed a titre ≥ 40 after one vaccination, compared to a total percentage of 80.2% after the second vaccination. This booster effect has previously been shown in chickens and turkeys after AIV vaccination with two weeks interval [387], and is expected to be long-lasting [388,388]. However, the necessity for a booster requires vaccination to occur soon after detection of an outbreak, as some time is required to build up immunity.

The GMT after 2 vaccinations were significantly different between orders (Figure 1). Galliformes, the order for which the vaccine had been specifically produced, but also Phoenicopteriformes, reacted to vaccination with the highest GMT. Differences in neutralising antibody response to vaccination have been demonstrated between domestic avian species, with chickens producing highest titres [191]. More than 70% of birds from the orders Anseriformes, Galliformes, and Phoenicopteriformes developed titres ≥ 40 in response to vaccination (Figure 1), and the vaccine may therefore be considered efficacious in these orders.

The vaccine dose given to Struthioniformes, which are considered to be very susceptible to AIV infection [369], and have significantly higher mean

weight than the other orders [Figure 1: 29.7 kg (95% CI: 17,7 - 41,6)], was likely too low. The humoral immune response to AIV infection in chickens is known to be associated with the virus doses administered [374,389]. Therefore, based on the low % seroprotection and low GMT, which is inversely correlated with published mean body weight (Figure 1), Struthioniformes are expected to respond better to a higher vaccine dose.

Route of vaccine administration (s.c. or i.m.) had no large effect on antibody response, therefore mode of administration was not regarded as a factor influencing antibody response in the statistical analysis of the results. However, formal statistical comparison between s.c. and i.m. administration with regard to antibody response was not possible, as administration was not at random, but differed per zoo, and distribution of orders among zoos was not similar.

The detection of HPAI H7N7 virus infection in mallards and swans kept in the same enclosure as infected chickens and guinea fowl demonstrates that this strain of HPAI H7N7 virus can be transmitted from poultry to other avian species. This illustrates the potential threat of transmission of HPAI H7N7 from poultry to other avian species.

In conclusion, vaccination is expected to be a useful and necessary tool for the protection of valuable exotic birds in zoos against HPAI H7 virus infection, and is a suitable alternative for confinement and pre-emptive culling when implemented together with virologic monitoring and strict bio-security measures at zoos within, or close to the infected area. The administration of twice 0.25 ml to birds < 1.5 kg, and twice 0.5 ml to birds ≥ 1.5 kg, either by i.m. or s.c. route, is efficacious for Anseriformes, Galliformes, and Phoenicopteriformes, but possibly not for the other orders. Because of a negative correlation between antibody response and mean body weight, a higher dose may be required in Struthioniformes. A booster vaccination is essential for efficacy, therefore vaccination of zoo birds should commence soon after detection of an outbreak in poultry in the area. We recommend further research into the efficacy of vaccinating all orders of exotic birds against H7 and H5 AIV with a shorter interval before booster vaccination, and with higher doses for birds with high mean body weight.

## ACKNOWLEDGEMENTS

Appendix 1: Vaccinated species in zoos, geometric mean titres (GMT) 30 – 60 days after 2 vaccinations with an inactivated H7N1 vaccine, and the percentage of birds from each species with a post-vaccination titre of ≥ 40

| Order | | | | Species | |
|---|---|---|---|---|---|
| | n | GMT | % ≥ 40 | Common name | Scientific name |
| Anseriformes | 66 | | | | |
| | 3 | 773 | 100 | Bahama pintail | *Anas bahamensis bahamensis* |
| | 1 | 320 | 100 | bar-headed goose | *Anser indicus* |
| | 3 | 6873 | 100 | black swan | *Cygnus atratus* |
| | 1 | 240 | 100 | common goldeneye | *Bucephala clangula* |
| | 2 | 560 | 100 | common pochard | *Aythia ferina* |
| | 7 | 743 | 100 | common shelduck | *Tadorna tadorna* |
| | 1 | 960 | 100 | common shoveler | *Anas clypeata* |
| | 2 | 1600 | 100 | domestic duck | *Anas platyrhynchos domesticus* |
| | 1 | 480 | 100 | Egyptian goose | *Alopochen aegypticus* |
| | 4 | 900 | 100 | emperor goose | *Anser canagicus* |
| | 3 | 400 | 100 | Eurasian wigeon | *Anas Penelope* |
| | 3 | 106 | 33 | European eider | *Somateria mollissima mollissima* |
| | 3 | 100 | 100 | European goosander | *Mergus merganser* |
| | 2 | 170 | 50 | falcated duck | *Anas falcate* |
| | 5 | 392 | 100 | fulvous whistling duck | *Dendrocygna bicolor* |
| | 1 | 1920 | 100 | mallard | *Anas platyrhynchos* |
| | 6 | 467 | 66 | Mandarin duck | *Aix galericulata* |
| | 1 | 80 | 100 | maned duck | *Chenonetta jubata* |
| | 7 | 714 | 100 | red-crested pochard | *Netta rufina* |
| | 5 | 636 | 100 | smew | *Mergus albellus* |
| | 4 | 330 | 75 | tufted duck | *Aythya fuligula* |
| | 1 | 20 | 0 | white-winged wood duck | *Cairina scutulata .* |

| | | | | |
|---|---|---|---|---|
| Charadriiformes | 4 | | | |
| | 2 | 320 | 50 | black-legged kittiwake | *Rissa tridactyla pollicaris* |
| | 1 | 20 | 0 | common puffin | *Fratercula arctica* |
| | 1 | 60 | 100 | pied avocet | *Recurvirostra avosetta* |
| Ciconiiformes | 7 | | | |
| | 1 | 160 | 100 | black stork | *Ciconia nigra* |
| | 3 | 773 | 100 | black-headed ibis | *Threskiornis melanocephalus* |
| | 1 | <20 | 0 | glossy ibis | *Plagadis falcinellus* |
| | 1 | 480 | 100 | little egret | *Egretta garzetta* |
| | 1 | <20 | 0 | marabou stork | *Leptopilos crumeniferus,* |
| Columbiformes | 6 | | | |
| | 5 | 624 | 60 | rock dove | *Columba livia* |
| | 1 | 80 | 100 | Scheepmakers' crowned pigeon | *Goura scheepmakeri sclaterii* |
| Coraciiformes | 2 | | | |
| | 1 | 480 | 100 | black and white casqued hornbill | *Bycanistes subcylindricus* |
| | 1 | 960 | 100 | blue-winged kookaburra | *Dacelo leachii* |
| Strigiformes | 2 | | | |
| | 1 | 80 | 100 | Eurasian eagle owl | *Bubo bubo bubo* |
| | 1 | 20 | 0 | snowy owl | *Nyctea scandiaca* |
| Galliformes | 56 | | | |
| | 1 | 80 | 100 | Andean guan | *Penelope montagnii montagnii* |
| | 1 | 640 | 100 | Congo peacock | *Afropavo congensis* |
| | 1 | 40 | 100 | crested woodpartridge | *Rollulus roulroul* |
| | 41 | 1871 | 93 | domestic chicken/red junglefowl | *Gallus gallus* |
| | 6 | 10 | 17 | helmeted guineafowl | *Numida meleagris* |
| | 1 | <20 | 0 | koklass | *Pucrasia macrolopha* |
| | 1 | 80 | 100 | Lady Amherst's pheasant | *Chrysolophus amherstiae* |

| | | | | | |
|---|---|---|---|---|---|
| | 1 | 160 | 100 | Palawan peacock pheasant | *Polyplectron emphanum* |
| | 1 | 1920 | 100 | red-billed curassow | *Crax blumenbachi* |
| | 2 | 240 | 100 | wild turkey | *Meleagris gallopavo* |
| Gruiformes | 9 | | | | |
| | 4 | 1350 | 100 | Demoiselle crane | *Anthropoides virgo* |
| | 2 | <20 | 0 | Manchurian crane | *Grus japonensis* |
| | 3 | 520 | 66 | South African crowned crane | *Balearica regulorum regulorum* |
| Pelecaniformes | 6 | | | | |
| | 2 | 60 | 50 | dalmatian pelican | *Pelecanus crispus* |
| | 3 | 206 | 66 | Eastern white pelican | *Pelecanus onocrotalus* |
| | 1 | 80 | 100 | great cormorant | *Phalacrocorax carbo* |
| Phoenicopteriformes | 20 | | | | |
| | 6 | 236 | 100 | Chilean flamingo | *Phoenicopterus chilensis Hyb* |
| | 4 | 350 | 100 | greater flamingo | *Phoenicopterus ruber ruber* |
| | 10 | 3044 | 100 | European flamingo | *Phoenicopterus roseus* |
| Psittaciformes | 4 | | | | |
| | 1 | 160 | 100 | blue-eyed cockatoo | *Cacatua ophthalmica* |
| | 1 | <20 | 0 | long-billed corella | *Cacatua tenuiros tris tenuirostris* |
| | 1 | 160 | 100 | Mexican military macaw | *Ara militaris mexicana* |
| | 1 | 320 | 100 | Mitchell's cockatoo | *Cacatua leadbeateri* |
| Sphenisciformes | 9 | | | | |
| | 5 | 76 | 80 | Humboldt penguin | *Sphenicus humboldti* |
| | 4 | 90 | 100 | jackass penguin | *Spheniscus demersus* |
| Struthioniformes | 20 | | | | |
| | 2 | 640 | 50 | emu | *Dromaius novaehollandiae* |
| | 17 | 558 | 50 | nandu/rhea | *Rhea Americana* |
| | 1 | <20 | 0 | ostrich | *Struthio camelus* |

Appendix 2: Free-ranging domestic and wild birds swabbed for avian influenza

| Order | Species | | |
|---|---|---|---|
| | n | Common name | Scientific name |
| Anseriformes | 59 | | |
| | 1 | black swan | *Cygnus atratus* |
| | 1 | domestic goose | *Anser sp* |
| | 9 | domestic duck | *Anas platyrhynchos domesticus* |
| | 1 | duck | *Anas sp* |
| | 4 | greylag goose | *Anser anser* |
| | 31 | mallard | *Anas platyrhynchos* |
| | 12 | mute swan | *Cygnus olor* |
| Charadriiformes | 4 | | |
| | 4 | black-headed gull | *Larus ridibundus* |
| | 3 | common gull | *Larus canus* |
| Ciconiiformes | 2 | | |
| | 2 | grey heron | *Ardea cinerea* |
| Falconiformes | 2 | | |
| | 2 | sparrowhawk | *Accipter nisus* |
| Galliformes | 10 | | |
| | 3 | pheasant | *Phasianus colchicus* |
| | 6 | wood pigeon | *Columba palumbus* |
| | 1 | quail | *Coturnix coturnix* |
| Gruiformes | 6 | | |
| | 3 | coot | *Fulica atra* |
| | 3 | moorhen | *Gallinula chloropus* |
| Passeriformes | 20 | | |
| | 6 | blackbird | *Turdus merula* |
| | 1 | bohemian waxwing | *Bombycilla garulus* |
| | 3 | carrion crow | *Corvus corone* |
| | 1 | fieldfare | *Turdus pilaris* |
| | 2 | jackdaw | *Corvus monedula* |
| | 5 | magpie | *Pica pica* |
| | 1 | rook | *Corvus frugilegus* |
| | 1 | song thrush | *Turdus philomelus* |
| Psittaciformes | 4 | | |
| | 1 | ring-necked parakeet | *Psitaculla krameri manillensis* |
| Strigiformes | 2 | | |
| | 1 | little owl | *Athene noctua* |
| | 1 | tawny owl | *Strix aluco* |
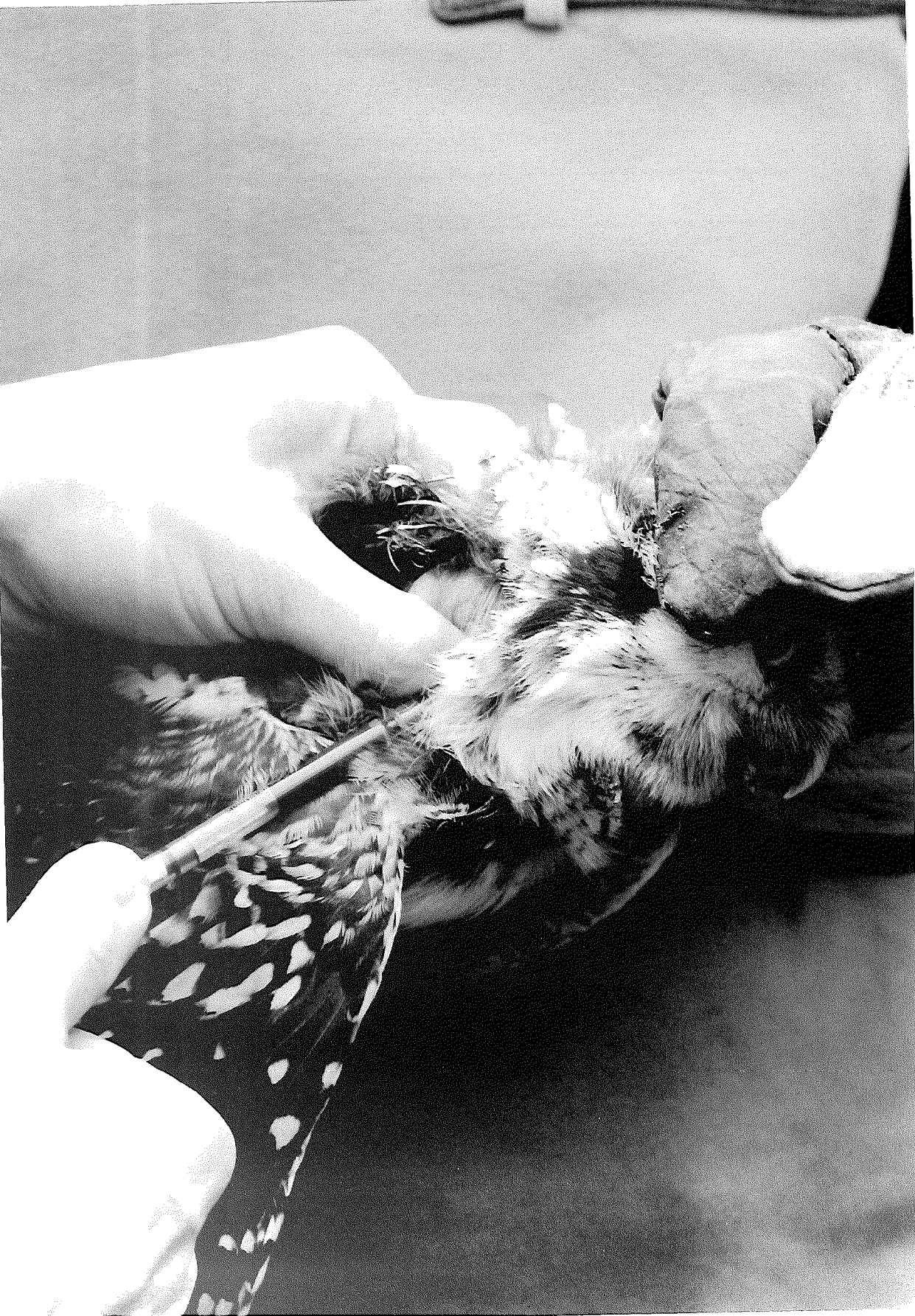
# 3.2.

**Vaccination against highly pathogenic avian influenza H5N1 virus in zoos using an adjuvanted, inactivated H5N2 vaccine**

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Highly pathogenic avian influenza (HPAI) H5N1 virus infections have recently caused unprecedented morbidity and mortality in a wide range of avian species. European Commission directive 2005/744/EC allowed vaccination in zoos under strict conditions, while reducing confinement measures. Vaccination with a commercial H5N2 vaccine with vaccine doses adapted to mean body weight per species was safe, and proved immunogenic throughout the range of species tested, with some variations between and within taxonomic orders. After booster vaccination the overall homologous geometric mean titre (GMT) to the vaccine strain, measured in 334 birds, was 190 (95% CI: 152-236), and 80.5 % of vaccinated birds developed a titre of ≥ 40. Titres to the HPAI H5N1 virus followed a similar trend, but were lower (GMT: 61 (95% CI: 49-76); 61% ≥ 40). The breadth of the immune response was further demonstrated by measuring antibody titres against prototype strains of 4 antigenic clades of currently circulating H5N1 viruses. These data indicate that vaccination should be regarded as a beneficial component of the preventive measures (including increased bio-security and monitoring) that can be undertaken in zoos to prevent an outbreak of and decrease environmental contamination by HPAI H5N1 virus, while alleviating confinement measures.

## INTRODUCTION

Avian influenza virus (AIV) is an Orthomyxovirus, which can be classified according to its pathogenicity and the antigenicity of its surface proteins haemagglutinin (H1-16) and neuraminidase (N1-9)[151]. Viruses containing the subtypes H5 and H7 may become highly pathogenic after introduction in poultry and cause outbreaks of highly pathogenic avian influenza (HPAI), in which mortality may be as high as 100% [160]. The change from a low pathogenic avian influenza (LPAI) phenotype to the HPAI phenotype is achieved by the introduction of basic amino acid residues into the cleavage site of the precursor haemagglutinin [153]. Aquatic wild avian species, in particular those belonging to the taxonomic orders Anseriformes (ducks, geese, swans) and Charadriiformes (shorebirds, gulls and terns) are generally considered to be the main natural reservoir of all LPAI viruses, including the LPAI ancestral viruses of HPAI strains [155,195]. However, an outbreak of HPAI H5N1 virus in Hong Kong in 2002 caused mortality in wild migratory avian species and resident waterfowl [372]. Isolates of this strain from 2002 caused systemic infections and severe neurological dysfunction in experimentally infected mallards (*Anas platyrynchos*) [163] confirming its high pathogenicity in ducks. Currently, the H5N1 subtype of HPAI virus has spread throughout Asia and into Europe and Africa, causing mortality in a wide range of avian species from different taxonomic orders: Anseriformes [165,168,170,372,390-392], Galliformes (turkeys, pheasants, quail and grouse)[391,392], Charadriiformes [165,168,170,372,391,392], Phoenicopteriformes (flamingos)[372,393], Ciconiiformes (storks and herons)[372,394],

Pelecaniformes (pelicans and cormorants)[394], Falconiformes (diurnal birds of prey)[395,396], Strigiformes (owls)[395,396], Struthioniformes (ostriches, emus)[391], Columbiformes (doves and pigeons)[372,394], Passeriformes (perching birds) [372,392,394,397,398], and Psittaciformes (parrots)[395]. This unprecedented spread of outbreaks, with a broadening of host range including mammalian species: leopards, tigers [176,216], domestic cats [177,178,399], domestic dogs [212], mustelids [400], viverrids [218], and humans (with 256 human cases and 151 deaths to date (18th of October 2006) [401]), has major implications for animal and human health around the globe, spawning concern for a new pandemic.

HPAI is classified by the World Organisation for Animal Health (OIE) as a notifiable disease, for which the European Union has a non-vaccination policy. Standard measures in the face of a HPAI virus outbreak are long term confinement or large scale pre-emptive culling of birds. However, this would be detrimental to the welfare and breeding programmes of valuable and endangered avian species in zoos. As an alternative, the Dutch Zoo Federation proposed vaccination in zoos in 2005, as they successfully did in 2003 during an outbreak of HPAI H7N7 virus, when vaccination with an inactivated H7N1 vaccine was safe and produced high serum haemagglutination antibody titres [402]. European Commission decision 2005/744/EC subsequently allowed vaccination as an additional preventive measure to prevent spread of HPAI H5N1 virus from wild birds to zoo birds under rigorous surveillance and control requirements, while reducing confinement measures.

Inactivated monovalent and polyvalent AIV water-in-oil emulsion vaccines have previously been demonstrated to be effective in reducing or preventing morbidity and mortality and reducing viral shedding [183,188] after HPAI virus challenge in chickens and turkeys [187]. Such a vaccine effectively interrupted virus transmission in poultry in the face of an outbreak in Hong Kong in 2002 [181]. In addition to vaccination, increased bio-security measures, in combination with increased monitoring by PCR and sentinel birds are imperative in combating HPAI virus infections. The use of the "differentiating infected from vaccinated animals" (DIVA) strategy, which uses a heterologous vaccine (in which the N subtype differs from that of the field strain) makes it possible to distinguish vaccinated birds from infected birds, while maintaining acceptable efficacy [190].

The goals of this study were to evaluate the safety and efficacy of an inactivated H5N2 vaccine in a wide selection of avian species in zoos, and to discuss the role of vaccination in conjunction with increased bio-security and monitoring as an alternative to large scale pre-emptive culling and confinement in case of an outbreak of HPAI H5N1 virus infection.

## MATERIALS AND METHODS

### Vaccination

An inactivated, commercial, water in oil adjuvanted H5N2 (A/duck/Pottsdam/1402/86) vaccine (Nobilis influenza® H5N2, Intervet International, Boxmeer, the Netherlands), inducing at least 6 $\log_2$ haemagglutination inhibition (HI) units according to potency test was used. The vaccine strain had a homology of 90% to the HA gene of the H5N1 field strain (A/turkey/Turkey/1/05) on the basis of nucleotide sequence (1530 base pairs, including basic cleavage site), and 92.4% on the basis of amino acids. The vaccine was produced and assayed according to the requirements in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter 2.7.12. Further details may be obtained from the manufacturer. More than 3000 birds were vaccinated in the 10 Dutch zoos participating in this study. The birds were vaccinated twice with 6 weeks interval via the subcutaneous route. The vaccine dose administered was adapted to body weight: birds with a body weight <1.4 kg were given 0.25 ml, 1.4 - 7 kg were given 0.5 ml, >7 – 12 kg were given 0.75 ml, >12 – 44 kg were given 1.25 ml, and those > 44 were given 2.5 ml. Published mean body weights of the species were used, rather than body weights of individual birds [375,376].

### Serology

Blood was collected from the right jugular vein or the ulnar vein (left or right) using manual restraint on the day of first vaccination (n=376: 76 species from 14 taxonomic orders) and between 30 - 60 days following the second vaccination [n= 334: 70 species from 14 taxonomic orders (Appendix 1)], approximately 10% of the total number vaccinated in 10 participating zoos. Blood collection on the day of second vaccination was not mandatory, and was only performed in 2 of 10 zoos (n= 109: 44 species from 8 taxonomic orders).

To evaluate the humoral immunologic response to vaccination, homologous H5 specific antibody titres were determined by HI test, following standard procedures, using turkey erythrocytes [377,378]. Known positive and negative sera were used as controls. Undetectable titres (<10) were regarded as 5 for calculation of geometric mean titres (GMT) with 95% confidence intervals (CI). Vaccine-induced HI titres of ≥ 40 were considered a measure of efficacy [402]. To evaluate the vaccine-induced immune response against the recently circulating HPAI H5N1 virus strain, A/turkey/Turkey/1/05 specific antibody titres were determined by HI test as above. To test the breadth of the antibody response, post-vaccination sera with a homologous antibody titre from 32 birds (18 species from 9 taxonomic orders: Anseriformes, Ciconiiformes, Galliformes, Gruiformes, Pelecaniformes, Phoenicopteriformes, Psittaciformes, Sphenisciformes, and Struthioniformes) were titrated against prototype virus strains representing antigenic variants of H5: A/mallard/Netherlands/3/99

(representing contemporary LPAI strains), A/Vietnam/1194/04 (representing H5N1 clade 1), A/Indonesia/5/05 (representing H5N1 clade 2, subclade 1) and A/turkey/Turkey/1/05 (representing H5N1 clade 2 subclade 2) [403]. Antigenic cartography methods as previously described [404] were then used to quantify the magnitude and breadth of the antibody response.

A virus neutralisation (VN) assay was performed on post-vaccination serum from 41 randomly selected birds from 9 taxonomic orders (Anseriformes, Ciconiiformes, Galliformes, Gruiformes, Pelecaniformes, Phoenicopteriformes, Psittaciformes, Sphenisciformes and Struthioniformes) with different HI titres. VN assays were performed essentially as described previously [343]. Briefly, serial dilutions of the respective serum samples, which had been heat-inactivated for 30 min at 56°C, were incubated 1 h at 37°C with $100TCID_{50}$ of A/turkey/Turkey/1/05 (H5N1). The mixture was then transferred to MDCK monolayers in microtitre plates. After incubation for 1 h, the cells were washed with PBS and incubated in infection medium for 5 days. The supernatants of the cultures were then tested for HA activity.

Differences of GMT-values between dose or weight groups were tested by One-Way ANOVA, rate differences by the Pearson $\chi^2$-test. Statistical analyses were performed using SPSS for Windows version 10.0.

## RNA isolation and virus detection
Cloacal swabs were collected from birds with pre-vaccination antibody titres, and selected birds in surrounding enclosures. RNA isolation and reverse transcription polymerase chain reaction were performed as described previously [195].

# RESULTS

## Safety
Mortality due to catching or handling stress reported during the vaccination campaign was low: 5 out of more than 3000 birds that were vaccinated twice. One penguin had a subcutaneous abscess at the site of vaccination, and died despite antibiotic treatment. No other vaccination-related adverse effects were reported. Indirect losses in some zoos consisted of a drop in reproduction due to disturbed behavioural patterns during confinement in some species following the vaccination campaign.

## Prevalence of pre-vaccination anti-H5 antibodies
Of 376 birds tested, 370 were seronegative for H5 AIV (98.4%) by HI test using the homologous antigen. Six birds (1.6%), from three orders [Anseriformes (n=4), Phoenicopteriformes (n=1) and Sphenisciformes (n=1)] and four different zoos were seropositive: greater flamingo, *Phoenicopterus roseus* (titre: 30), Humboldt penguin, *Spheniscus humboldti* (titre: 40), black swan, *Cygnus atratus*, (titre: 30), mute swan, *Cygnus olor,* (titre: 40),

common shelduck, *Tadorna tadorna* (titre: 20) and European eider, *Somateria mollissima* (titre: 60).

| Response after 1st vaccination | | | | Response after 2nd vaccination | | | |
|---|---|---|---|---|---|---|---|
| Dose (ml) | n | GMT (95%CI) | n (%) ≥40 | Dose (ml) | n | GMT (95% CI) | n (%) ≥40 |
| 0.25 | 51 | 31 (19-50) | 26 (51.0%) | 0.25 | 109 | 206 (141-303) | 89 (81.7%) |
| 0.50 | 49 | 39 (22-71) | 24 (49.0%) | 0.50 | 190 | 197 (147-264) | 154 (81.1%) |
| | | | | 0.75 | 13 | 62 (17-225) | 8 (61.5%) |
| > 0.50 | 9 | 72 (10-508) | 5 (55.6%) | 1.25 | 17 | 180 (75-431) | 14 (82.4%) |
| | | | | 2.50 | 5 | 151 (8-2914) | 4 (80.0%) |
| All | 109 | 37 (25-53) | 55 (50.5%) | all | 336 | 190 (152-236) | 269 (80.5%) |
| P | | 0.460 | 0.136 | P | | 0.370 | 0.533 |

Table 1. Serum antibody responses measured by haemagglutination inhibition test are shown as geometric mean titres (GMT) and % of animals with a titre equal to or greater than 40. Blood was collected at the time of second vaccination and 30-60 days later. There were no significant differences in responses of the different dose groups.

## Sero-reponse after H5 vaccination

For 109 birds, titres after one vaccination (at the time of the second vaccination) were determined. On average, GMT was 37 (95% CI: 25-53), and 50.5% of birds reached a serum antibody titre ≥ 40 against the homologous antigen (Table 1). The majority of these birds received vaccine doses of 0.25 ml and 0.5 ml. There were no significant differences in antibody response between doses (P=0.136). For 334 birds, titres 30-60 days after 2nd vaccination were determined. On average, the birds reached a post-vaccination GMT of 190 (95% CI: 152-236), and 80.5% had a titre ≥ 40 against the homologous antigen. The majority of these birds received vaccine doses of 0.25 ml and 0.5 ml. There were no significant differences in antibody response between doses. In accordance, serum antibody titre after the second vaccination was not significantly dependent on weight (P=0.633, linear regression). Three of four birds with a pre-vaccination antibody titre tested showed an increase in antibody titre after vaccination.
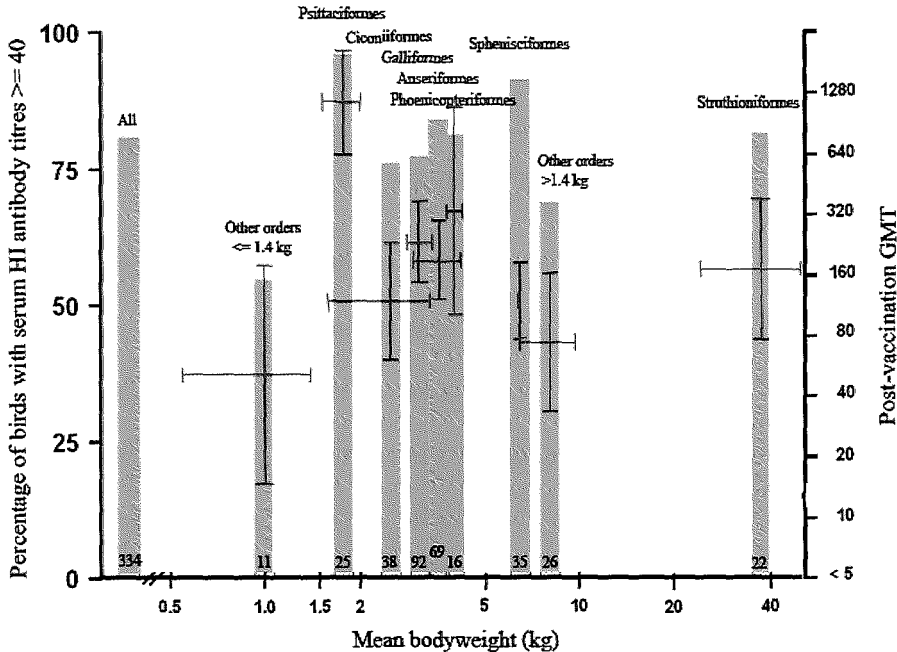
Figure 1. Humoral immune response of avian species in zoos following vaccination with an inactivated H5N2 vaccine with dose adjusted to mean bodyweight. Depicted is the percentage of birds per taxonomic orders with serum antibody haemagglutination inhibition (HI) titre ≥40, and geometric mean titres (GMT) vs. mean body weight of different avian taxonomic orders. An inactivated H5N2 vaccine with dose adjusted to average body weight was used, administered twice with 6 weeks interval. Titres shown were measured 30 to 60 days following the second vaccination. Taxonomic orders of which sample size was too low for statistical analysis are grouped in "other orders ≤1.4 kg" (Charadriiformes, Columbiformes, and Passeriformes) and "other orders >1.4 kg" (Coraciiformes, Gruiformes, Pelecaniformes, and Strigiformes). Red points with vertical lines: post-vaccination GMT with 95% confidence interval (CI). Horizontal red lines: 95% CI of mean body weight in kg. Blue bars: % of birds with a post-vaccination titre ≥40. Black numbers in bars: numbers of birds per order.

## Breadth of response

For 325 birds, serum antibody titres after second vaccination against both the H5N2 vaccine component and the HPAI H5N1 strain circulating in Europe (A/turkey/Turkey/05) were determined. The vaccine induced antibodies against the HPAI strain, although titres were 3.1 times lower than homologous titres. On average, the birds reached a post-vaccination GMT of 61 (95% CI: 49-76), and 61.2% had a titre ≥ 40. (Figure 2). The degree of agreement (within a 4-fold margin) between the post-vaccination results of HI test using A/turkey/Turkey/1/05 as antigen   and VN test against A/turkey/Turkey/1/05 was 73.2%.
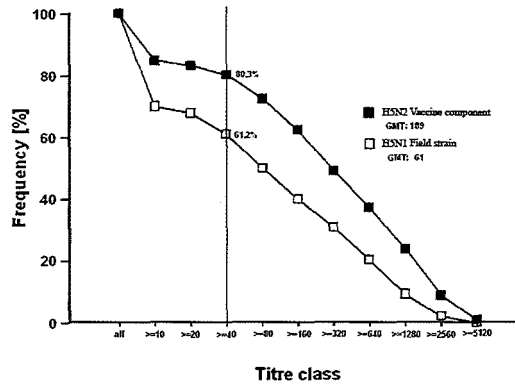
Figure 2. Comparison of serum haemagglutination inhibition antibody titres against avian influenza H5N2 vaccine virus and avian influenza H5N1 field virus following vaccination. Haemagglutination inhibition titres against both the vaccine component (H5N2: A/duck/Pottsdam/1402/86) and the HPAI field strain (H5N1: A/turkey/Turkey/05) were determined in 325 birds, 30-60 days following 2 vaccinations with 6 weeks interval.
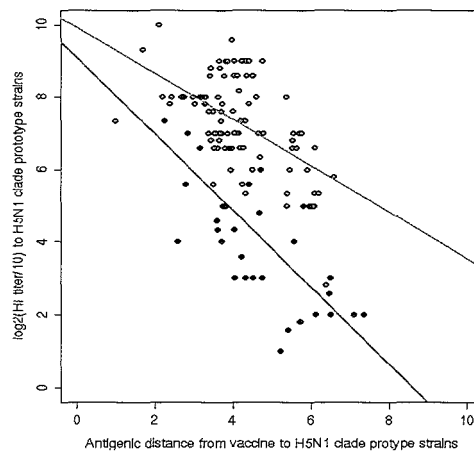


Figure 3. Post-vaccination serum haemagglutination inhibition antibody titres as a function of the antigenic distance between vaccine and prototype strains of 4 H5N1 clades. Sera from 32 vaccinated zoo birds were titrated against prototype strains of 4 H5N1 clades [403]. Antigenic distances to the prototype strains were determined from an antigenic maps of H5 strains made using antigenic cartography [404]. The birds clustered in 2 groups: group 1 (green); Galliformes, Anseriformes, Phoenicopteriformes, Struthioniformes and Psittaciformes versus group 2 (purple); Ciconiiformes, Gruiformes, Pelecaniformes and Sphenisciformes), based on the breath of the antibody responses. Lines show linear regression through points of the same colour. The group 1 (green) line has slope -0.7 intercept 10.41; the group 2 (purple) line has slope -1.07, intercept 9.47. The slope difference is statistically significant (p=0.021).

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We further tested the breadth of the antibody response using sera from 32 birds belonging to 7 orders. Inspection of the HI data revealed that the immune response to prototype strains from 4 antigenic "clades" [403] divided the 7 taxonomic orders into 2 groups (group 1; Galliformes, Anseriformes, Phoenicopteriformes, Struthioniformes and Psittaciformes versus group 2; Ciconiiformes, Gruiformes, Pelecaniformes and Sphenisciformes). We created antigen maps [404] with the HI data to compare the breadth of antibody response between the two groups. Group 1 birds were found to have a statistically significant broader antibody response than the group 2 birds (P=0.021, linear regression) (figure 3).

**Virus detection**

No AIV antigen was detected in collected cloaca swabs.

## DISCUSSION

In the present paper, we have shown that vaccination of zoo birds with an adjuvanted inactivated H5N2 vaccine (Nobilis influenza® H5N2, Intervet, Boxmeer, the Netherlands) is safe and produces an immune response. In total, 80.5% of the vaccinated birds produced a titre of ≥ 40 after booster vaccination, with a post-vaccination GMT of 190. This was similar to the results obtained after vaccination of birds in zoos with an inactivated H7N1 vaccine produced by the same manufacturer (81.5%, GMT 190)[402]. A booster vaccination was required to produce serum antibody titres in 80.5% of vaccinated birds (50.5% after single vaccination) again similar to H7 vaccination. In our study vaccination was considered to be efficacious when HI titres were ≥ 40 [or 2 log2 (HI titre/10)], this in contrast to published protective titres of > 16 in poultry [181] to account for the unknown interspecies variability, and the impossibility of performing virus challenges in the valuable and endangered species in zoos.

There were no significant differences in antibody titres between dose groups, suggesting that the chosen dose regimen adjusted to mean body weight per species was adequate (table 1). The dose regimen was based on the results obtained after H7 vaccination, where antibody response significantly decreased with an increase in weight. The heaviest taxonomic order (Struthioniformes) had a good immune response to H5 vaccination, with a larger percentage reaching an antibody titre of 40 or higher, and higher GMTs than after H7 vaccination when vaccine dose was up to a five-fold lower. However, as no alternate vaccine dose regimens were tried, this difference between responses to H5 and H7 vaccination cannot be attributed to the used doses with absolute certainty.

Differences in responses between and within taxonomic orders were seen (Appendix 1), as previously reported [405]. In Figure 1 the lowest responses are seen in two groups composed of several taxonomic orders: "others ≤ 1.4 kg" (including orders Charadriiformes, Columbiformes,

Coraciiformes, and Passeriformes) and "others > 1.4 kg" (including orders Gruiformes, Pelecaniformes, and Strigiformes). This was primarily the effect of only three orders that seemed to show a lower antibody response [Pelecaniformes (n=9, %≥40: 55.6%), Passeriformes (n=2, %≥40: 50%), and Columbiformes (n=5, %≥40: 20%), although the numbers per order were very low. Pigeons have previously been described as being resistant, or at least less sensitive, to infection with HPAI virus [391,392], and have failed to produce antibodies after infection [406]. However, higher susceptibility of pigeons to more recent HPAI H5N1 virus strains has been shown [213,407]. Although the order of Galliformes showed favourable overall antibody responses, guinea-fowl, *Numida meleagris,* (n=24) reacted with low GMT (26) and %≥40 (37.5%), despite having been given a vaccine dose of 0.5ml. Guinea-fowl have been documented with clinical disease after infection with HPAI H5N1 virus [408], showing respiratory and gastrointestinal signs. The apparent failure of these species to respond sufficiently to H5 vaccination therefore needs to be studied in further detail.

Antibody titres against the HPAI virus strain circulating in Europe at the time of vaccination showed a similar trend, although generally a 3-fold lower than to the homologous strain, with 61% developing a titre ≥ 40. Post-vaccination HI titres and VN titres against the HPAI field strain showed a degree of agreement of 73.2%. The breadth of the immune response was demonstrated by antibody titres against prototype strains for 4 H5N1 clades. These responses divided the 7 taxonomic orders tested into two groups (group 1; Galliformes, Anseriformes, Phoenicopteriformes, Struthioniformes and Psittaciformes versus group 2; Ciconiiformes, Gruiformes, Pelecaniformes and Sphenisciformes) (Figure 3). ), Group 1 birds showed the broadest immune response with high antibody titers against the prototype strains of all 4 H5 clades and a predicted (by extrapolating the regression line) protective response against future strains up to 12 antigenic units from the current vaccine. In contrast, group 2 birds had low HI antibody titers against the prototype strain of the most antigenically distant clade (A/Indonesia/5/2005).

The longevity of serum antibody titres upon vaccination in these species is unknown. Ducks have been documented with antibodies up to 10 months post-vaccination, and were protected from challenge infection at this time, but the longevity of antibodies in geese was much shorter [186]. In Singapore, a small sub-sample of vaccinated zoo birds showed persistence of serum antibodies when tested 6 months post H5 vaccination [405,405]. Re-vaccination 6-10 months post-vaccination may therefore be required to maintain protective titres among the large variety of avian species in zoos. We recommend further research into the longevity of serum antibody titres upon vaccination in different exotic species.

Pre-vaccination antibody titres to H5 were found in 6 birds, all housed in outdoor enclosures with ponds (4 Anseriformes, 1 Phoenicopteriformes, 1 Sphenisciformes). Titres were low (range 20-60), no virus excretion was found, and the birds were from different zoos, therefore not suggesting acute

infection or outbreak. These titres may therefore be attributed to previous incidental infections with low pathogenic H5 viruses obtained from free-ranging avian species. It appeared that pre-exposed birds also show an increase in titre following vaccination, although the number tested was very small.

The advantages of vaccination are that it reduces the risk of infection (a higher dose is required for infection), and concurrently reduces morbidity, mortality and shedding of virus. Therefore outbreaks are less likely to occur, and easier to contain without the need for large-scale culling or confinement, especially in zoos where levels of bio-security, quarantine measures and veterinary care are high. Additionally, derogations to bio-security measures (such as an alleviation of confinement measures) can be made in vaccinated zoo birds (European Commission Decision 2005/94/EC), provided that such derogations do not endanger disease control. Disadvantages can be "masking" of infection: delaying detection of the virus, and the potential loss of birds during large scale catching and handling. Direct losses experienced during or just after vaccination were low and attributed mainly to injury and stress, while indirect losses (a drop in reproduction rate) were reported for some species. To minimise losses, future vaccination campaigns should ideally be conducted when animals are scheduled to be handled for other reasons, e.g. when moved indoors for winter, which would minimise stress, be at a favourable time relative to the breeding season, and provide enough time to build up immunity before the birds go back outside and bird migration in spring has started.

In conclusion, vaccination with an inactivated H5N2 vaccine is expected to be a useful and necessary component of the preventive measures (including increased bio-security and monitoring) which can be undertaken to protect valuable birds in zoos against H5N1 virus infection, and would obviate the need for large scale culling and confinement in case of an outbreak. The dose regimen used, where dose was adapted to body weight, is generally immunogenic throughout the range of species tested and aimed against a broad variety of H5 AIV strains, although a booster vaccination is necessary to produce high GMT and % of animals with a titre ≥40.

## ACKNOWLEDGEMENTS

Appendix 1: Humoral immune response of avian species in zoos, vaccinated twice (6 weeks interval) with an inactivated H5N2 vaccine with dose adjusted to average body weight. Geometric mean titres (GMT) and the percentage of birds from each species with a post-vaccination serum haemagglutination inhibition (HI) titre of ≥ 40 shown were measured 30 – 60 days after the second vaccination.

| Order | Species | | HI antibody titres | | |
| | Common name | Scientific name | n | GMT | % ≥ 40 |
|---|---|---|---|---|---|
| **Anseriformes** | | | 69 | 192 | 84.1 |
| | Bahama pintail | *Anas bahamensis* | 3 | 96 | 66.7 |
| | bar-headed goose | *Anser indicus* | 1 | 2240 | 100 |
| | barnacle goose | *Branta leucopsis* | 4 | 569 | 100 |
| | black swan | *Cygnus atratus* | 4 | 599 | 100 |
| | black-necked swan | *Cygnus melanocoryphus* | 1 | 1920 | 100 |
| | Canada goose | *Branta canadensis* | 1 | 200 | 100 |
| | cinnamon teal | *Anas cyanoptera* | 2 | 847 | 100 |
| | common shelduck | *Tadorna tadorna* | 9 | 324 | 82 |
| | common shoveler | *Anas clypeata* | 1 | 200 | 100 |
| | coscoroba swan | *Coscoroba coscoroba* | 2 | 1693 | 100 |
| | emperor goose | *Anser canagicus* | 4 | 139 | 100 |
| | Eurasian wigeon | *Anas penelope* | 2 | 40 | 50 |
| | European eider | *Somateria mollissima* | 2 | <10 | 0 |
| | European goosander | *Mergus merganser* | 3 | 80 | 100 |
| | fulvous whistling duck | *Dendrocygna bicolor* | 1 | 120 | 100 |
| | greylag goose | *Anser anser* | 4 | 268 | 100 |

| | | | | |
|---|---|---|---|---|
| Hawaiian goose | *Branda sandvicensis* | 1 | <10 | 0 |
| hooded merganser | *Mergus cucullatus* | 2 | 196 | 100 |
| mallard | *Anas platyrhynchos* | 1 | 280 | 100 |
| Mandarin duck | *Aix galericulata* | 5 | 33 | 60 |
| marbled teal | *Marmaronetta angustirostris* | 1 | 2240 | 100 |
| red-crested pochard | *Netta rufina* | 3 | 183 | 66.7 |
| rosy-billed pochard | *Netta peposaca* | 4 | 260 | 100 |
| smew | *Mergus albellus* | 2 | 160 | 100 |
| trumpeter swan | *Cygnus buccinator* | 2 | 905 | 100 |
| whistling swan | *Cygnus columbianus* | 2 | 14 | 100 |
| wood duck | *Aix sponsa* | 2 | 259 | 100 |
| **Charadriiformes** | | **3** | **306** | **100** |
| pied avocet | *Recurvirostra avosetta* | 3 | 306 | 100 |
| **Ciconiiformes** | | **38** | **119** | **76.3** |
| bald ibis | *Geronticus eremita* | 2 | 2217 | 100 |
| black stork | *Ciconia nigra* | 1 | 70 | 100 |
| black-crowned night heron | *Nycticorax nycticorax* | 8 | 277 | 87.5 |
| black-headed ibis | *Threskiornis melanocephalus* | 3 | 207 | 66.7 |
| glossy ibis | *Plagadis falcinellus* | 2 | 20 | 50 |
| little egret | *Egretta garzetta* | 7 | 89 | 71.4 |
| marabou stork | *Leptopilos crumeniferus,* | 3 | 32 | 66.7 |

| | | | | |
|---|---|---|---|---|
| | puna ibis | *Plegadis ridgwayi* | 10 | 64 | 70 |
| | white stork | *Ciconia ciconia* | 2 | 320 | 100 |
| **Columbiformes** | | | **5** | **14** | **20** |
| | rock dove | *Columba livia* | 4 | 18 | 25 |
| | crested pigeon | *Ocyphaps lophotes* | 1 | <10 | 0 |
| **Coraciiformes** | | | **1** | **560** | **100** |
| | blue-winged kookaburra | *Dacelo leachii* | 1 | 560 | 100 |
| **Galliformes** | | | **92** | **236** | **77.2** |
| | chukar partridge | *Alectoris chukar* | 1 | 200 | 100 |
| | Congo peacock | *Afropavo congensis* | 7 | 874 | 100 |
| | red junglefowl | *Gallus gallus* | 50 | 582 | 92 |
| | helmeted guineafowl | *Numida meleagris* | 24 | 26 | 37.5 |
| | Himalayan monal pheasant | *Lophophorus impeyanus* | 2 | 57 | 50 |
| | Malay great argus | *Argusianus argus* | 1 | 480 | 100 |
| | Palawan peacock pheasant | *Polyplectron emphanum* | 1 | 180 | 100 |
| | Reeve's pheasant | *Syrmaticus reevesi* | 2 | 28 | 50 |
| | wild turkey | *Meleagris gallopavo* | 4 | 1002 | 100 |
| **Gruiformes** | | | **15** | **109** | **80** |
| | black crowned crane | *Balearica pavonina* | 2 | 283 | 100 |
| | common trumpeter | *Psophia crepitans* | 1 | 40 | 100 |
| | Manchurian crane | *Grus japonensis* | 2 | 40 | 50 |
| | sandhill crane | *Grus canadensis* | 2 | 960 | 80 |
| | South African crowned crane | *Balearica regulorum regulorum* | 6 | 283 | 100 |
| | white-naped crane | *Grus vipio* | 2 | 14 | 50 |
| **Passeriformes** | | | **2** | **28** | **50** |
| | raven | *Corvus corax* | 2 | 28 | 50 |

| | | | | | |
|---|---|---|---|---|---|
| **Pelecaniformes** | | | **9** | **37** | **55.6** |
| | dalmatian pelican | *Pelecanus crispus* | 2 | 32 | 50 |
| | Eastern white pelican | *Pelecanus onocrotalus* | 7 | 39 | 57.1 |
| **Phoenicopteriformes** | | | **16** | **332** | **81.3** |
| | Chilean flamingo | *Phoenicopterus chilensis* | 7 | 128 | 57.1 |
| | greater flamingo | *Phoenicopterus roseus* | 9 | 697 | 100 |
| **Psittaciformes** | | | **25** | **1152** | **96** |
| | blue-fronted macaw | *Amazona aestiva* | 7 | 403 | 86 |
| | hyacinth macaw | *Anodorhynchus hyacinthinus* | 2 | 1810 | 100 |
| | Panama amazon | *Ara ochrocephala panamensis* | 1 | 2560 | 100 |
| | scarlet macaw | *Ara macao* | 15 | 1678 | 100 |
| **Sphenisciformes** | | | **35** | **119** | **91.4** |
| | Humboldt penguin | *Sphenicus humboldti* | 32 | 119 | 90.6 |
| | jackass penguin | *Spheniscus demersus* | 3 | 115 | 100 |
| **Strigiformes** | | | **2** | **89** | **50** |
| | snowy owl | *Nyctea scandiaca* | 2 | 89 | 50 |
| **Struthioniformes** | | | **22** | **173** | **81.8** |
| | emu | *Dromaius novaehollandiae* | 1 | 240 | 100 |
| | nandu/rhea | *Rhea Americana* | 16 | 177 | 81.3 |
| | ostrich | *Struthio camelus* | 5 | 151 | 80 |
| **all** | | | **334** | **190** | **80.5** |

# 3.3.

# Longevity of serum antibodies after vaccination against highly pathogenic avian influenza (H5 and H7) in zoos

*Submitted*

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Highly pathogenic avian influenza (HPAI) viruses of the H5 and H7 subtypes are highly contagious viruses that can cause up to 100% mortality in infected poultry. Although HPAI was thought to affect only poultry species, since 2002 a large number of non-domestic bird species have been diagnosed with HPAI infection [409]. HPAI is classified by the World Organisation for Animal Health (OIE) as a notifiable disease, for which the European Union has a non-vaccination policy. Standard measures in the face of a HPAI virus outbreak are long term confinement or large scale pre-emptive culling of birds. However, since this would be detrimental to the welfare and breeding programmes of valuable and endangered avian species in zoos, vccination of zoo birds as an additional preventive measure against HPAI virus infection (while reducing confinement measures) in Belgian, Dutch and German zoos was first allowed during an outbreak of HPAI H7N7 virus in poultry in 2003 (Decision 2003/291/EC). In 2005, Decision 2005/744/EC allowed vaccination in European zoos against the encroaching HPAI H5N1 subtype.

In the present paper we demonstrate serum HI antibody titres of different non-domestic avian species housed in Rotterdam Zoo detected two years after two vaccinations with the H7N1 vaccine and one year after two vaccinations with the H5N2 vaccine, as well as the antibody response to one vaccine dose one year after the first two H5N2 vaccinations.

## MATERIALS AND METHODS

During the 2003 HPAI H7N7 outbreak in poultry, birds in Dutch zoos were vaccinated twice with six weeks interval using a whole inactivated oil-adjuvanted vaccine (Nobilis influenza®, Intervet International, Boxmeer, the Netherlands) based on influenza virus A/chicken/Italy/473/99 (H7N1), with high homology to the field strain HPAI H7N7 A/chicken/Netherlands/1/03 (97.4% nucleotide and 98.7 % amino acid sequence identity). This resulted in the induction of antibody titres ≥40 in 81.5% of the vaccinated birds, with an overall GMT of 190 [402]. Homologous H7 specific antibody titres (using H7N1 A/chicken/Italy/473/99 as antigen) were determined two years later in 48 birds when a new vaccination campaign was started in Dutch zoos against the H5 subtype. These birds were not re-vaccinated with the H7N1 vaccine.

In 2005, birds in Dutch zoos were vaccinated twice with six weeks interval using a whole inactivated adjuvanted H5N2 vaccine (Nobilis influenza® H5N2, Intervet International, Boxmeer, the Netherlands), with vaccine doses adapted to mean body weight per species, using data collected during the H7N1 vaccination campaign. The vaccine strain (A/duck/Pottsdam/1402/86) had a homology of 90% to the HA gene of the H5N1 field strain (A/turkey/Turkey/1/05) on the basis of nucleotide sequence (1530 base pairs, including basic cleavage site), and 92.4% on the basis of amino acids. Vaccination was safe, and proved immunogenic throughout the range of species tested, with some variations between and within taxonomic orders. After booster vaccination the overall homologous GMT to the vaccine strain, was 190, and

80.5% of vaccinated birds developed a titre of ≥ 40. Titres to the HPAI H5N1 virus followed a similar trend, but were lower (GMT: 61; 61%≥ 40) [410]. In both studies there was high agreement between post-vaccination antibody titres determined by serum HI test, and virus neutralisation titres.

One year after the initial two vaccinations, the birds were re-vaccinated with a single dose of the same H5N2 vaccine and dose-weight regimen as the previous year [410], Blood was collected from the right jugular vein or the ulnar vein (left or right) at the time of revaccination and 4 weeks later, using manual restraint. Serum samples from 72 previously vaccinated birds from 8 taxonomic orders could be evaluated 4 weeks after re-vaccination (25 Anseriformes, 12 Ciconiiformes, 4 Galliformes, 6 Gruiformes, 3 Pelecaniformes, 7 Phoenicopteriformes, 11 Sphenisciformes and 3 Struthioniformes). Only 44 of these (15 Anseriformes, 10 Ciconiiformes, 3 Galliformes, 4 Gruiformes, 1 Pelecaniformes, 6 Phoenicopteriformes, 3 Sphenisciformes and 2 Struthioniformes) could be evaluated at all three times of blood collection as some samples were not suitable for testing (e.g. auto-haemolysis, not enough serum, etc)

HI serum antibody titres were determined in laboratory 1[1] for the responses to the first two vaccinations, but one year post-vaccination and 4 weeks after revaccination had to be determined in the national veterinary reference laboratory (laboratory 2[2]), therefore serum samples were split to compare results from both labs. To evaluate the humoral immunologic response to vaccination, homologous and heterologous H5 specific antibody titres (A/duck/Pottsdam/1402/86 in lab 1 and A/Ost/Den/74420/96 in lab 2) were determined by HI test, following standard procedures, using turkey erythrocytes [377,378]. Undetectable titres: <10 (lab 1) and < 4 (lab 2) were regarded as 5 and 2 respectively for calculation of GMT. Antibody titres of 16, 32 and 40 are known to protect domestic ducks and chickens from infection [181,183,186], therefore these are used as correlates of protection, as vaccination/ challenge experiments cannot be carried out in most zoo bird species. Titres considered protective are ≥ 40 (as in previous studies in zoo birds) for the results of lab 1, and ≥ 32 for lab 2, due to the different titration methods used in the 2 labs.

## RESULTS

In all Dutch zoos, two years after H7N1 vaccination, H7 specific serum HI antibody titres from both 2003 and 2005 were available from the same 48 birds (figure 1.). Within this period, titres clearly decreased: while 81% of birds had a positive titre (≥ 10) and 75% a high positive titre (≥ 40) in 2003, these figures were 19% and 10%, respectively, in 2005. The GMTs decreased from 95 in 2003 to 7 in 2005. As these birds were not revaccinated with an H7 vaccine, the effect of revaccination two years after the initial two

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vaccinations is not known.
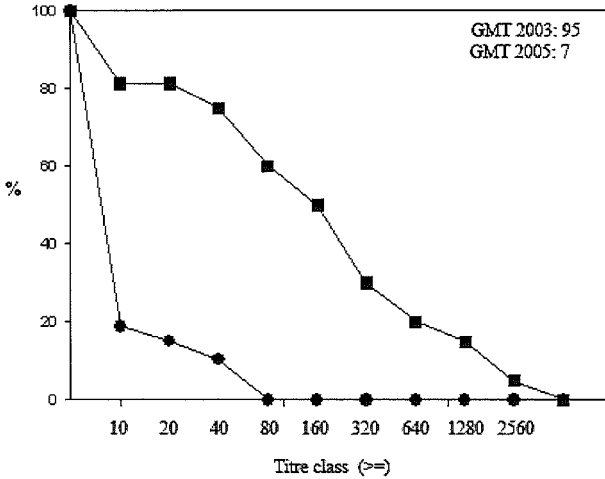


Figure 1. Persistence of H7 specific HI serum antibodies, 2 years after vaccination. Forty-eight birds that were vaccinated with an H7N1 vaccine in 2003 were tested for H7-specific serum HI antibody titres in 2003 and 2005. Within this period, titres clearly decreased: While 81% of birds had a positive titre (≥10) and 75% a high positive titre (≥ 40) in 2003, these figures were 19% and 10%, respectively, in 2005. The GMT decreased from 95 in 2003 to 7 in 2005. Squares depict HI titres 4 weeks after two vaccinations; circles depict HI titres two years later.
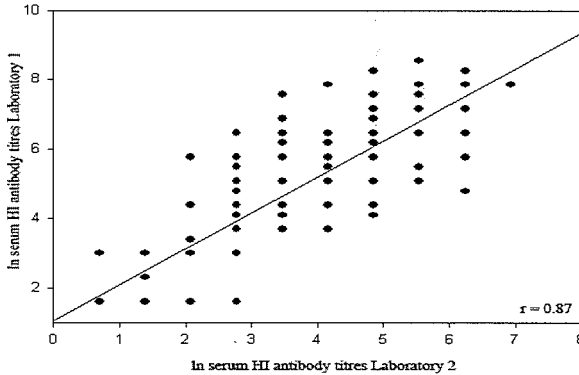


Figure 2. Serum samples from previously vaccinated and unvaccinated birds (n=144) collected at time of re-vaccination (one year after the initial 2 vaccine doses) and 4 weeks later were tested for H5 specific serum HI antibody titres in 2 labs and natural logarithms of these titres were compared. The correlation coefficient is 0.87.

H5 specific serum antibody titres determined in the two labs showed a correlation coefficient of 0.87 (Figure 2.). As antibody titres determined at laboratory 2 had to be reported to the Dutch government, these titres are used in the results from the time of re-vaccination and 4 weeks later.

H5 specific antibody titres had decreased substantially one year after vaccination (n = 62; GMT 10; 28 % ≥ 32), compared to the total GMT 4 weeks after the initial 2 vaccinations (n = 51; GMT: 74; 69% ≥ 40). Four weeks after revaccination with one vaccine dose, H5 specific HI antibody titres had increased to comparable levels of the previous year (n = 72; GMT 59; 74% ≥ 32).

This decline in H5 specific HI serum antibody titres one year after the initial two vaccinations and boost effect by one vaccine dose was reflected in the serum samples from 44 birds, from 8 different taxonomic orders, which could be tested at every time of blood collection (figure 3.). A larger percentage of these 44 birds have a serum HI antibody titre ≥ 32 four weeks after re-vaccination than 4 weeks after the initial two vaccinations, and the GMT after revaccination is lower, but comparable to the GMT after 2 vaccine doses one year before (89 vs 66). The GMT one year after two vaccinations (at the time of revaccination) was 12.
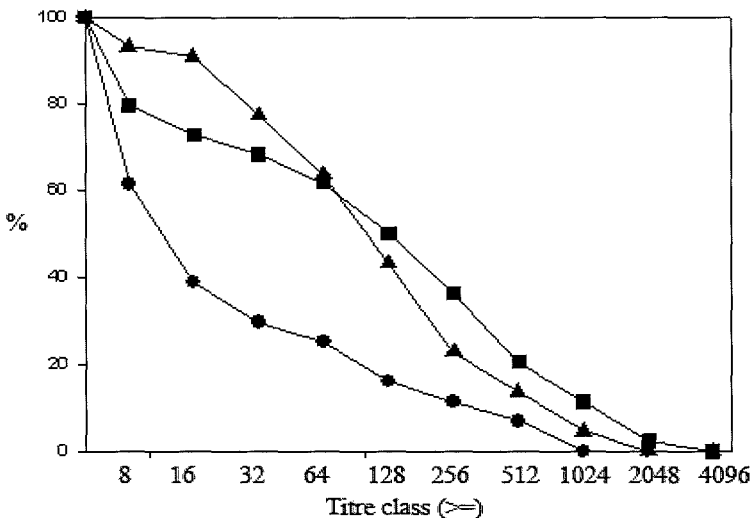


Figure 3. H5 specific serum HI antibody titres 4 weeks after 2 vaccinations in 2005 (squares), at time of re-vaccination one year later (circles), and 4 weeks after re-vaccination (triangles) were available for 44 birds from 8 different taxonomic orders in Rotterdam Zoo. The graph depicts the percentage of birds per serum HI antibody titre class (≥ titre).

As previously reported for the initial 2 vaccine doses [410], there are differences in responses between taxonomic orders. In all orders (except for Phoenicopteriformes), GMTs per taxonomic order had decreased to values <32 one year after vaccination. One vaccine dose boosted antibody levels such that 6 out of 8 taxonomic orders tested had a GMT ≥ 32 four weeks after revaccination (figure 4.). Pelecaniformes and Gruiformes reacted to revaccination with lower antibody titres, as previously reported for the initial two vaccinations, although the number of Pelecaniformes tested was low (n=3).

In conclusion, to maintain high levels of antibodies in birds in zoological collections, the initial vaccination against HPAI (twice, with dose adjusted to mean bodyweight per species) should be repeated one year later with a single dose.
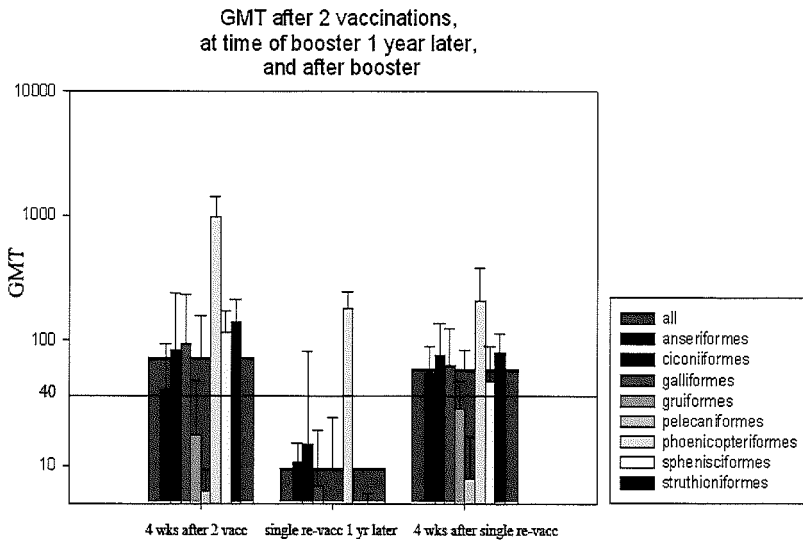


Figure 4. GMTs determined in Rotterdam Zoo, 4 weeks after 2 vaccinations, at the time of re-vaccination one year later, and 4 weeks after re-vaccination. Large bars depict total GMT at times of blood collection (n= 51, 62 and 72 respectively), smaller bars depict GMT per taxonomic order. Vertical lines: 95% CI; Horizontal line: antibody titre 40.

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# 3.4.

Protection of rock pigeons (*Columba livia*)
against highly pathogenic influenza (H5N1)
by an inactivated H5N6 vaccine

*Submitted*

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Chapter 3.4.

The currently ongoing outbreaks of highly pathogenic avian influenza (HPAI) caused by the H5N1 virus subtype is unprecedented in its duration, spread and host range, which includes humans. Feral and domestic rock pigeons (*Columba livia*) are abundant throughout the world and live in close proximity to humans. Consequently their susceptibility to infection with HPAI viruses has been subject of discussion. Experimental intra-tracheal and intra-oesophageal infection with $10^8$ $TCID_{50}$ of HPAI virus of the H5N1 subtype from two different antigenic clades (A/Indonesia/5/05 or A/turkey/Turkey/1/05) induced systemic infection with low morbidity and mortality in rock pigeons. Clinical signs of infected animals were limited to depression, general malaise, and neurological signs (ataxia, tremors, opisthotonus, torticollis). At 3 days post-inoculation (dpi) immunohistochemistry revealed the presence of viral nucleoprotein in lungs, airsac and brain. Virus could be isolated from several organs at 3 dpi, and viral RNA was demonstrated by RT-PCR in several organs at 3 and 27 dpi of animals infected with either of the two viruses. Beyond 4 dpi no virus was isolated from oropharyngeal swabs, and no virus was isolated from cloacal swabs throughout the duration of the experiment. Vaccination of pigeons with a commercial inactivated adjuvanted H5N6 vaccine for poultry was safe and largely limited extra-respiratory infection, prevented development of severe clinical signs and mortality, and limited oropharyngeal virus shedding to 1 dpi, with either of the two virus strains. Thus, vaccination effectively reduced the possible risk posed by pigeons infected with HPAI H5N1 viruses

## INTRODUCTION

Avian influenza viruses (AIV) belong to type A influenza viruses of the Orthomyxoviridae family, and can be classified on the basis of the antigenic properties of their surface glycoproteins haemagglutinin (H) and neuraminidase (N), and on the basis of their pathogenicity for chickens after intravenous inoculation. Currently, 16 H (H1-H16) subtypes and 9 N subtypes (N1-N9) have been identified which are found in many different combinations [151,160]. Aquatic avian species (of the taxonomic orders Anseriformes, Charadriiformes) are considered the main natural reservoir for AIV, and generally experience little if any morbidity and mortality after infection. Highly pathogenic avian influenza (HPAI) viruses are restricted to the H5 and H7 subtypes, and have been isolated mainly from Galliformes (chickens, turkeys, grouse, pheasants and quails) in which they can cause acute generalised disease, of which the mortality in poultry may be as high as 100%. Although outbreaks of HPAI were historically restricted to poultry flocks, in 2002 an outbreak of HPAI virus of the H5N1 subtype caused severe disease in wild migratory birds and resident waterfowl in Hong Kong, with high mortality [163,372]. Since 2002, HPAI viruses of the H5N1 virus subtype have made an unprecedented spread from South-

East Asia throughout Asia and into the middle East, Europe and Africa, with a broadening of host range including a large number of avian species: to date at least 105 species from 14 different taxonomic orders [164] and several mammalian species, including humans.

Rock pigeons (*Columba livia*) are abundant throughout the world, mainly as feral pigeons in cities, kept as pets, and are selectively bred as racing pigeons which fly large distances. Controversy has arisen over the susceptibility of pigeons to AIV infection. Pigeons have previously been described as being resistant, or largely resistant, to infection with HPAI viruses of the H7 and H5 subtypes [391,392,411], and failed to produce antibodies after experimental infection [406]. However, higher susceptibility of pigeons to more recent HPAI virus strains of the H5N1 subtype has been documented [213,372,394,411,412], with neurotropism of the virus shown by antigen detection by means of immunohistochemistry in only the central nervous system. Bird-to-bird transmission has not been documented and sentinel chickens housed with experimentally infected pigeons did not become infected [407]. Infected feral and domestic pigeons would pose a potential threat to poultry, certain mammalian species and humans if HPAI H5N1 virus shedding occurs, since feral pigeons are abundant in cities, and domestic pigeons are kept in large numbers in close contact with humans.

Vaccination is a useful means of reducing the horizontal spread of avian influenza viruses in poultry [179,180], and has proven to be effective in protecting poultry from HPAI under field conditions [181]. However, H5 and H7 vaccination of pigeons and other species of the taxonomic order Columbiformes induced relatively low serum antibody responses compared to other taxonomic orders tested in zoos, suggesting that vaccination would be less efficacious in these species [402,410].

The aim of this study was (1) to determine safety and efficacy of a whole inactivated adjuvanted H5N6 vaccine against infection with one of two strains of HPAI from different antigenic clades, and (2) to determine the tissue tropism, associated disease and virus excretion in pigeons infected with either of these viruses.

## MATERIALS AND METHODS

### Animals and experimental design

The experimental protocol was approved by an independent animal ethics committee, and all experiments were performed under bio-safety level 3 conditions. Forty-eight male and female rock pigeons from the same breeder were housed together in an indoor aviary from the age of approximately 4 weeks onward, and given coloured leg-bands to randomly create 4 groups of 12 birds each. Two groups were vaccinated subcutaneously at the age of 4-5 weeks and 6 weeks later with 0.25 ml of an inactivated adjuvanted H5N6 avian influenza virus vaccine for poultry (Nobilis Influenza® H5N6, Intervet, Boxmeer, the Netherlands). The vaccine strain used (A/duck/

Potsdam/2243/84) had a homology of 88% and 87% to A/Indonesia/5/05 and A/turkey/Turkey/1/05 on the basis of nucleotide sequence of the HA gene (1692 base pairs, excluding the basic cleavage site), and 91.5% and 91.5% on the basis of amino acids respectively. The vaccine was produced and assayed according to the requirements made in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (chapter 2.7.12) and induces at least 6 $\log_2$ haemagglutination inhibition (HI) units according to the potency test in chickens (further details may be obtained from the manufacturer). The control groups were sham-vaccinated with 0.25 ml of phosphate buffered saline (PBS). Three weeks after the second vaccination each group was moved to separate negatively pressurised bio-security level 3 isolators. Four weeks after the second vaccination, two groups (one vaccinated and one sham-vaccinated) were inoculated with A/Indonesia/5/05 (A/Indonesia), and two groups (one vaccinated and one sham-vaccinated) with A/turkey/Turkey/1/05 (A/turkey). Inoculation was performed with allantoic fluid containing 1 x 10$^8$ TCID$_{50}$/ml HPAI H5N1 virus divided over trachea (0.5ml) and oesophagus (0.5ml). At this time transponders for temperature registration were implanted subcutaneously. Records were kept for clinical signs (daily by a qualified veterinarian), temperature (daily) and body weight (every 3 days) until 27 days post-inoculation (dpi).

Blood (0.5-1ml) was collected from the right jugular vein under manual restraint at the times of vaccination, on the day of infection, 14 dpi, and at time of euthanasia (3 dpi or 27 dpi). Blood collection tubes with clot activator and serum separator (MiniCollect®, Greiner Bio-One) were centrifuged, and serum was stored at -70ºC. Cloacal and oropharyngeal samples were collected with cotton swabs and stored at −70°C in transport medium consisting of Hanks' balanced salt solution, 10% vol/vol glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, and 250 µg/ml gentamicin (MP Biomedicals, Zoetermeer, the Netherlands). Swabs were taken at times of vaccination, and daily for two weeks post infection. Hereafter swabs were collected twice a week. Three dpi, 5 pigeons from each group were euthanised by bleeding after isoflurane anaesthesia. Remaining animals were euthanised when moribund, or at 27 dpi. Necropsies were performed on all animals for gross pathology, histopathology, immunohistochemistry and virological examination.

After starting the vaccinations, intercurrent *Salmonella* (*Salmonella enterica,* serovar Typhimurium) and *Trichomonas* infections were diagnosed when all the pigeons were housed together. In spite of immediate start of anti-bacterial and trichomonacidal treatments, three animals were lost due to these infections. Consequently one group of 12, and three groups of 11 pigeons were left for challenge infection. All pigeons were clinically healthy and tested negative to these pathogens at the time of infection.

## Serology

To evaluate humoral immune responses to vaccination and infection, A/duck/Potsdam/2243/84 (vaccine strain), A/turkey and A/Indonesia specific antibody titres were determined in duplicate by haemagglutination inhibition (HI) test, following standard procedures, using turkey erythrocytes [377,378]. Undetectable titres (<10) were regarded as 5 for calculation of geometric mean titres (GMT) with 95% confidence intervals (CI). The percentage of pigeons with a titre ≥ 40 was calculated for comparison with previously published vaccination of species from the taxonomic order Columbiformes in zoos [402,410].

## Viruses

Virus strains representing 2 antigenic variants [404] were used: A/turkey/Turkey/1/05 from H5N1 clade 2, subclade 2; and A/Indonesia/5/05 from H5N1 clade 2, subclade 1 [413]. Stocks were produced by second passage in embryonated chicken eggs, and allantoic fluids were harvested after 3 days. Infectious virus titres were determined as 50% tissue culture infective dose ($TCID_{50}$) in Madin-Darby Canine Kidney (MDCK) cells (ATCC: Product CCL-34 (NBL-2)) as described previously [414].

## Histopathologic and immuno-histochemical examination

Necropsies and tissue sampling were performed according to a standard protocol. Samples were collected from lung, air sac, oesophagus, proventriculus, duodenum, pancreas, jejunum, ileum, caecum, colon, liver, spleen, kidney, adrenal glands, cerebrum, cerebellum, and brain stem. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were cut to a thickness of 4 µm, de-waxed, and stained with hematoxylin and eosin for histological evaluation. Duplicate sections were stained by an immunohistological method using a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody for detection of influenza viral antigen, and an IgG2a isotype-control [415].

## Virological examination

Duplicate samples of the same tissues collected for histopathology and immunohistochemistry were stored at -70°C until virological examination. Tissue samples were weighed and homogenised with a homogeniser (Kinematica Polytron, Lucerne, Switzerland) in 3 ml of infection medium (Eagles Minimal Essential Medium [EMEM], bovine serum albumin [fraction V 7.5%, 1:25], 4 µg/ml trypsin, 2 mM l-glutamin, 100 U/ml penicillin, 100 µg/ml streptomycin, 7.5% $NaHCO_3$, 1 M Hepes). The homogenised solution (200 µl) was then added to 300 µl lysis buffer for RNA isolation, and stored at -70°C.

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) were performed as described previously [380]. RNA was isolated by using a MagnaPure LC system with the MagnaPure LC total nucleic acid

isolation kit (Roche Diagnostics, Almere, the Netherlands), and influenza A virus was detected by using a real-time RT-PCR assay [416]. Amplification and detection were performed with an ABI7700 machine with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) by using 20 μl eluate in an end volume of 50 μL.

Infectious virus titres were determined for all swabs collected, and all influenza A virus RT-PCR–positive homogenised tissue samples. To this end ten-fold serial dilutions were inoculated on MDCK cells in triplicate to determine $TCID_{50}$ per ml or gram tissue as described previously [414].

# RESULTS

## Adverse effects, clinical signs and mortality

None of the birds exhibited general or local adverse effects that could be attributed to vaccination. All birds, regardless of vaccination status showed signs of general malaise after tracheal and oesophageal challenge infection with $10^8$ $TCID_{50}$ HPAI H5N1 virus (A/turkey or A/Indonesia): depression, ruffled plumage, minor increase in body temperature) for 1 dpi. In all groups a minor mean rise in body temperature (up to 0.5°C) was seen during the first 2 dpi, although mean temperatures remained between 42 - 43°C for 27 dpi (results not shown). Fluctuations in mean body weight were not substantially different between the groups during the experiment.

### Sham vaccinated pigeons

One out of 6 sham-vaccinated pigeons inoculated with A/turkey exhibited minor neurological signs (tremors) at 4 dpi and progressive severe neurological signs (torticollis, hemiparalysis of the wings, ataxia, and eventually unable to stand) at 5 dpi, when it was euthanised. Three out of 6 sham-vaccinated pigeons showed minor clinical signs (ruffled feathers, tremors) from 1 dpi until 7 dpi. Inoculation of sham-vaccinated pigeons with A/Indonesia caused no mortality, and low morbidity with minor clinical signs (ruffled feathers, tremors) in 2 out of 7 pigeons from 1 dpi until 7 dpi. At 7 dpi in one of these A/Indonesia inoculated pigeons the clinical signs became severe (opisthotonus/torticollis, ataxia, tremors – worsening with excitement, depression, and ruffled plumage). This pigeon was able to stand and feed -therefore not euthanised- and slowly recovered with remaining occasional tremors, ataxia and opisthotonus until 27 dpi.

### Vaccinated pigeons

Tracheal and oesophageal challenge infection of vaccinated pigeons with $10^8$ $TCID_{50}$ HPAI H5N1 virus (A/turkey or A/Indonesia) resulted in signs of general malaise (depression, ruffled plumage, minor increase in body temperature) for 1 dpi, and no mortality.

## Serology

At the time of challenge infection (0 dpi), vaccinated animals had responded to 2 vaccinations with high serum HI antibody titres and high percentage seroconversion to the homologous H5N6 antigen used in the vaccine (GMTs: 132 and 217; 80% and 100% for A/Indonesia and A/turkey respectively) (Figure 1.), but with low serum HI antibody titres against the heterologous HPAI H5N1 virus strains (GMTs of the A/turkey and A/Indonesia inoculated groups using A/turkey in the HI test: 22 and 11, with 36% and 30% sero-conversion respectively; and GMTs of A/turkey and A/Indonesia inoculated groups using A/Indonesia in the HI test: 5 and 5, with 0% seroconversion). Three dpi, the GMTs were comparable to those in pre-infection samples in all groups, and all but one vaccinated bird had detectable antibody titres to the vaccine antigen, followed at 14 dpi by an increase in GMT, and 100% seroconversion in the vaccinated birds.
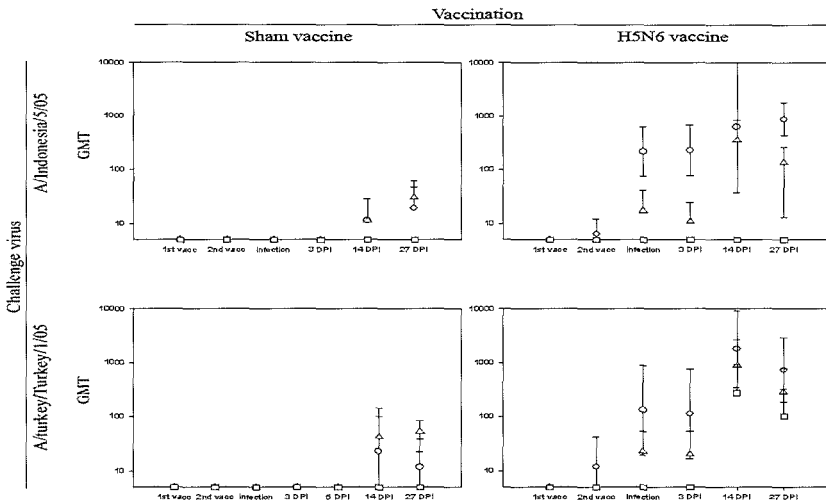


Figure 1. Geometric mean titres (GMT) of vaccinated pigeons from 1st vaccination to 27 days post-inoculation (dpi) with a high dose ($10^8 TCID_{50}$) of either A/Indonesia/5/05 or A/turkey/Turkey/1/05 (H5N1). HI tests were performed with different antigens. Circles: A/duck/Potsdam/2243/84 (vaccine strain); triangles: A/turkey/Turkey/1/05; squares: A/Indonesia/5/05. Lines depict standard deviation.

All sham-vaccinated pigeons were sero-negative at the time of inoculation and three dpi to all three antigens used in the HI test. Inoculation of sham-vaccinated pigeons with A/turkey induced higher GMTs and percentages of sero-conversion at 14 and 27 dpi than A/Indonesia inoculation, using A/turkey (14 dpi: 40% vs 14%; 27 dpi: 80% vs 28%) and the vaccine strain (14 dpi: 40% vs 0%; 27 dpi: 40% vs 14%) as antigens in the HI test. The GMTs were higher against the vaccine strain after inoculation, and no A/Indonesia-

specific antibodies were observed in sham-vaccinated pigeons inoculated with either strain.

## Viral RNA detection (RT-PCR) in swabs

Oropharyngeal swabs from all sham-vaccinated, and 90% of vaccinated birds tested positive by RT-PCR at 1 dpi, regardless of virus strain used for inoculation (figure 2.). Virus load in oropharyngeal swabs declined after 1 dpi, and was detectable up to 5 dpi in vaccinated birds and 9 dpi in sham-vaccinated birds, regardless of virus used for inoculation. No oropharyngeal swabs were positive by RT-PCR after 9 dpi.



Figure 2. Virus detection by means of RT-PCR in oropharyngeal swabs taken from sham- vaccinated and vaccinated pigeons, after intra-tracheal and intra-oesophageal infection with $10^8 TCID_{50}$ of either A/Indonesia/5/05 or A/turkey/Turkey/1/05 (H5N1). Swabs were taken up to 27 days post infection, but were all negative after day 9. Bars represent the geometric mean virus detection (40-Ct value), error bars indicate standard deviation, and % represents the percentage of RT-PCR positive animals per group. To calculate geometric means, 0.1 was used as 40-Ct for the negative samples.

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Cloacal swabs of sham-vaccinated birds were positive by RT-PCR for up to 11 and 9 dpi after A/Indonesia and A/turkey inoculation respectively. Geometric mean values of virus detection (40-Ct value) were low ($\leq 1$) on each day with positive viral RNA detection (on 2, 6, 8, 9 and 11 dpi for A/Indonesia, and on 8 and 9 dpi for A/turkey). Vaccinated birds had low RT-PCR positive cloacal swabs (geometric means $\leq 1$ on each day) only on 2 DPI after A/Indonesia, and on 2 and 9 DPI after A/turkey inoculation.
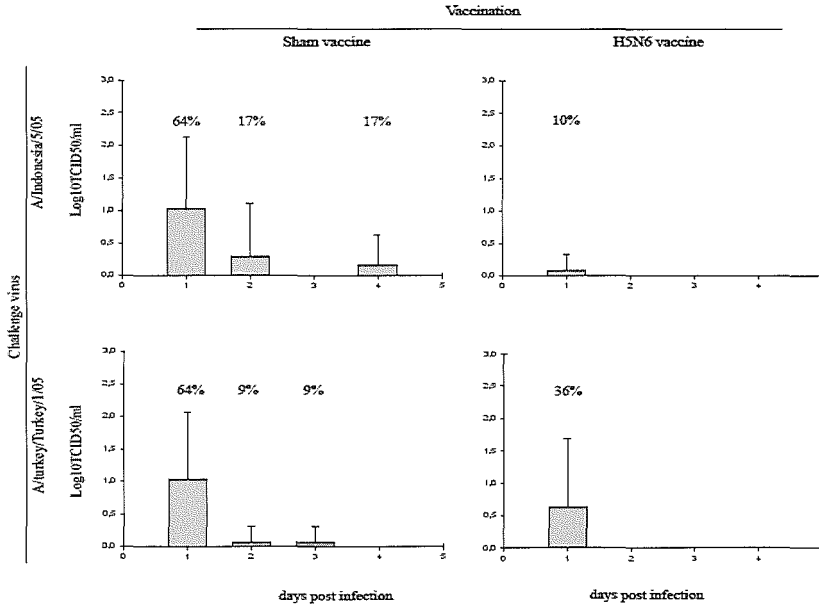


Figure 3. Geometric mean virus isolation from oropharyngeal swabs taken from sham-vaccinated and vaccinated pigeons, after intra-tracheal and intra-oesophageal infection with $10^8 TCID_{50}$ of either A/Indonesia/5/05 or A/turkey/Turkey/1/05 (H5N1). Swabs were taken up to 27 days post infection, but were all negative after day 4. Cloacal swabs were taken during the same period, but no virus could be isolated and results are therefore not shown. Bars represent virus titre: geometric means of Log10TCID50/ml, error bars represent the standard deviation, and % represents the percentage of virus isolation positive animals per group.

## Virus isolation from swabs

Virus was isolated from the oropharynx of the majority of birds from all groups at 1 dpi (figure 3.). Virus was isolated at low titres from a minority of sham-vaccinated birds up to 3 dpi or 4 dpi (A/turkey and A/Indonesia respectively). No virus was isolated from vaccinated birds after 1 dpi. No virus was isolated from any cloacal swabs at any time after inoculation, regardless of vaccination status.

## Viral RNA detection by RT-PCR in organs

At 3 dpi, viral RNA was detected by RT-PCR in all organs tested (brain, lung air sac, pancreas, spleen, liver and duodenum) from sham-vaccinated animals. Vaccinated animals inoculated with A/Indonesia (figure 4.) tested positive in all organs by RT-PCR except brain and duodenum, those inoculated with A/turkey (figure 5.) tested positive in all organs except pancreas. Geometric mean viral RNA load (represented as 40-Ct value) per organ, and the percentage of positive animals per group were generally lower for the vaccinated groups. In one sham-vaccinated A/turkey inoculated pigeon euthanised at 5 dpi high viral RNA loads were detected in all organs except pancreas and duodenum. At 27 dpi, viral RNA was detected in lungs from all groups, regardless of vaccination status, although geometric mean titres and % positive birds was lower for the vaccinated, A/turkey inoculated group. The only other organ in which viral RNA was detected by RT-PCR at 27 dpi was brain for sham-vaccinated animals, and liver for vaccinated birds after A/turkey inoculation.

## Virus Isolation and quantification from organs

Virus could be isolated from several organs in sham-vaccinated birds at 3 dpi (figure 6.). Virus could only be isolated from the lungs of vaccinated birds at 3 dpi. For the lungs, the percentage of birds positive by virus isolation, and geometric mean $TCID_{50}$ per gram lung tissue was lower in the vaccinated groups than in the sham-vaccinated groups (A/turkey: 20% vs 60%; geometric mean $TCID_{50}$/g: $10^{0.18}$ vs$10^{0.92}$; A/Indonesia: 80% vs 80%; geometric mean $TCID_{50}$/g:$10^{1.57}$ vs $10^{1.86)}$. The highest virus titres ($TCID_{50}$/g tissue) isolated from sham-vaccinated pigeons at 3 dpi was $10^{7.0}$ (air sac, A/turkey infection) and $10^{4.3}$ (lung, A/Indonesia infection). The highest virus titres isolated from vaccinated pigeons at 3 DPI was$10^{3.9}$ (lung, A/Indonesia infection) and $10^{2.0}$ (lung, A/turkey infection). The one sham-vaccinated pigeon infected with A/turkey that was euthanised due to severe neurological signs at 5 dpi was positive by virus isolation in the brain ($10^{6.46}$ $TCID_{50}$/g) and air sac ($10^{3.66}$ $TCID_{50}$/g). No virus was isolated at 27 dpi from any organs of any of the pigeons, regardless of vaccination status.
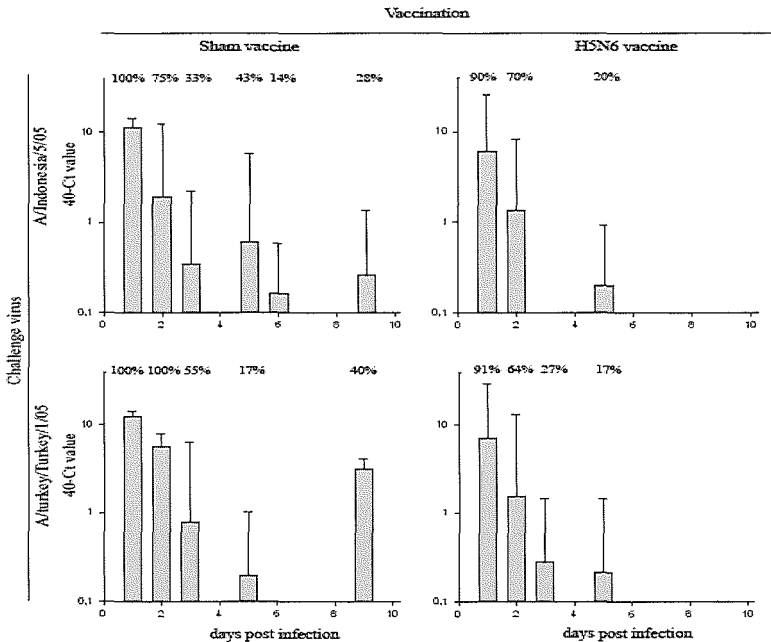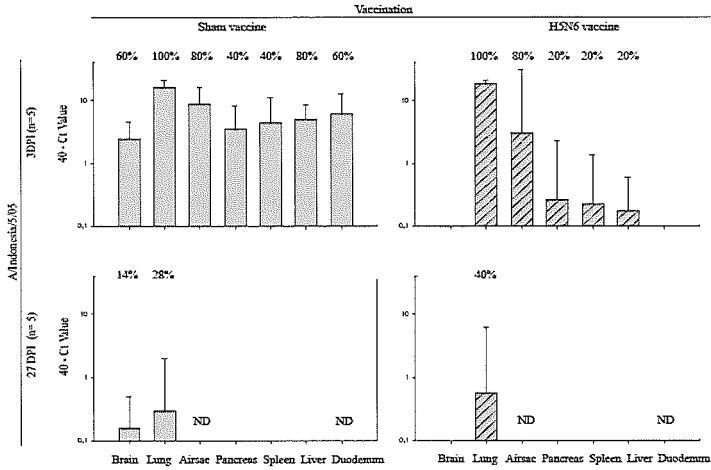
Figure 4. Virus detection by means of RT-PCR in organs taken from sham-vaccinated and vaccinated pigeons: 3 and 27 days after tracheal and intra-oesophageal inoculation with $10^8 TCID_{50}$ of A/Indonesia/5/05 (H5N1). Bars represent geometric mean of 40-Ct values, error bars represent the standard deviation, and % represents the percentage of RT-PCR positive animals per group.
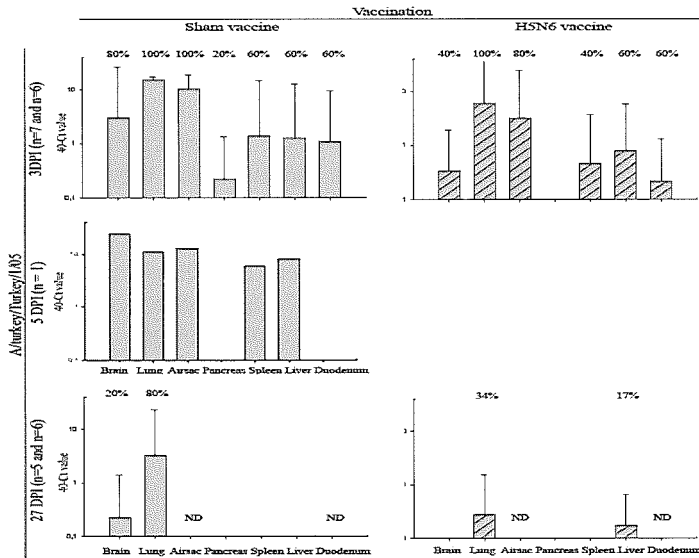


Figure 5. Geometric mean virus detection by means of RT-PCR in organs taken from sham-vaccinated and vaccinated pigeons: 3, 5 and 27 days after tracheal and intra-oesophageal inoculation with $10^8 TCID_{50}$ of A/turkey/Turkey/1/05 (H5N1). Bars represent geometric mean of 40-Ct values, error bars represent the standard deviation, and % represents the percentage of RT-PCR positive animals per group.
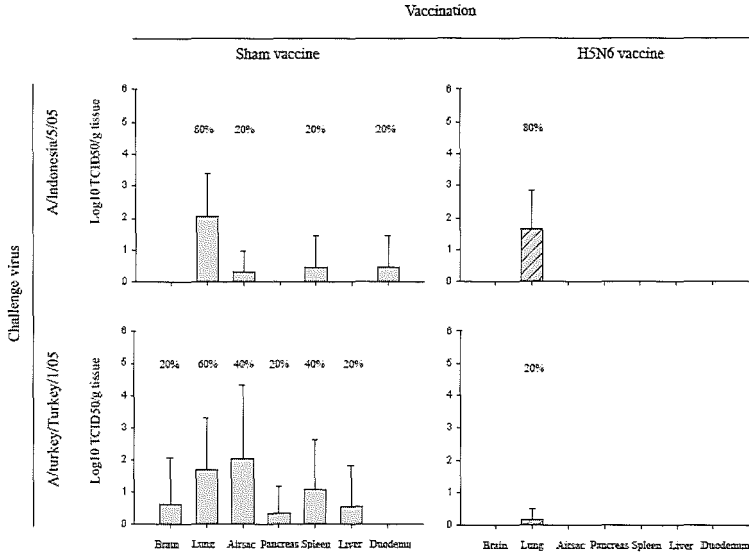
Figure 6. Virus isolation ($TCID_{50}$/g tissue) from organs taken from sham-vaccinated, and vaccinated pigeons, 3 days after intra-tracheal and intra-oesophageal inoculation with a high dose of either A/Indonesia/5/05 or A/turkey/Turkey/1/05 (H5N1). Bars represent geometric mean $log_{10}TCID_{50}$/g tissue, error bars represent the standard deviation, and % represents the percentage of virus isolation positive animals per group.
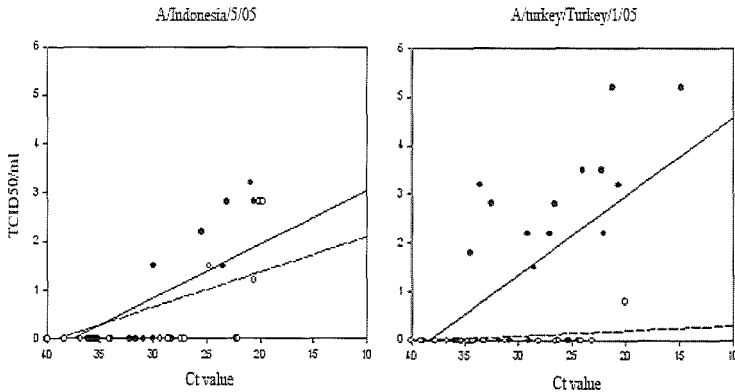


Figure 7. Vaccination reduces viability of highly pathogenic avian influenza virus in organs of infected pigeons. Graphs depict virus titre ($log_{10}TCID_{50}$/g) vs PCR virus detection (Ct value) in organs taken from sham-vaccinated (n=5) and vaccinated pigeons (n=5), 3 days after intra-tracheal and intra-oesophageal inoculation with $10^8TCID_{50}$ of either A/Indonesia/5/05 or A/turkey/Turkey/1/05 (H5N1). Black circles and solid line represent values and regression line for sham-vaccinated animals, white circles and dotted line represent values and regression line for vaccinated animals.

## Gross Pathology

All animals (vaccinated and sham-vaccinated) necropsied at 3 dpi showed consolidation of the ventrocaudal left lung, of varying extent (10 to 30% of lung volume). No changes were seen in the right lung. Opaque air sacs were seen in sham-vaccinated (A/Indonesia: 40%, A/turkey: 20%) and vaccinated (A/turkey: 40%) pigeons at 3 dpi. Splenomegaly was seen in sham-vaccinated and vaccinated pigeons after A/Indonesia infection (40% and 40% at 3 dpi, and 71 and 80% at 27 dpi respectively. No further gross lesions were seen at 27 dpi. One sham-vaccinated, A/turkey infected pigeon necropsied at 5 dpi had subcutaneous oedema of the head and neck, consolidation of the left lung, and demarcated brittle grey liver (approximately 90% of the surface area).

## Immunohistochemistry

Lung and air sacs were the only organs in which nucleoprotein was detected by means of immunohistochemistry (IHC) at 3 dpi (Table 1.). Immunohistochemistry positive cells were found mainly in the parabronchi, and were more widely distributed throughout lungs of sham-vaccinated birds infected with A/Indonesia compared to vaccinated birds. No difference in distribution was seen between lungs of vaccinated and sham-vaccinated birds infected with A/turkey. At 5 dpi, nucleoprotein was detected in the cerebellum of one pigeon that was euthanised with severe neurological signs, but no nucleoprotein was detected in any other organs. No nucleoprotein was detected by IHC in organs from any groups at 27 dpi.

## Histopathology

At 3 dpi a consistent histopathological finding in both the A/Indonesia and A/turkey infected pigeons was a bronchopneumonia with mononuclear infiltrates and (peri-) vasculitis. There were no remarkable differences in severity of bronchopneumonia between the vaccinated and sham-vaccinated groups infected with A/turkey, but vaccinated A/Indonesia infected pigeons were affected to a lesser extent than sham-vaccinated pigeons. A meningo-encephalitis with peri-vascular cuffing, gliosis and neuronal necrosis was seen in brains of 3/5 sham-vaccinated, A/turkey infected pigeons, but not in vaccinated animals. No histopathological changes were seen in the brains of A/Indonesia infected pigeons, regardless of vaccination status.

At 27 dpi histopathological findings were far less severe, and no differences in severity were observed between vaccinated and sham-vaccinated groups. In the A/turkey infected group, meningo-encephalitis was seen in one sham-vaccinated and one vaccinated pigeon, with gliosis and peri-vascular cuffing respectively. In the A/Indonesia infected group, one sham-vaccinated pigeon showed meningo-encephalitis with peri-vascular cuffing and gliosis.

| | Cerebrum | Cerebellum | Lung | Air sac |
|---|---|---|---|---|
| Sham-vaccinated A/Indonesia/5/05 3 dpi | | | | |
| Pigeon 1 | - | - | ++ | - |
| Pigeon 2 | - | - | ++ | + |
| Pigeon 3 | - | - | ++ | + |
| Pigeon 4 | - | - | ++ | |
| Pigeon 5 | - | - | +++ | + |
| Vaccinated A/Indonesia/5/05 3 dpi | | | | |
| Pigeon 1 | - | - | - | - |
| Pigeon 2 | - | - | - | - |
| Pigeon 3 | - | - | ++ | - |
| Pigeon 4 | - | - | + | - |
| Pigeon 5 | - | - | + | - |
| Sham-vaccinated A/turkey/Turkey/1/05 3 dpi | | | | |
| Pigeon 1 | - | - | - | - |
| Pigeon 2 | - | - | - | ++ |
| Pigeon 3 | - | - | - | - |
| Pigeon 4 | - | - | + | - |
| Pigeon 5 | - | - | + | - |
| Sham-vaccinated A/turkey/Turkey/1/05 5 dpi ** | | | | |
| Pigeon 1 | ++ | - | - | + |
| Vaccinated A/turkey/Turkey/1/05 3 dpi | | | | |
| Pigeon 1 | - | - | - | - |
| Pigeon 2 | - | - | + | - |
| Pigeon 3 | - | - | + | - |
| Pigeon 4 | - | - | - | - |
| Pigeon 5 | - | - | | - |

Table 1. Average distribution* of Influenza A nucleoprotein, as determined by immunohistochemistry in (non-) vaccinated pigeons, 3 or 5 days after intra-tracheal and intra-oesophageal infection with $10^8$TCID50 of either A/Indonesia/5/05 or A/turkey/Turkey/1/05 (H5N1). No nucleoprotein was detected in other organs tested 3 dpi (pancreas, spleen, liver, kidney, oesophagus, proventriculus, duodenum, jejunum, ileum, caecum, colon, testis, ovary, adrenal gland), or in any organ 27 dpi.*- = none, + = infrequent; ++ = common; +++ = widespread.** This pigeon was euthanised because of severe clinical signs

## DISCUSSION

In the present paper we have shown that simultaneous intra-tracheal and intra-oesophageal infection with an extremely high dose of HPAI virus of the H5N1 subtype (A/Indonesia/5/05 or A/turkey/Turkey/1/05) induces systemic infection in pigeons, with low morbidity and mortality. Clinical signs of infected animals were depression, general malaise, and neurological signs (ataxia, tremors, opisthotonus, torticollis). Severe clinical signs were seen in one out of six pigeons after A/turkey -, and one out of seven pigeons after A/Indonesia inoculation, and started at 4 dpi and 7 dpi respectively. The systemic infection was demonstrated by the presence of nucleoprotein by IHC in the lungs, air sacs, and brain, and histopathological changes in the lungs and brains.

Virus was detected by means of RT-PCR (brain, lung, air sac, pancreas, spleen, liver and duodenum), and virus could be isolated from several organs at 3 dpi (A/Indonesia: lung, air sac, spleen, duodenum; A/turkey: brain, lung, air sac, pancreas, spleen, and liver). Immunohistochemistry was less sensitive (brain, lung and airsac were positive) than RT-PCR or virus isolation to measure influenza virus infection, as IHC detects viral antigen in cells – and thus requires replication of virus, whereas virus isolation measures infectious virus, and RT-PCR detects presence of viral RNA.

Our findings of IHC positive cells of the parabronchi in the lungs, the air sacs and brain, and the (transient) bronchopneumonia induced by A/turkey and A/Indonesia inoculation suggest that primary replication took place deep in the lungs and air sacs. The parabronchi, which are closely surrounded by blood capillaries and are involved in gaseous exchange, probably allow for passage of virus into the bloodstream, and subsequent haematogenic spread to the brain. After penetration of the blood-brain barrier, further replication can occur in glial cells and neurons, as described in chicks inoculated via the air sac route [417]. Neurotropism of HPAI virus (H5N1) in pigeons has previously been described [407] without IHC positive cells in the respiratory tract, although these pigeons were euthanised later than 3 dpi, and route of inoculation and virus strain used were different. Whereas endothelial tropism appears to play an important role in the pathogenesis of HPAI in chickens, and death at 1-2 dpi is associated with lung oedema, congestion, and the presence of viral antigen predominantly in vascular endothelial cells, but also parenchyma of multiple organs [408,418,419], no viral antigen was detected in endothelial or parenchymal cells of H5N1 infected pigeons. The virus isolation described in several organs in the present study may thus reflect the presence of virus in the blood of organs other than the lungs and brains during the viraemic phase. The difference in cell-tropism and pathogenesis of H5N1 virus infection in pigeons compared to e.g. chickens may explain the lower susceptibility of this species.

The presence of infectious virus in the oropharynx was observed in a number of sham-vaccinated pigeons (A/turkey: 64%; A/Indonesia: 58%) at

1 dpi, and continued for 3 and 4 dpi after A/turkey and A/Indonesia infection respectively. The maximum titre isolated at 1 dpi was $1 \times 10^{3.2}$ TCID$_{50}$/ml, and it cannot be excluded that this was residual virus from the inoculation ($1 \times 10^8$ TCID$_{50}$/ml), and does therefore not confirm virus replication on the first day. Oropharyngeal swabs were positive by RT-PCR in sham-vaccinated birds until 11 dpi and 9 dpi after A/Indonesia and A/turkey infection respectively. Cloacal swabs were positive in a small number of pigeons by RT-PCR, with low individual levels and geometric means, and a small peak in sham-vaccinated birds on 8 and 9 dpi. No infectious virus was isolated from cloacal swabs, regardless of vaccination status. Virus excretion predominantly from the respiratory tract after HPAI H5N1 virus infection has been previously described in various species of ducks [162,420] in contrast to viral shedding in ducks upon LPAI virus infection, which occurs mainly from the gastrointestinal tract [159]. Previous attempts to isolate virus from oropharyngeal swabs taken from pigeons after experimental HPAI virus (H5N1: A/chicken/HongKong/220/97) have not been successful [391].

Vaccination of pigeons with the inactivated, adjuvanted H5N6 vaccine (Nobilis H5N6, Intervet, Boxmeer, the Netherlands) was safe, and produced protection against severe clinical disease after inoculation with viruses from 2 different antigenic clades. Furthermore it induced complete protection against the presence of extra-respiratory detectable infectious virus (Figure 6), with IHC positive cells and virus isolation at 3 DPI from only the lungs, and it largely reduced the systemic spread of virus as detected by RT-PCR. Although geometric mean virus detection by RT-PCR (40-Ct value) 3 dpi was higher in some organs of the vaccinated groups compared to the sham-vaccinated groups (A/Indonesia infection: lung: 18.4 vs 15.2; air sac: 3.1 vs 2.5; A/turkey infection: lung 19.7 vs 15.5), the percentage of birds from which virus could be isolated from the lungs and geometric mean virus titres were much lower in vaccinated birds than in sham-vaccinated birds.

Protection against systemic infection appeared to be more effective against the A/turkey strain than against the A/Indonesia strain. Although virus was detected by RT-PCR in organs of some vaccinated birds inoculated with A/Indonesia, the level of correlation with virus isolation from these organs was much lower than that in birds infected with A/turkey (figure 7). At the time of inoculation, vaccinated birds had high GMTs and percentage seroconversion measured in the HI test, especially compared to serum HI antibody titres induced in Columbiformes after H5N2 vaccination [410]. Vaccination induced highest GMTs against the homologous vaccine virus strain, and high titres against A/turkey (figure 1.). In sham-vaccinated birds GMTs induced by infection were much lower than those induced by vaccination, and highest against A/turkey. An explanation for the low correlation between virus isolation and virus load by RT-PCR is that antibodies induced by vaccination effectively neutralised virus, while neutralised virus could still be detected by means of RT-PCR (figure 7). However, the absence of high titres of virus specific antibodies does not mean that pigeons would not be protected from clinical disease. Two

weeks post inoculation with A/turkey, only 40% of sham-vaccinated pigeons had a virus specific antibody titre while morbidity was low, and all but one survived inoculation with a high dose of virus. None of the sham-vaccinated birds inoculated with A/Indonesia produced virus specific antibodies, while all of these birds survived, and clinical signs were seen in two out of seven specimens. Experimental infections of ducks with recent H5N1 strains have failed to induce antibody titres detectable by HI test in some infected birds [420].

Antibodies specific to A/Indonesia were absent in all groups throughout the duration of the experiment, except for 1 vaccinated bird at 14 DPI after A/turkey inoculation (antibody titre: 1620), and one more from the same group at 27 dpi (antibody titres: 560 and 30). At 14 and 27 dpi the serum antibody titre of these birds determined by HI test using vaccine strain or A/turkey were much higher (7680 or 640 and 1280 or 140 respectively). It has previously been reported that vaccination of certain taxonomic orders of birds with an inactivated adjuvanted H5N2 vaccine induces low HI antibody titres against the A/Indonesia virus strain [410]. Additionally, an asymmetry in antibody recognition pattern, with low A/Indonesia-specific antibodies detectable by HI test compared to other virus strains used, was observed in mice after MVA-HA vaccination and in ferrets after infection with the original influenza viruses [413,421].

It may be concluded that on the basis of the histopathological data, the H5N6 vaccine did not prove to be protective against the (transient) bronchopneumonia induced by A/turkey and A/Indonesia infection. However, some protective effect was seen in brains of vaccinated, A/turkey inoculated pigeons at 3 dpi. Surprisingly, at 3 dpi histopathological signs of meningo-encephalitis were seen in sham-vaccinated, A/turkey inoculated pigeons that did not show severe neurological signs. At 3 dpi no histopathological signs of meningo-encephalitis were seen in A/Indonesia inoculated pigeons, regardless of vaccination status. Sham-vaccinated pigeons that exhibited severe clinical neurological signs had demonstrable meningo-encephalitis at 5 dpi (A/turkey infection) and 27 dpi (A/Indonesia infection). Infection of the brain with A/Indonesia may occur later than with A/turkey (severe neurological signs after A/Indonesia inoculation became apparent at 7 dpi, compared to 4 dpi after A/turkey inoculation) and could explain the histopathological and immuno-histochemical differences seen between these virus strains at 3 dpi.

Virus was isolated from the oropharynx in a limited number of vaccinated pigeons (A/Indonesia: 10%; A/turkey: 36%) for 1 dpi (Figure 3). Oropharyngeal swabs were positive by RT-PCR in vaccinated birds up to 5 dpi (A/Indonesia infection), compared to 11 dpi in sham-vaccinated pigeons. Virus detected and isolated at 1 dpi (maximum virus titre isolated: $10^{3.2}$ $TCID_{50}$/ml) may be residual virus from the inoculation one day prior to swab collection (inoculation dose was $10^8$ $TCID_{50}$/ml), and does not confirm replication on the first day. Cloacal swabs were positive in a small number of vaccinated pigeons by RT-PCR at two dpi (A/turkey and A/Indonesia) and 9 dpi (A/Indonesia), although

viral loads were extremely low. No infectious virus was isolated from cloacal swabs, regardless of vaccination status. Vaccination thus effectively reduces the threat of H5N1-infected pigeons as a source of virus by excretion.

Consolidation of only the caudo-ventral left lung in all inoculated pigeons at 3 dpi can be attributed to the mode of inoculation, as birds in the isolator at the time of inoculation were held in a left-lateral position – and therefore the virus inoculum was deposited in the left lung.

Although previous studies have shown the low susceptibility of pigeons to HPAI H5N1 virus infection, with 21% mortality after infection with a strain from 2003 [407], morbidity and mortality in the present study are even lower, using the same virus doses for challenge. A possible immune-modulating effect of the *Salmonella* and *Trichomonas* infection prior to HPAI virus challenge in the present study cannot be excluded. Several bacteria species and cholera toxin can activate macrophages non-specifically and enhance cytokine production, causing subsequent enhancement of the adaptive immune responses against influenza viruses in mice [422-424]. The effect of *Salmonella* and *Trichomonas* infection on the innate immunity of pigeons against influenza viruses has not been documented.

In conclusion, although the susceptibility of pigeons to high dose challenge with these two HPAI H5N1 virus strains is relatively low, with low morbidity and mortality, virus spreads systemically to different organs - virus was isolated from organs up to 5 dpi, and limited shedding of virus via the respiratory route takes place for up to 4 days. Vaccination is safe, and prevents severe clinical signs and mortality, limits extra-respiratory infection of both virus strains, provides partial protection against lung infection and disease (after A/Indonesia infection), and limits virus shedding to 1 dpi, although the presence of infectious virus in lung and air sacs at 3 dpi could still pose a risk to predators and scavengers.

## ACKNOWLEDGEMENTS
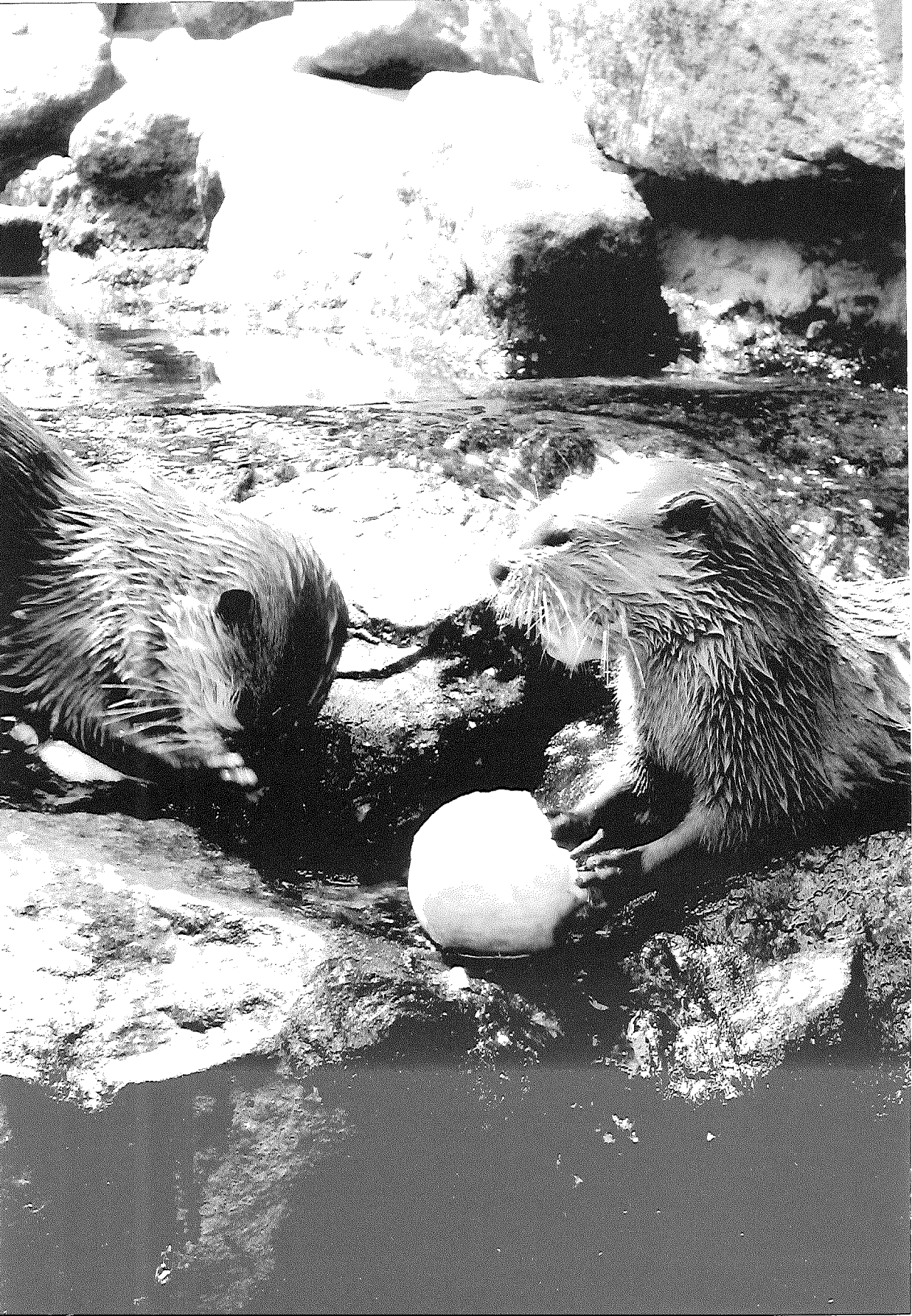
# 4.

# Summarising Discussion

In part adapted from:

*Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related with the vaccination against avian influenza of H5 and H7 subtypes as a preventive measure carried out in Member States in birds kept in zoos under Community approved programmes. The EFSA Journal (2007)* [200] *(first draft prepared by J. Philippa, in close collaboration with J.Pujols Romeu, W. Schaftenaar, M. Hartmann, R. Fouchier, E. Sos, O.Ribó, H.Fernandez and A. Osterhaus).*

Infectious diseases can have great impact on free-ranging and captive populations of non-domestic animals. Our understanding of the prevalence of infectious diseases in free-ranging animals is limited. Early detection by laboratory and syndromic surveillance plays a major role in managing outbreaks of such infectious diseases. Surveillance for certain viruses, especially those with zoonotic potential like avian influenza viruses, is currently being conducted on a large scale in free-ranging species. From these studies we have learned that free-ranging birds play a crucial role in the persistence of avian influenza and that a large number of avian and mammalian species are susceptible to infection with these viruses. Preventive vaccination is one of the tools that can be used to combat infections in individual animals and large scale outbreaks. However, little is known about the efficacy of vaccines, which have been produced for domestic animals, in non-domestic species. This thesis focuses on the efficacy of preventive vaccination of captive non-domestic species against two groups of viruses which have caused large outbreaks in free-ranging and captive animal populations: morbilliviruses and highly pathogenic avian influenza (HPAI) viruses.

## Serologic surveys for antibodies to selected pathogens in free-ranging species

Since the 1980's, emerging and re-emerging infectious diseases have made an enormous impact on public and animal health. Although emergence and re-emergence of infectious diseases can in part be attributed to improved interest or attention and diagnostic methods, the foremost cause should be sought in changes in human behaviour. These changes in human behaviour have resulted in, amongst others, a shrinking or fragmented wildlife habitat which has changed population numbers and in some areas has caused increased population densities. An increased population density can result in increased inter- and intra-species contacts and exposure to new pathogens, dependent on contact rates and pathogen transmission. The ability of a pathogen to infect multiple hosts, including hosts in other taxonomic orders, poses a direct threat of the "spill-over" of infectious agents from reservoir (domestic and non-domestic) animal populations to other susceptible species. The translocation of wildlife for rehabilitation, conservation, agriculture, or hunting can bring an additional inherent risk of exposure of free-ranging species to new infectious agents by connecting populations that were previously separated.

Early detection of outbreaks, by improved surveillance in wild and domestic animals for (zoonotic) pathogens is critical for the management of emerging infections [10,11,16]. Improved surveillance for infectious diseases in animals can thus aid in disease management of both human and (free-ranging) animal populations. There are many examples of projects in which free-ranging animals are trapped or otherwise caught for reasons other than serum collection, and additional collection and storage of serum samples from

these animals can prove to be of use in identification of causative agents of outbreaks, or retrospective epidemiological studies. Serologic surveys can provide information on the prevalence of viral infections in species of free-ranging non-domestic animals. Although the resulting morbidity or mortality caused by these infections is often not known, these surveys may help to guide further studies on the impact of infectious diseases on free-ranging populations, and provide information to (future) rehabilitation and translocation projects.

Such a collection of stored sera or whole blood samples, collected from several species of monitored or hunted free-ranging terrestrial carnivores and marine mammals in Canada, were used to evaluate the prevalence of antibodies to selected pathogens in these species (Chapter 2.2.1.). Between 1984 and 2000, samples were taken at several locations in Canada from cougars (*Felis concolor*), lynxes (*Felis lynx*), American badgers (*Taxidea taxus*), fishers (*Martes pennanti*), wolverines (*Gulo gulo*), wolves (*Canis lupus*), black bears (*Ursus americanus*), grizzly bears (*Ursus arctos*), polar bears (*Ursus maritimus*), walruses (*Odobenus rosmarus*), and belugas (*Delphinapterus leucas*). We documented the presence of antibodies against a number of viruses in species in which these infections have not been reported before: antibodies to CAV in walruses, to PDV in black bears, grizzly bears, polar bears, lynxes, and wolves, to DMV in grizzly bears, polar bears, walruses and wolves, to CPIV in black bears and fishers, and to DRV in belugas and walruses. We found regional differences in the prevalences of the different pathogens in some of the species tested. These may be related to differences in population densities of the species tested, or in the contact rate with other species (including domestic species) that may act as sources of infection. For example, the wolverines were sampled in very remote areas, where less exposure to sources of infection may be a possible explanation for the absence of antibodies to any of the pathogens tested for. In several species the antibody response was directed at more than one of the morbilliviruses tested for (CDV, PDV, and DMV). The presence of antibodies to the two morbilliviruses which are usually associated with marine mammals (PDV and DMV) in terrestrial species was surprising. All the morbilliviruses exhibit cross-reactivity in serological assays, but titres are highest against the homologous virus [269]. Titres indicated infection of some of the terrestrial species with not only CDV, but also PDV and DMV.

Another use of serum banks was to study the potential risk of viral disease on a small population of endangered European mink (*Mustela lutreola*), in an area where future re-introduction of this species may be performed (Chapter 2.2.2.). The European mink, a small semi-aquatic mustelid, has withdrawn dramatically from its former territory during the last century and is currently listed as endangered by the IUCN. Reasons implicated for the decline include excessive trapping, change or loss of habitat, interspecies competition by the larger American mink (*Mustela vison*), and mortality from infectious diseases. Breeding programmes have been set up in Estonia and

Spain with the intention of releasing European mink into its former territory, to aid in the conservation of this species. In order to investigate the possible role of selected pathogens in the decline of the endangered European mink, and the risk of infection of captive-bred European mink to be released in the area, a serologic survey was conducted. Sera were collected from 481 free-ranging individual animals of five mustelid species trapped between March 1996 and 2003 in monitoring projects or pest-control programmes in eight departments of South-western France. Antibodies to CDV were detected in all species, i.e., in 8.7% of 127 European mink, 20% of 210 polecats (*Mustela putorius*), 5% of 112 American mink, 33% of 21 stone marten (*Martes foina*) and 5% of 20 pine marten (*Martes martes*). Prevalences were significantly higher in stone marten and polecats, possibly in relation with their habitat which is in closer proximity to humans (and therefore domestic carnivores which may have acted as a source of the virus) than the other species tested. Antibodies to CAV were detected in all species but the pine marten, with seroprevalence ranging from 2 to 10%. Seroprevalence of CPIV was 1% in European mink, 1% in American mink and 5% in polecats, and was not detected in *Martes* species. The detection of antibodies to RV in three animals was surprising, as the sampling area is considered to be rabies-free, but may rather be due to spill-over of closely related bat-lyssa viruses, which cannot be distinguished by the serological methods used. Alternatively it may be due to non-specific reactions in the ELISA used. Indeed normally the cut-off used in this assay is rather 100 than 50, which was used here. Therefore a non-specific nature of this borderline titre is a more likely explanation. The high prevalence and number of species with documented antibodies to CDV suggests that this pathogen could have significant effects on the highly susceptible free-ranging mustelid populations, and has several implications for the conservation and re-introduction of the endangered European mink: (1) European mink to be released into areas with endemic CDV, or where prevalence is high due to an external source, should be vaccinated before release; (2) domestic dogs and hunting hounds, likely sources of virus for free-ranging carnivores, should be vaccinated in designated release areas; (3) pest-trapping programmes (which sometimes accidentally trap European mink), should use proper disinfection of traps to exclude traps as sources of infection.

The above mentioned sero-surveys in Canada and France have shown that CDV is common pathogen in many free-ranging carnivore species. All families of the taxonomic order Carnivora are susceptible to infection with CDV, a morbillivirus which is among the most significant infectious agents of domestic dogs and many non-domestic species in terms of morbidity and mortality. These vary per species, but case-fatality rate in naïve susceptible species can be as high as 100%. Outbreaks of CDV and the closely related PDV have caused large scale epidemics in naive (and highly fragmented) populations of terrestrial carnivores and marine mammals.

Although antibodies to morbilliviruses were found in sera from all the locations in Canada, and throughout the sampled area in France, suggesting

that they are widespread among free-ranging carnivores, there have been no mass mortalities associated with CDV infection in free-ranging animals in Canada or France, although there certainly is CDV-infection associated mortality. This may be explained by low susceptibility to infection or the low population density of some of the species tested (e.g., bears), which decreases the likelihood of a mass mortality. Mortalities of some of the more susceptible species (e.g., the mustelids) may go by unnoticed due to their small size. Alternatively, the levels of seroprevalence suggests that CDV is endemic (i.e., it is able to persist in a population for a long time without the need for introducing new infectious animals from external populations) in certain species and test areas, in which case mortality would be spread more evenly throughout the year, and go by unnoticed.

## Clinical signs of PDV infection in harbour seals

In contrast to the above-studies species in Canada and France, in which no mass mortalities due to morbillivirus infections were recorded, the Northern European harbour seal (*Phoca vitulina*) population experienced two mass mortality events caused by PDV infection: one in 1988, and one in 2002. Interestingly, at the time of writing (July 2007) there are indications that increased mortality due to a morbillivirus infection has recently taken place at the same location in Denmark as where the previous two epidemics started. During the most recent PDV epidemic that occurred in 2002, when 22 000 seals died, the clinical signs were recorded for 20 harbour seal pups that were admitted to the Seal Rehabilitation and Research Centre (SRRC) with clinical disease suggestive of PDV-associated disease. They were diagnosed by RT-PCR at necropsy to have indeed died from PDV infection (Chapter 2.1.). Clinical signs are recorded for every animal that is admitted to the SRRC, and due to a higher presumed prevalence of neurological signs than expected from published data and experiences from the 1988 PDV epidemic, the clinical signs of seals with a confirmed PDV infection were evaluated to confirm this presumption. The most prominent clinical signs were respiratory signs of variable extent in 100%, conjunctivitis in about 70%, and neurological signs in about 50% of the infected seals. Severe neurological signs were one of the euthanasia criteria during the epidemic, and a large number of juvenile seals that were euthanised on humane grounds outside the SRRC could not be included in this study, because a full data set (including haematology and serum biochemistry) was not available. Documented clinical signs during the 1988 epidemic suggested a much lower occurrence of neurological signs, confirming the suspicion of a higher prevalence of neurological signs in 2002. At time of admittance, a higher percentage of seals had a positive serum immunoglobulin (IgG) titre compared to IgM titres, suggesting that seals were in a relatively late stage of infection, and making the detection of IgG titres a useful diagnostic method. This was possible because the seroprevalence of PDV-specific serum antibodies in the harbour seal population had declined

to negligible levels after the 1988 epidemic. High levels of IgG at admittance were not correlated to absence of clinical signs or longer survival. Reported lymphoid depletion in dead seals collected during the epidemic was not reflected in the total mononuclear leukocyte count of seals upon admittance. Haematological tests further showed absolute granulocytosis, thrombocytosis, anaemia, and an increase in total white blood cell count in most of the animals evaluated.

## Vaccination against morbillivirus infections

In zoos and rehabilitation projects, infection risks can be minimised by reducing contact with unvaccinated wild or domestic species through proper fencing, the use of strict hygiene, and implementation of quarantine protocols for animals before they enter into the collection. In rehabilitation centres, health and immunological status of animals upon admittance are likely to be compromised, and the high population densities cause higher intra-, and often inter-species contact rates than in natural situations. When quarantine and hygienic protocols are not strictly adhered to, the risk of infection increases. Preventive vaccination cannot be a substitute for proper quarantine and hygienic measures, but it is one of the tools that lowers infection risks, and can be used to prevent infections in individual animals, as well as large scale outbreaks.

A problem faced with vaccination of non-domestic carnivores against morbilliviruses – a major cause of mortality in these species - is the variation between and within species in their susceptibility to modified-live virus (MLV) vaccines. Many species have been documented with CDV vaccine-induced canine distemper with often lethal consequences. Currently the commercially available vaccines in the EU are all MLV vaccines. Safer alternatives would be inactivated vaccines, which do not cause infections, but their ability to induce an adequate immune response is much lower than that of their attenuated counterparts. Currently there are no inactivated CDV vaccines commercially available. Other safe alternatives are experimental subunit and recombinant vaccines, the latter of which is commercially available and is authorised for use in ferrets in the USA, but not in the EU.

A prime example of the beneficial effects of stringent hygiene and quarantine measures in combination with safe and effective vaccination was seen in the SRRC during the PDV outbreak in 2002. Harbour seals were vaccinated using a CDV immuno-stimulating complex (ISCOM) vaccine (developed during the PDV outbreak in 1988) upon entry into quarantine units, which they left after 30 days if they show no clinical signs of disease and after vaccine-induced antibodies had been determined. The close antigenic relationship between CDV and PDV provides cross-protection against both of these morbilliviruses. The duration of protective immunity following this vaccination in seals is unknown, but it is intended to last for at least the duration of stay in the rehabilitation centre. Seals that had left the quarantine units,

but which were still present in the SRRC while PDV-infected animals were admitted (into quarantine units), remained free from PDV-related morbidity and mortality throughout the duration of the epidemic.

## Vaccination of European mink against CDV

The endangered European mink is highly susceptible to infection with CDV, and MLV vaccine-induced CDV infections have been reported in this species [126,323]. Safe and effective vaccination against CDV in European mink would be especially useful in breeding centres aiming at re-introduction of the species, where the population density is high, while the vaccine-induced immunity obtained would benefit survival of these animals upon release into CDV-endemic areas. However, currently there is no safe and effective commercially available CDV vaccine for use in highly susceptible non-domestic species like the European mink. We evaluated the safety and efficacy of the experimental CDV vaccine used in seals (CDV-ISCOM, 10 µg/ml, ErasmusMC, Rotterdam, The Netherlands) and a recombinant vaccine (Purevax®, Merial, Duluth, USA) which is commercially available outside the EU, in European mink (Chapter 2.3.1.). Both vaccines were safe and induced antibodies to CDV, although the ISCOM did so sooner, and geometric mean titres (GMT) were generally higher than those induced by the recombinant vaccine. One year after the third vaccination, antibodies were still present in both groups, although GMTs were higher in the ISCOM vaccinated group, and percentage of animals with a high VN titre ($\geq 80$) was 100%, compared to much lower percentages in the recombinant vaccine group.

Serum antibody titres measured by ELISA followed a similar trend as those measured by VN test, but were generally higher. The VN test is regarded the gold standard method for determination of immunity to morbilliviruses[339] because it measures functional neutralising antibodies, directed at the haemagglutinin (H) and fusion (F) surface proteins of the virus [425]. The ELISA, which is based on Vero cell culture-grown detergent-treated virus antigens, detects antibodies directed at the H and F surface proteins of the virus, but it also detects antibodies against the nucleoprotein (NP) antigen and possibly cell components. Antibodies to NP do not contribute directly to neutralisation and are therefore missed in a VN test; nevertheless, antibodies against these structures are abundantly produced in response to infection or vaccination with conventional vaccines [357], although not in response to the ISCOM- or recombinant vaccinations. Using an ELISA with Vero cell lysate to determine background optical densities excludes serum antibodies directed at the Vero cell components from the results. The ELISA furthermore detects antibodies directed at a large range of epitopes on the H and F proteins, not only the conformational epitopes, and titres will thus be higher than those measured in the VN test.

These results suggest that while both vaccines induce a humoral response, the CDV-ISCOM vaccine would protect European mink better

against CDV infection based on the induction of higher antibody titres in this species. However, the contribution of other protective mechanisms induced by vaccination with either of these vaccines, such as induction of cellular immunity should also be considered. Obviously, final conclusions about efficacy of these vaccines could come from challenge experiments, which are not appropriate given the endangered status of this species,

## Vaccination of African wild dogs against CDV

The African wild dog (*Lycaon pictus*) is one of the most endangered species of the taxonomic family Canidae. They once ranged widely throughout 39 sub-Saharan African countries, but currently their population is estimated at 3 000 – 5 500, in 15 countries. Their dramatic decline is largely due to human persecution (snaring, shooting, and road accidents), habitat fragmentation, reduced prey availability, competition with other carnivores, and diseases. A fragmented wildlife habitat has altered population numbers and densities, and resulted in increased inter-species contacts (including with unvaccinated domestic dogs) and exposure to new pathogens such as CDV and rabies.

We evaluated the safety and efficacy of the same CDV-ISCOM and recombinant vaccine (Purevax®) in African wild dogs (Chapter 2.3.2.) as we have done for the European mink. For both vaccines, two vaccine doses were required to induce antibody titres, although the GMT and percentage of animals with a high antibody titre tested by VN was very low (ISCOM group: GMT 8 and 8%; recombinant group: GMT 28 and 25%). One year post-vaccination the percentage of animals with high VN serum antibody titres was higher in the recombinant group than in the ISCOM group (39% vs 0%). The increase in GMT and percentage of animals with high titres one year after three vaccinations, compared to those after two vaccinations, suggest that the antibody responses of the animals were boosted by the administration of the third vaccine dose, although no blood was collected at the time of the expected peak in antibody titres.

These results indicated that both vaccines were safe but, using vaccine-induced VN antibodies as a correlate of protection, neither vaccine was sufficiently efficacious (the recombinant vaccine was slightly more efficacious). Surprisingly, vaccination did induce satisfactory antibody titres detected by ELISA (GMT: 80; 85% ≥ 80 and GMT: 84; 69% ≥ 80 for the recombinant and ISCOM groups respectively), suggesting that ISCOM vaccination does induce CDV specific antibodies in African wild dogs, although they do not neutralise virus in the VN test. As previously stated in the discussion on antibody titres in European mink, the ELISA measures antibodies directed at a much larger range of epitopes than the VN test, which is regarded as the gold standard method for determination of immunity to morbilliviruses. However, the observed discrepancy between VN and ELISA titres after ISCOM vaccination of African wild dogs was not observed in similar vaccination evaluations in European mink vaccinated with the same batch of CDV-ISCOM vaccine, as discussed

previously (Chapter 2.3.1). There was no interruption of the cold chain required for preservation of the CDV-ISCOM vaccine as was previously suggested as one of the possible causes of vaccine failure in African wild dogs [13]. The antigen concentration used in the ISCOM may have been too low, although this concentration has proven to be efficacious in terms of VN serum antibody titre induction in many other non-domestic species. It therefore appears that the reason for this discrepancy should be sought in the intrinsic capability/ inability of the African wild dog to produce neutralising antibodies in response to CDV-vaccination. The population bottleneck created by the decimated and fragmented populations of African wild dogs may have resulted in a lack of genetic variability of this species, which subsequently can have had negative consequences for the immune system. This may have led to a sub-optimal response to a vaccine that may also have sub-optimal characteristics for this species.

## Vaccination of carnivores in Rotterdam Zoo against CDV

In Rotterdam Zoo several CDV-susceptible species are housed, which are vaccinated with CDV-ISCOM using standard protocols: juvenile animals are vaccinated 3 times at 3 week intervals, starting at around 10 weeks of age, and re-vaccinated annually with one vaccine dose. Adult animals of unknown vaccination status are vaccinated twice with 3-4 weeks interval, and re-vaccinated annually. At time of vaccination a blood sample is collected and CDV-specific antibodies are determined by ELISA and VN test. To evaluate the CDV-ISCOM vaccine-induced antibody responses, only those animals with no prior vaccination history were used, as prior vaccination may influence the efficacy of the CDV-ISCOM, and influences the results. No comparisons could be made with the efficacy of the recombinant vaccine in these species, as its use is not permitted in the EU.

Red pandas (Ai*lurus fulgens*), members of the taxonomic family Ailuridae, are highly susceptible to CDV infection, and were reported with vaccine-induced distemper in 1976 [120]. In Rotterdam Zoo pandas had previously been vaccinated with a CDV-ISCOM, and data were available for 16 animals (Figure 1: triangles). The GMTs measured by ELISA were low, and a number of animals did not seroconvert at all. However, during the last five years the red pandas reacted to vaccination with higher antibody titres, and results are therefore depicted separately from those from previous years. The total antigen concentration of the CDV-ISCOM previously used was 5 µg/ml, whereas the CDV-ISCOMS used in the last 5 years contained 10 µg/ml, and higher titres were induced, measured by both ELISA and VN (Figure 1: circles). Other species discussed in the following section were vaccinated with the vaccine with higher antigen concentration (10 µg/ml).

Maned wolves (*Chrysocyon brachyurus*) of the taxonomic family Canidae, are very susceptible to CDV infection, and have been reported with vaccine-induced CDV infection previously. CDV-ISCOM vaccination could be

evaluated in two animals in Rotterdam Zoo (Figure 2).

Figure 1.
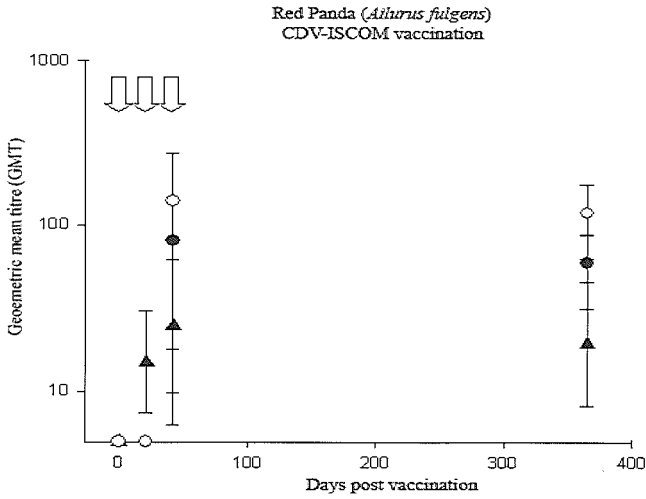


Figures 1 to 5. Antibody titres induced by CDV-ISCOM vaccination, with an antigen concentration of 10µg/ml, in several species: red pandas, *Ailurus fulgens* (Figure 1), maned wolves, *Chrysocyon brachyurus* (Figure 2), Asiatic small-clawed otters, *Amblonyx cinereus* (Figure 3), European otters, Lutra lutra (Figure 4) and Malay civets, *Viverra tangalunga* (Figure 5). The animals were kept in Rotterdam Zoo, and vaccinated three times with three weeks interval, then yearly with one vaccine dose. Blood was collected at times of vaccination. White circles depict serum antibodies measured by ELISA, black circles depict serum antibody titres measured by virus neutralisation test, and black triangles depict serum antibody titres measured by ELISA in response to vaccination with a CDV-ISCOM vaccine with an antigen concentration of 5µg/ml.

Figure 2.
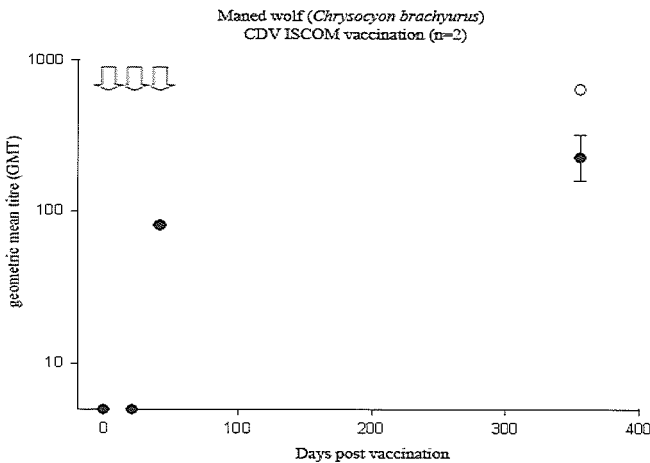
CDV infections have been described in different species of the subgroup Lutrinae (family Mustelidae): captive and free-ranging Eurasian otters (*Lutra lutra*) [426-428], Asiatic small-clawed otters (*Aonyx cinerea*), (W. Schaftenaar pers. comm), as well as North American river otters (*Lutra canadensis*), and serologic surveys have shown the presence of antibodies against CDV in free-living North American river otters [273,429]. We evaluated three Asiatic small-clawed otters (Figure 3) and two European otters (Figure 4) in Rotterdam Zoo.

Figure 3.



Asiatic small-clawed otters (*Amblonyx cinereus*)
CDV-ISCOM vaccination (n=3)

Figure 4.



European otter (*Lutra lutra*)
CDV-ISCOM vaccination (n=2)
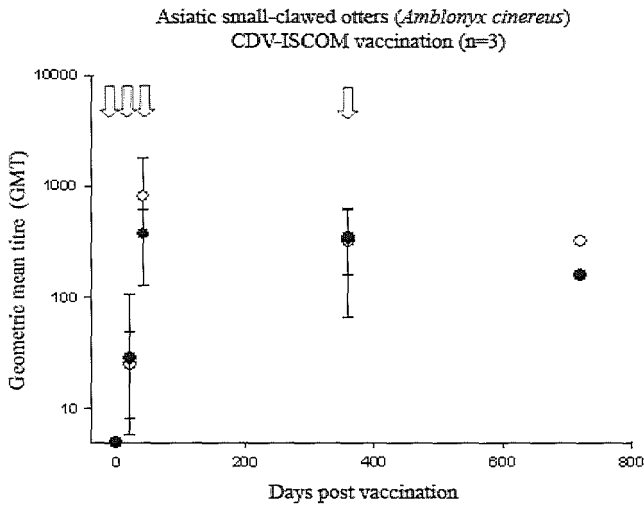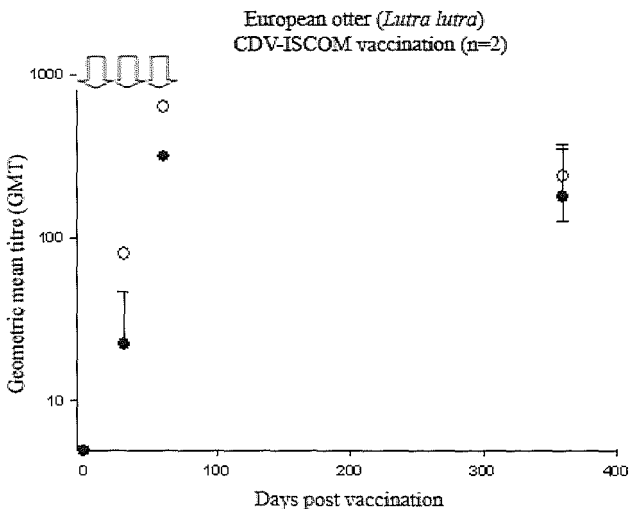
The Malay civet (*Viverra tangalunga*) is a member of the taxonomic family Viverridae, which has previously been documented to be susceptible to CDV infection [115]. In Rotterdam Zoo we evaluated 5 Malay civets for three years after first CDV-ISCOM vaccination, and three animals for 4 years (Figure 5).

Figure 5.



Malay civet (*Viverra tangalunga*)
CDV ISCOM vaccination
n=5 (3yrs) and n=3 (4yrs)
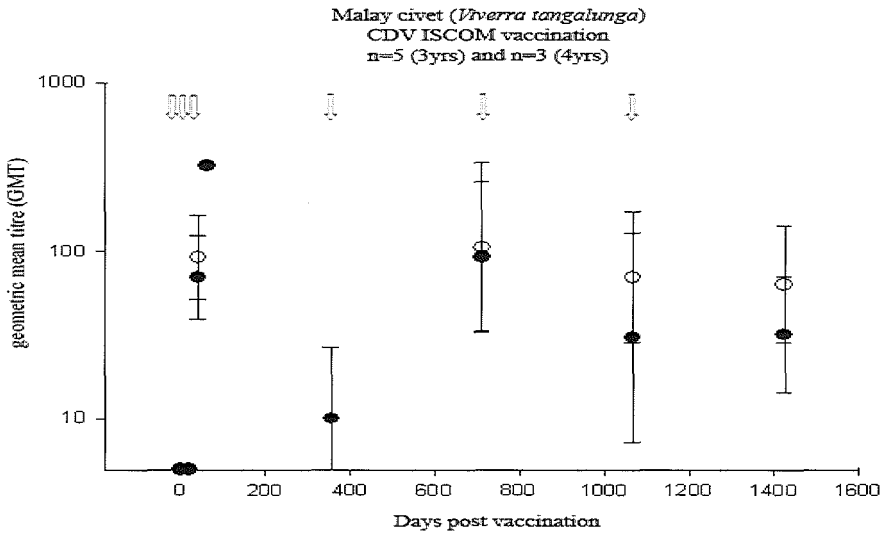
In general, most species needed two vaccinations before antibody titres were induced. The two otter species were the only species that had detectable antibodies after one vaccination. Antibody titres ≥ 40 were induced by two vaccinations in all animals. From the limited data obtained in Rotterdam Zoo, three weeks after the third vaccination, the antibody titre had increased further. One year after the initial three vaccinations, antibody levels dropped substantially in most species evaluated. Re-vaccination with one yearly vaccine dose following the initial vaccinations boosted the GMT to higher values, and antibodies remained present using yearly revaccination. GMT determined by ELISA was generally higher than, or equal to those determined by VN. Big discrepancies between ELISA and VN titres, as were seen in African wild dogs, were not seen in the species evaluated in Rotterdam Zoo.

## Conclusions: morbilliviruses in non-domestic carnivores

In this thesis the presence of antibodies against a number of viral pathogens in populations of free-ranging non-domestic carnivores, both in France and Canada has been shown. The prevalence of antibodies against CDV was the most prominent of the viruses tested, and it is known that this virus, and the closely related PDV and DMV have caused major epidemics in non-domestic species. The high prevalence of antibodies against CDV suggests that CDV is endemic in certain areas and species. An infectious agent that does not persist in the individual host can only become endemic if the population size is large enough (the critical population size), as the pool of susceptible individuals in small populations is exhausted rapidly, which leads to extinction of the infection. Whether a disease can become endemic furthermore depends on e.g., the basic reproduction number, length of latency and infectious period, as well as seasonal effects [430]. Fragmentation of habitat as a result of human interaction can favour the emergence of morbillivirus epidemics in wildlife by creating fragmented (smaller) populations, and additionally by forcing different species to co-inhabit certain areas, which may expose naive populations to an external source of morbillivirus, causing "virgin soil" epidemics. The ability of CDV to infect multiple host species increases the likelihood of its transmission to naïve populations. Domestic dogs, which have increased contact rates with free-ranging species due to an encroachment of human habitation on the natural habitat of these species, have been implicated as possible sources of CDV infection in a number of epidemics in naive populations of free-ranging carnivores and marine mammals (e.g., African wild dogs, African lions, black-footed ferrets, and Caspian seals [15,92,95,105,344]).

Human actions can additionally influence the occurrence of epidemics in free-ranging populations through well-intentioned re-introduction and translocation programmes which do not take the incidence of infectious agents into account. For these programmes to be maximally effective, it is important to know the status of infectious agents in the designated area, so that animals can obtain suitable levels of vaccine-induced protection before release. Equally important is a screening of the animals before release to ensure that they do not introduce a new pathogen into the designated release area.

Although serological studies assess immunological responses to exposure to infectious agents, no information is obtained concerning the disease agents, or their ability to cause clinical disease signs. Future studies should thus focus on the isolation and identification of these viruses in order to improve our understanding of their epidemiology, pathogenicity, and impact (with regards to morbidity or mortality) on free-ranging species. Clinical signs documented during a PDV epidemic in harbour seals were substantially different from those recorded during an epidemic 14 years earlier, emphasising the additional importance of careful documentation of clinical signs.

Early detection of outbreaks may aid in managing outbreaks of

infectious diseases, and can be complemented by vaccination of domestic and rehabilitated animals as well as free-ranging animals under certain conditions. Management of CDV epidemics in free-ranging species should include vaccination of domestic dogs in the area, and should try to restrict contact rates between domestic and non-domestic species to reduce the infection risk from this source. The same holds true for rabies virus outbreaks. Attention should be payed to the possibility of (reduced) excretion of attenuated CDV by vaccinated dogs, which are vaccinated with a MLV vaccine, and could potentially still infect susceptible non-domestic species.

We evaluated two candidate CDV vaccines for use in non-domestic species. The CDV-ISCOM vaccination was safe and efficacious in terms of inducing adequate levels of VN antibody titres in most species tested, although numbers per species tested was low for some, and differences in response to CDV vaccination were seen in these studies. Antibody titres remained at acceptable levels with yearly re-vaccination with one dose. Virus neutralising antibodies were induced by the CDV-ISCOM in all species except the African wild dog. Although the CDV-ISCOM vaccine performed well in all the other non-domestic species tested, neither this vaccine, nor a recombinant canarypox-based vaccine that is authorised for use in the USA, induced VN antibody levels indicative of protection in African wild dogs. This suggests an intrinsic inability of this species's immune system to react adequately to these vaccines.

These studies give an increased knowledge of the effect of preventive vaccination against CDV in non-domestic carnivores, but currently no safe and efficacious vaccine is authorised in the EU for use in non-domestic species. The ISCOM is an experimental vaccine. The safe and efficacious canarypox-vectored vaccine against CDV is currently not authorised in the EU, because it is a genetically-modified organism, even though other canarypox-vectored vaccines have been authorised successfully in the EU. To protect susceptible non-domestic species in the EU against CDV infection, this, or another safe and effective CDV vaccine, should be authorised as soon as possible for use in non-domestic species in EU member states.

## Vaccination of non-domestic avian species against HPAI viruses

In the European Union, routine vaccination of poultry against avian influenza viruses is currently not practised as this would interfere with international trade agreements. Instead, eradication measures during an outbreak in poultry include (long-term) confinement, humane killing and safe disposal of carcasses of all poultry on the infected farm, and, depending on the poultry density in the area and the epidemiological situation, pre-emptive culling of poultry on neighbouring farms and emergency vaccinations (Directive 92/94/EEC). Since 2003, more than 220 million birds have been culled to eradicate H5N1 HPAI outbreaks.

The standard measures used to prevent and eradicate HPAI virus outbreaks in poultry (long-term confinement and large scale preventive culling) would be detrimental to the welfare, conservation status and breeding programmes of zoo birds, which often are irreplaceable, valuable and endangered avian species (IUCN Red list, http://www.iucnredlist.org/). Directive 2005/94/EC foresees a derogation from killing of birds provided the birds can be brought inside and are subjected to virus detection tests (after the last death/positive finding, 2 tests at an interval of 21 days have to be performed according to the diagnostic manual Decision 2006/437/EC). However, most zoos do not have the capability to suitably confine their entire bird collections for extended time, and many species would not be able to adjust to confinement and increased stress with subsequent welfare problems and increased exposure to pathogens resulting in disease (e.g., aspergillosis, bumblefoot) [192-194].

Instead of confinement, vaccination of zoo birds against HPAI virus was allowed as an additional preventive measure (while reducing confinement measures) in Belgian, Dutch and German zoos during an outbreak of HPAI H7N7 virus in poultry in 2003 (Decision 2003/291/EC). Similarly, in 2005, Decision 2005/744/EC allowed vaccination in European zoos against the encroaching H5N1 subtype. Since then, targeted preventive vaccination campaigns as an alternative to indoor confinement have also been authorised in poultry. In the Netherlands, voluntary vaccination of hobby poultry and free-range laying hens was allowed (Decision 2006/147/EC). In France, it was allowed for domestic ducks and geese (Decision 2006/148/EC). These campaigns were subject to rigorous surveillance and control requirements.

During the HPAI H7N7 outbreak in poultry in 2003, birds in Dutch zoos were vaccinated twice with six weeks interval using a whole inactivated oil-adjuvanted vaccine, based on influenza virus A/chicken/Italy/473/99 (H7N1), with high homology to the field strain HPAI H7N7 A/chicken/Netherlands/1/03 (97.4% nucleotide and 98.7 % amino acid sequence identity of the H gene). This resulted in the induction of antibody titres $\geq 40$ (used as a correlate of protection in this study) in 81.5% of the vaccinated birds, with an overall GMT of 190. Birds of the taxonomic orders Anseriformes, Galliformes and Phoenicopteriformes showed higher GMT, and larger percentages developed

a serum HI antibody titre ≥ 40 than those of the other orders. Furthermore, a decrease in antibody response with an increase in body weight > 1.5 kg was shown. The high agreement between post vaccination antibody titres determined by serum HI test (using the vaccine strain), and VN titres (using the field strain), was used as a further measure of immunogenicity. The broad efficacy demonstrated in a large variety of taxonomic orders illustrated the value of vaccination as an additional preventive measure against HPAI virus infection (Chapter 3.1.).

In 2005, the Dutch zoos were the first to implement Decision 2005/744/EC to provide protection against the encroaching HPAI H5N1 subtype. Birds were vaccinated with an inactivated adjuvanted H5N2 vaccine with vaccine doses adapted to mean body weight per species, using data collected during the H7N1 vaccination campaign. The vaccine strain (A/duck/Pottsdam/1402/86) had a homology of 90% to the H gene of the H5N1 field strain (A/turkey/Turkey/1/05) on the basis of nucleotide sequence (1530 base pairs, including basic cleavage site), and 92.4% on the basis of amino acids. Vaccination was safe, and proved immunogenic throughout the range of species tested, with some variations between and within taxonomic orders. After booster vaccination the overall homologous GMT to the vaccine strain, measured in 334 birds, was 190 (95% CI:152–236), and 80.5% of vaccinated birds developed a titre of ≥ 40. Titres to the HPAI H5N1 virus followed a similar trend, but were lower (GMT: 61 (95% CI: 49–76); 61%≥ 40) (Chapter 3.2.).

The breadth of the immune response was further demonstrated by measuring antibody titres against prototype strains of four antigenic clades of currently circulating H5N1 viruses. Antigenic distances to the prototype strains were determined using antigenic cartography [404]. Antigenic cartography uses the antigenic properties of influenza viruses in combination with epidemiological and genetic data, and is used to select virus strains for use as human pandemic (H5N1) vaccine candidates [431]. Influenza vaccines whose haemagglutinins are antigenically similar to circulating strains provide the highest level of protection from infection in humans [432]. The birds clustered in two groups based on the breadth of antibody responses. Group 1 (Anseriformes, Galliformes, Phoenicopteriformes, Psittaciformes and Struthioniformes) showed a very broad response to vaccination, with predicted protection against future strains up to 12 antigenic units from the current vaccine. Group 2 (Ciconiiformes, Gruiformes, Pelecaniformes and Sphenisciformes) had low HI antibody titres against the prototype strain of the most antigenically distant clade (A/Indonesia/5/05).

In 2006, a working group of animal health and welfare experts was established by the European Food Safety Authority (EFSA), to provide a scientific assessment of the preventive vaccination against avian influenza of H5 and H7 subtypes carried out in zoos in Member States (MS). The total number of birds vaccinated, as reported by 12 MS, was 44721. Individual data from 4718 birds (374 species from 19 taxonomic orders) were submitted. Not all of these could be used for every evaluation: pre-vaccination titres could be

evaluated for 3039 birds; titres after first vaccination were evaluated for 1429 birds, and post-second vaccination titres for 2296 birds.

Differences in vaccination schedules, doses and routes, differences in methodology and antigens used in the HI tests between laboratories (due to the absence of international reference standards, and the absence of inter-laboratory standardisation of methodology), the use of different vaccines[1] in different taxonomic orders and the sometimes incomplete reporting of results, limited the evaluation of some of the data provided by EU MS. Cut-off points varied with laboratory, and titres considered a measure of adequate immune response were 8, 16, 32, 40, and 64. Most countries used dilution series starting at 4 or 8, therefore results were evaluated for titres 16 and 32 (documented surrogate markers for protection in chickens [154,181,183,186]), and undetectable titres were regarded as 4 for calculation of GMT. In the absence of (and unfeasibility of obtaining) vaccination/challenge data in often endangered zoo bird species, the evaluation had to be based on extrapolation of serological data from poultry and limited other bird species.

The H5 and H7 vaccines registered for poultry in the EU showed differences in efficacy, measured as serum HI antibodies induced by two doses of vaccine (Annex 1). Three of the five vaccines evaluated induced relatively high GMT and high percentage seroconversion in the vast majority of vaccinated birds. The HI titres induced by vaccination showed marked differences between and within taxonomic orders. Both routes of vaccination (i.m. and s.c.) were effective in inducing HI serum antibody responses, and for most avian species the poultry dose was suitable. In some larger species higher doses, adjusted to body weight, induced higher serum antibody titres. (e.g., for ostriches a 10-fold increase of the poultry dose: 10 x 0.25 ml. However, extremely high doses at a single site of injection (e.g., vaccination of ostriches with 10 ml of vaccine) appeared to have a negative effect on the induction of serum antibody titres, and induced local adverse reactions.

There were indications that one vaccination was sufficient to induce high serum antibody titres in at least two taxonomic orders of birds. However, a second vaccine administration ensured seroconversion in the majority of birds of most species. Limited data indicated that antibody titres persisted in several species for six months after vaccination. Adverse effects and mortality associated with vaccination were low and were mainly attributable to handling stress or trauma. Differences in adverse effects reported from different zoos highlight the importance of proper skilled handling.

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[1]
Vaccine A: H5N9 (A/turkey/Wisconsin/68).
Vaccine B: H5N2 (A/duck/Pottsdam/1402/86).
Vaccine C: H5N2 (A/chicken/Mexico/232/94/CPA).
Vaccine D: H5N9 (A/chicken/Italy/22A/98).
Vaccine E: H5N9 (A/chicken/Italy/22A/H5N9/1998).

## Longevity of serum antibodies in non-domestic avian species upon vaccination against HPAI viruses

One year after vaccination with the H5N2 vaccine, birds in Dutch zoos were revaccinated with the same vaccine. Antibody titres one year after the initial two vaccinations and the effect of one booster vaccination at this time were evaluated. In Rotterdam Zoo, 72 previously vaccinated birds could be evaluated for the effect of one booster vaccination (Chapter 3.3.). For 44 birds, serum samples were available from 4 weeks after the initial two vaccinations the previous year, at the time of revaccination, and 4 weeks later. Birds which had been vaccinated with the H7 vaccine two years prior to the H5N2 revaccination were additionally tested for the presence of H7-specific antibodies.

Serum collected at the time of revaccination had to be tested at the National veterinary reference laboratory, while previously this was done at the ErasmusMC. In order to compare titres, the correlation between results from these two laboratories was determined by sub-sampling 141 serum samples and conducting the tests at both laboratories. The correlation coefficient of the results from the two laboratories was high (CC=0.87).

Serum antibody titres of the birds tested in Rotterdam Zoo had clearly decreased in one year time: while 80% of birds had a positive titre ($\geq$ 8) and 68% a high positive titre ($\geq$ 32) after 2 vaccinations, these figures were 61% and 30% respectively one year later. Four weeks after re-vaccination these figures increased to 93% and 77% respectively. Although a larger percentage of these 44 birds had a serum HI antibody titre $\geq$ 32 after re-vaccination, the GMT was lower than GMT after 2 vaccine doses one year before (88 vs 66).

As previously reported for the initial 2 vaccine doses, there were differences in responses between taxonomic orders. Of all birds tested (8 taxonomic orders) 4 orders did not have a GMT > 5 one year after vaccination, and only one order (Phoenicopteriformes) had a GMT > 40. Four weeks after revaccination 6/8 taxonomic orders tested had a GMT > 40.

GMTs had decreased even further two years after vaccination, as was shown by the H7 specific serum HI antibody titres (Chapter 3.3.). As these birds were not revaccinated with an H7 vaccine, the effect of revaccination two years after the initial vaccinations is not known.

## Vaccination of pigeons against HPAI H5N1 virus.

Rock pigeons (*Columba livia*) and other orders of the taxonomic order Columbiformes have previously been described as being resistant, or at least less sensitive, to infection with HPAI virus of the H7 and H5 subtypes, and have failed to produce high levels of antibodies after infection or vaccination. However, higher susceptibility of pigeons to more recent HPAI virus strains of the H5N1 subtype has been documented, and transmission of virus to cats which fed on infected pigeons has been shown, although excretion of virus or bird-bird transmission has not been documented. Feral pigeons are ubiquitous near human settlements, and large numbers of pigeons are kept as pets or as racing pigeons, thus potentially posing an infection risk to humans if virus excretion would occur.

We inoculated vaccinated and unvaccinated rock pigeons with high doses of highly pathogenic avian influenza virus of the H5N1 subtype from two different antigenic clades: A/Indonesia/5/05 (A/Indonesia) or A/turkey/ Turkey/1/05 (A/turkey) via the intra-tracheal and intra-oesophageal route (Chapter 3.4.). The vaccine strain used (A/duck/Potsdam/2243/84; H5N6) had a homology of 87% and 88% to A/turkey and A/Indonesia on the basis of nucleotide sequence of the H gene (1692 base pairs, excluding the basic cleavage site), and 91.5% and 91.5% on the basis of amino acids respectively. Low morbidity and mortality were observed in unvaccinated pigeons, while pigeons vaccinated with an inactivated adjuvanted H5N6 vaccine were protected from developing clinical signs. Clinical signs of infected animals were depression, general malaise, and neurological signs (ataxia, tremors, opisthotonus, torticollis). Virus could be re-isolated from several organs at 3 days post-inoculation (dpi) (A/Indonesia: lung, airsac, spleen, duodenum; A/turkey: brain, lung, airsac, pancreas, spleen, and liver), and immunohistochemistry showed the presence of nucleoprotein in the lung, airsac and brain. By RT-PCR virus could be detected in several organs at 3 and 27 dpi. Infectious virus was isolated from oropharyngeal swabs for up to 3 (A/turkey) and 4 dpi (A/Indonesia); cloacal swabs remained negative throughout the duration of the experiment.

Vaccination was safe, and prevented severe clinical signs and mortality, largely limited extra-respiratory infection of both virus strains, provided partial protection against lung infection and disease (after A/Indonesia infection), and limited virus shedding to one day after experimental infection (which may be due to residual virus after the extremely high inoculation dose). However, infectious virus was re-isolated from lung and air sacs at 3 dpi, and in one bird that died with severe neurological signs it was re-isolated from the brain at 5 dpi.

## Conclusions: Vaccination of non-domestic avian species against HPAI viruses

Bio-security measures remain the first line of protection of zoo birds against the introduction of AI viruses and should be implemented in zoos. These bio-security measures should include strict hygiene and quarantine measures, but should also exclude the possibility of introducing AI viruses through feed animals such as day old chicks, other poultry or their products. Clinical and virological monitoring of captive and wild birds in zoos should be practised for early detection of introduced viruses by wild birds, domestic birds, or their products. Strict bio-security measures will also reduce the risk of subsequent infection of wild birds from zoo birds. Wild birds have been documented to be susceptible to HPAI virus infection, and could potentially play a role in the spread of HPAI virus, although the majority of avian influenza viruses detected in free-ranging birds have been LPAI viruses. If bio-security measures cannot sufficiently protect zoo birds from exposure to HPAI viruses coming from wild birds (based on an overall risk assessment which includes welfare aspects) vaccination with vaccines against HPAI of H5 and H7 subtypes authorised for use in poultry should be used to protect these zoo birds. In designing AI vaccination programmes and schedules for zoo birds, recent data on wild bird migration and prevalence of AI virus infections in wild birds should be taken into account. Vaccination against AI viruses of the H5 and H7 subtypes with current inactivated oil-adjuvanted poultry vaccines is safe and, in most taxonomic orders of zoo birds, effective in terms of inducing HI serum antibody titres. AI vaccines should be administrated in a way that elicits high HI antibody titres in the vast majority of the zoo birds vaccinated, i.e., by adjusting dose to average body weight. Although there are indications that one vaccination might suffice for some species, a second vaccine dose ensures high titres in the vast majority of species. Unless it is demonstrated that one vaccine administration is sufficient, two administrations are recommended. The H5 and H7 vaccines currently registered for poultry in the EU show differences in the performance in terms of HI response in zoo birds after two doses. There appears to be no difference due to route of vaccination (s.c. or i.m.), so route can be adjusted to the bird species to be vaccinated. In order to maintain high titres in the captive populations in zoological collections, annual re-vaccination seems to be required, as antibody titres decrease significantly in most taxonomic orders, and high titres are seen after a single annual booster dose.

Standard protocols for vaccination, sampling, testing and reporting should be established at EU level to optimise evaluation and future implementation of AI vaccination programmes for zoo birds (see example in Annex 2). Inter-laboratory standardisation of serological assays to detect AI serum antibodies in birds should be established. There is a need for the preparation of international standard reference sera to facilitate this standardisation.

Mortality and adverse effects were low in all zoos evaluated in EU MS, and mainly attributed to handling stress and trauma. Zoos can, and should therefore try to minimise these losses in the execution of HPAI vaccination programmes. To minimise indirect losses due to decreased breeding results, AI vaccination during breeding seasons should be avoided whenever possible. Mortality due to catching and handling stress can be reduced by handling the birds less. Once the efficacy of a vaccination protocol has been validated for certain species using certain vaccines, measurement of post-vaccination HI serum antibody titres should no longer be mandatory by the EU. These birds will then only have to be handled for vaccination, and not 4 weeks later. Further research should be carried out to establish effective vaccination schedules, routes, and dose regimens in different zoo bird species. This may, amongst others, lead to a reduction in the number of booster vaccinations needed in certain species. Novel generation vaccines which may be administered in the form of an aerosol (as is used in vaccination of poultry against Newcastle disease virus) may prove to be useful in non-domestic species, and would eliminate the need for handling the birds.

The vaccination campaigns against HPAI virus have focused on protecting birds in zoological collections. However, a large number of mammalian species, including tigers and leopards, have also been documented with HPAI virus infection with recent H5N1 subtypes. There is currently no commercial vaccine available to protect mammals from HPAI H5N1 virus infection. A recombinant fowlpox-vectored vaccine expressing the H5 gene has been shown to produce high antibody titres against heterologous H5N1 virus antigen in cats after booster vaccination [433], and may prove to be useful in prophylactic vaccination programs of mammals in the future. Until then, these animals have to be protected by bio-security measures such as excluding the introduction of AIV through raw poultry used as feed.

Vaccination has proven to be an effective tool in curtailing outbreaks of HPAI virus in poultry, and although infection with subsequent virus excretion can still occur, this is drastically decreased in vaccinated animals compared to unvaccinated animals. We have shown by challenge infections of pigeons with HPAI strains from 2 different antigenic clades that although pigeons are not very susceptible to infection, the occurrence of morbidity and mortality is decreased by vaccination. Additionally, vaccination reduced spread of virus to the different organs, and reduced excretion of the virus. However, the presence of viable virus in lung and air sacs of vaccinated pigeons at 3 DPI means that infected pigeon carcasses of vaccinated birds could still pose an infection risk to predators and scavengers.

The broad vaccine efficacy in the different avian taxonomic orders illustrates that vaccination against avian influenza is a useful tool for the protection of non-domestic avian species in zoos, which allows for an alleviation of confinement measures – and is therefore beneficial to the health and welfare of these birds. However, increased bio-security measures in combination with virological monitoring remain imperative in combating outbreaks of HPAI.

The European vaccination campaigns in zoos should be seen as an additional protective measure to protect the (often irreplaceable, valuable and endangered) species in the zoological collections from infection by wild birds and other sources, while reducing confinement measures, and concurrently reducing the possibility of the zoos becoming a source of infection for wild birds. However, poultry farms should be regarded as the major sources of HPAI virus, as their birds have documented high susceptibility, excrete large amounts of virus, and population densities are high. Increased bio-security measures on poultry farms, reduced contact with wild birds (while not compromising the poultry welfare), culling and vaccination of poultry will be the most important steps in controlling large scale HPAI outbreaks such as the H5N1 outbreaks we are currently experiencing.

# GENERAL CONCLUSIONS

Laboratory and syndromic surveillance of free-ranging non-domestic animals plays a major role in early detection, identification and management of outbreaks of infectious diseases. In addition, this surveillance can be used in re-introduction or translocation projects, where knowledge of the infectious disease status of the area can lead to the necessary precautionary measures to make these projects maximally successful. Vaccination of non-domestic species can be an important additional measure to increased bio-security measures in preventing and managing virus infections in these animals. This thesis focused on two groups of viruses which have caused major outbreaks: morbilliviruses and highly pathogenic avian influenza viruses. In the EU there are currently no authorised safe and effective CDV vaccines that can be used in non-domestic species. An experimental CDV-ISCOM vaccine performs well in most species tested, based on the induction of high levels of VN serum antibody titres. A commercial canarypox-based recombinant vaccine that is authorised in the USA, is efficacious in terms of inducing VN serum antibody titres. However, neither of these vaccines induces adequate serum antibody responses in the African wild dog.

Vaccination of non-domestic avian species against AI viruses with poultry vaccines currently authorised in the EU induces adequate HI serum antibody responses after two vaccine doses in the majority of species and taxonomic orders tested. A yearly booster with one vaccine dose appears to be sufficient to maintain adequate antibody levels in these species.

Annex 1. HI serum antibody titres after first and second vaccination with an H5 vaccine in European zoos. Results are depicted for the different taxonomic orders, with the vaccines, intervals, and doses used, based on data submitted by EU Member States to the European Food Safety Authority (EFSA) [200].

| Order | Vaccine | Interval (wks) | Dose (ml) | Post-first vaccination titres | | | | Post-second vaccination titres | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | n | GMT | % ≥16 | % ≥32 | n | GMT | % ≥16 | % ≥32 |
| ALL | ALL | ALL | ALL | 1429 | 27 | 54 | 49 | 2296 | 89 | 82 | 76 |
| ALL | 1 | 6 | ALL | | | | | 12 | 36 | 83 | 58 |
| | 2 | 4-6 | ALL | 539 | 75 | 84 | 77 | 1550 | 142 | 91 | 85 |
| | 3 | 4-6 | ALL | 69 | 19 | 54 | 49 | 79 | 43 | 72 | 71 |
| | 4 | 4-6 | ALL | | | | | | | | |
| | 5 | 3 | ALL | 821 | 14 | 34 | 31 | 655 | 33 | 62 | 56 |
| Accipitriformes | 2 | 4-6 | 1 | 5 | 159 | 100 | 100 | 5 | 336 | 100 | 100 |
| Anseriformes | 2 | ALL | ALL | 190 | 78 | 87 | 83 | 307 | 192 | 95 | 92 |
| | 2 | 4-6 | 0.25 | 27 | 29 | 56 | 52 | 37 | 171 | 92 | 89 |
| | 2 | 4-6 | 0.5 | 17 | 18 | 47 | 41 | 45 | 233 | 89 | 87 |
| | 2 | 4-6 | 0.5 + 1 | 17 | 59 | 94 | 94 | 16 | 450 | 100 | 100 |
| | 2 | 4-6 | 0.75 | | | | | 2 | 905 | 100 | 100 |
| | 2 | 4-6 | 1 | 83 | 132 | 98 | 93 | 62 | 245 | 97 | 97 |
| | 2 | 4-6 | ns | 28 | 102 | 96 | 93 | 130 | 143 | 95 | 90 |
| | 2 | 4-6 | ALL | 172 | 76 | 86 | 81 | 292 | 191 | 95 | 91 |
| | 2 | ns | 1 | 17 | 95 | 100 | 94 | 15 | 213 | 100 | 100 |
| | 2 | ns | ns | 1 | 256 | 100 | 100 | | | | |
| | 2 | ns | ALL | 18 | 102 | 100 | 94 | 15 | 213 | 100 | 100 |
| | 3 | 4-6 | ALL | 9 | 20 | 67 | 56 | 13 | 61 | 85 | 85 |
| | 3 | 4-6 | 0.5 | 1 | 4 | 0 | 0 | 4 | 38 | 75 | 75 |
| | 3 | 4-6 | 1 | 8 | 25 | 75 | 63 | 9 | 75 | 89 | 89 |
| | 5 | 3 | ns | 144 | 111 | 79 | 77 | 134 | 116 | 88 | 82 |
| Bucerotiformes | 5 | 3 | ns | 25 | 4 | 4 | 4 | 16 | 5 | 6 | 6 |
| Charadriiformes | 2 | 4-6 | ALL | | | | | 6 | 99 | 100 | 100 |
| | 2 | 4-6 | 0.25 | | | | | 6 | 99 | 100 | 100 |
| | 5 | 3 | ns | 14 | 4 | 0 | 0 | 14 | 20 | 50 | 50 |
| Ciconiiformes | 2 | ALL | ALL | 91 | 113 | 88 | 84 | 191 | 207 | 93 | 92 |
| | 2 | 4-6 | 0.25 | 58 | 141 | 93 | 88 | 104 | 250 | 93 | 92 |
| | 2 | 4-6 | 0.5 | 17 | 25 | 59 | 53 | 25 | 120 | 85 | 84 |
| | 2 | 4-6 | 1 | 5 | 194 | 100 | 100 | 5 | 891 | 100 | 100 |
| | 2 | 4-6 | ns | 3 | 64 | 100 | 100 | 48 | 119 | 96 | 94 |
| | 2 | 4-6 | ALL | 83 | 98 | 87 | 82 | 183 | 192 | 93 | 92 |
| | 2 | ns | 0.5 | 8 | 470 | 100 | 100 | 8 | 1117 | 100 | 100 |
| | 3 | 4-6 | ALL | 24 | 34 | 67 | 67 | 24 | 93 | 92 | 88 |
| | 3 | 4-6 | 0.5 | 19 | 39 | 74 | 74 | 19 | 103 | 95 | 90 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 4-6 | 1 | 5 | 21 | 40 | 40 | 5 | 64 | 80 | 80 |
| | 5 | 3 | ns | 65 | 8 | 20 | 15 | 53 | 15 | 45 | 37 |
| Columbiformes | 2 | 4-6 | ALL | | | | | 13 | 25 | 77 | 46 |
| | 2 | 4-6 | 0.25 | | | | | 8 | 21 | 75 | 38 |
| | 2 | 4-6 | 0.5 | | | | | 1 | 4 | 0 | 0 |
| | 2 | 4-6 | ns | | | | | 4 | 64 | 100 | 50 |
| | 5 | 3 | ns | 57 | 5 | 12 | 7 | 48 | 7 | 21 | 15 |
| Coracciformes | 2 | 4-6 | ALL | 1 | 4 | 0 | 0 | 9 | 67 | 89 | 67 |
| | 2 | 4-6 | 0.25 | | | | | 1 | 560 | 100 | 100 |
| | 2 | 4-6 | 0.5 | | | | | 4 | 19 | 75 | 25 |
| | 2 | 4-6 | ns | | | | | 4 | 128 | 100 | 100 |
| | 5 | 3 | ns | 2 | 4 | 0 | 0 | 4 | 7 | 25 | 25 |
| Falconiformes | 1 | 6 | 0.3 | | | | | 3 | 22 | 66 | 33 |
| | 2 | 4-6 | ALL | 15 | 56 | 100 | 80 | 36 | 141 | 94 | 94 |
| | 2 | 4-6 | 0.5 | 9 | 51 | 100 | 78 | 18 | 84 | 89 | 89 |
| | 2 | 4-6 | 1 | 6 | 64 | 100 | 83 | 5 | 256 | 100 | 100 |
| | 2 | 4-6 | ns | | | | | 13 | 230 | 100 | 92 |
| | 5 | 3 | ns | 72 | 6 | 15 | 13 | 50 | 60 | 72 | 74 |
| Cuculiformes | 5 | 3 | ns | 1 | 4 | 0 | 0 | 2 | 4 | 0 | 0 |
| Galliformes | 1 | 4-6 | 0.3 | | | | | 5 | 37 | 80 | 60 |
| | 2 | ALL | ALL | 83 | 99 | 82 | 77 | 328 | 179 | 90 | 85 |
| | 2 | 4-6 | 0.25 | 6 | 30 | 50 | 50 | 25 | 255 | 92 | 92 |
| | 2 | 4-6 | 0.5 | 53 | 189 | 94 | 93 | 115 | 306 | 85 | 82 |
| | 2 | 4-6 | 0.75 | * | * | * | * | 4 | 1001 | 100 | 100 |
| | 2 | 4-6 | ns | 20 | 23 | 55 | 45 | 150 | 123 | 93 | 85 |
| | 2 | ns | ALL | 24 | 32 | 63 | 50 | 24 | 93 | 92 | 57 |
| | 2 | ns | 0.25 | 1 | 16 | 100 | 0 | 1 | 128 | 100 | 100 |
| | 2 | ns | 0.5 | 3 | 81 | 100 | 100 | 3 | 128 | 100 | 100 |
| | 2 | ns | ns | 20 | 23 | 55 | 45 | 20 | 54 | 90 | 60 |
| | 5 | 3 | ns | 47 | 8 | 23 | 17 | 58 | 42 | 78 | 69 |
| Gruiformes | 1 | 4-6 | 0.3 | | | | | 5 | 37 | 100 | 60 |
| | 2 | ALL | ALL | 40 | 26 | 80 | 60 | 91 | 85 | 95 | 88 |
| | 2 | 4-6 | ALL | 39 | 26 | 80 | 59 | 89 | 86 | 94 | 89 |
| | 2 | 4-6 | 0.25 | | | | | 1 | 40 | 100 | 100 |
| | 2 | 4-6 | 0.5 | 36 | 28 | 83 | 64 | 68 | 83 | 94 | 88 |
| | 2 | 4-6 | 0.75 | 2 | 9 | 50 | 0 | 2 | 40 | 50 | 50 |
| | 2 | 4-6 | ns | 2 | 23 | 50 | 50 | 20 | 104 | 100 | 100 |
| | 2 | ns | 0.5 | 1 | 32 | 100 | 100 | 2 | 45 | 100 | 50 |
| | 3 | 4-6 | 0.5 | 1 | 128 | 100 | 100 | | | | |
| | 5 | 3 | ns | 27 | 11 | 30 | 22 | 18 | 11 | 50 | 28 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Passeriformes | 2 | 4-6 | ALL | | | | | 10 | 27 | 80 | 70 |
| | 2 | 4-6 | 0.25 | | | | | 6 | 22 | 83 | 67 |
| | 2 | 4-6 | ns | | | | | 4 | 38 | 75 | 75 |
| | 5 | 3 | ns | 9 | 5 | 11 | 0 | 5 | 14 | 60 | 20 |
| Pelecaniformes | 2 | 4-6 | ALL | 20 | 21 | 65 | 50 | 56 | 42 | 73 | 63 |
| | 2 | 4-6 | 0.5 | 2 | 16 | 100 | 0 | 12 | 12 | 42 | 25 |
| | 2 | 4-6 | 0.75 | 2 | 1120 | 100 | 100 | 9 | 34 | 56 | 56 |
| | 2 | 4-6 | 1 | 8 | 9 | 38 | 38 | 13 | 98 | 92 | 85 |
| | 2 | 4-6 | ns | 6 | 32 | 100 | 83 | 19 | 60 | 84 | 63 |
| | 2 | ns | ns | 2 | 4 | 0 | 0 | 2 | 45 | 100 | 100 |
| | 3 | 4-6 | 3 | | | | | 1 | 64 | 100 | 100 |
| | 5 | 3 | ns | 26 | 17 | 42 | 39 | 32 | 16 | 41 | 41 |
| Phoenicopteriformes | 2 | ALL | ALL | 46 | 208 | 94 | 94 | 182 | 310 | 98 | 97 |
| | 2 | 4-6 | 0.5 | 30 | 218 | 93 | 93 | 65 | 376 | 97 | 94 |
| | 2 | 4-6 | 1 | 16 | 189 | 94 | 94 | 4 | 431 | 100 | 100 |
| | 2 | 4-6 | 1 + 0.5 | | | | | 12 | 575 | 100 | 100 |
| | 2 | 4-6 | ns | | | | | 101 | 251 | 99 | 99 |
| | 3 | 4-6 | 0.5 | 11 | 53 | 81 | 81 | 11 | 106 | 91 | 91 |
| | 5 | 3 | ns | 82 | 114 | 83 | 83 | 78 | 133 | 91 | 89 |
| Piciformes | 2 | 4-6 | ns | | | | | 1 | 4 | 0 | 0 |
| | 5 | 3 | ns | 2 | 4 | 0 | 0 | 2 | 4 | 0 | 0 |
| Psittacciformes | 1 | 4-6 | 0.3 | | | | | 1 | 4 | 0 | 0 |
| | 2 | 4-6 | ALL | 14 | 28 | 86 | 71 | 82 | 191 | 95 | 92 |
| | 2 | 4-6 | 0.25 | 14 | 28 | 86 | 71 | 39 | 600 | 100 | 97 |
| | 2 | 4-6 | ns | | | | | 43 | 67 | 90 | 86 |
| | 5 | 3 | ns | 185 | 5 | 11 | 7 | 86 | 12 | 47 | 38 |
| Sphenisciformes | 2 | 4-6 | ALL | 2 | 4 | 0 | 0 | 107 | 43 | 86 | 70 |
| | 2 | 4-6 | 0.5 | 2 | 4 | 0 | 0 | 34 | 130 | 97 | 97 |
| | 2 | 4-6 | ns | | | | | 73 | 26 | 81 | 59 |
| | 5 | 3 | ns | 21 | 7 | 19 | 10 | 16 | 8 | 38 | 0 |
| Strigiformes | 1 | 6 | 0.3 | | | | | 1 | 4 | 0 | 0 |
| | 2 | 4-6 | ALL | | | | | 29 | 44 | 79 | 66 |
| | 2 | 4-6 | 0.25 | | | | | 1 | 4 | 0 | 0 |
| | 2 | 4-6 | 0.5 | | | | | 5 | 25 | 40 | 20 |
| | 2 | 4-6 | ns | | | | | 23 | 56 | 91 | 65 |
| | 5 | 3 | ns | 12 | 4 | 0 | 0 | 11 | 7 | 18 | 18 |
| Struthioniformes | 1 | 6 | ns | | | | | 2 | 812 | 100 | 100 |
| | 2 | ALL | ALL | 31 | 30 | 61 | 48 | 96 | 65 | 70 | 64 |
| | 2 | 4-6 | ALL | 26 | 29 | 62 | 50 | 92 | 54 | 69 | 62 |
| | 2 | 4-6 | 1 | 13 | 79 | 77 | 77 | 16 | 100 | 81 | 75 |
| | 2 | 4-6 | 1.25 | 3 | 11 | 33 | 33 | 17 | 180 | 88 | 88 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 4-6 | 2 | | | | | 5 | 18 | 60 | 40 |
| 2 | 4-6 | 2.5 | 1 | 80 | 100 | 100 | 5 | 151 | 80 | 80 |
| 2 | 4-6 | ns | 9 | 9 | 44 | 11 | 49 | 30 | 55 | 49 |
| 2 | ns | 1 | 5 | 32 | 60 | 40 | 3 | 81 | 100 | 100 |
| 3 | 4-6 | ALL | 24 | 6 | 21 | 13 | 29 | 13 | 42 | 42 |
| 3 | 4-6 | 3 | * | * | * | * | 4 | 23 | 75 | 75 |
| 3 | 4-6 | 5 | 10 | 10 | 50 | 30 | 10 | 64 | 90 | 90 |
| 3 | 4-6 | 8 | * | * | * | * | * | * | * | * |
| 3 | 4-6 | 10 | 14 | 4 | 0 | 0 | 14 | 4 | 0 | 0 |
| 5 | 3 | ALL | 30 | 21 | 47 | 43 | 28 | 82 | 82 | 75 |
| 5 | 3 | 1 | 22 | 33 | 59 | 55 | 20 | 119 | 85 | 80 |
| 5 | 3 | ns | 8 | 6 | 12 | 12 | 8 | 32 | 75 | 63 |

Annex 2. Proposed standard protocol for reporting data on AI vaccination in zoo birds - European Food Safety Authority (EFSA) [200]

| General information | | | | | | |
|---|---|---|---|---|---|---|
| Country | Zoo | Vaccine | Route (specify if different in different species) | Weight – Dose regime used (Actual/estimated /average weight of species) | Vaccine interval | Interval from last vaccination to post-vaccination blood collection |
| | | | | | | |

| Individual bird information | | | | | | HI serum antibody titre | | |
|---|---|---|---|---|---|---|---|---|
| English name / local name | Latin name | Taxonomic Order | Individual identification | Weight | Vaccine Dose (ml) | Pre-vacc | Post-1st vacc | Post-2nd vacc |
| | | | | | | | | |

| Adverse Individual effects | | Mortality | |
|---|---|---|---|
| Local | general | Direct (catching/handling) | Delayed (specify cause of death) |
| | | | |

| Information required from the Laboratory | | | | |
|---|---|---|---|---|
| Virus strain of vaccine | Antigens (virus strains) used in HI test | Cut-off or end-titre point used as a measure of vaccine efficacy | Reference serum used | (reference to) Methodology |
| | | | | |

R.

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# NL.

## Nederlandse samenvatting

## Opkomende infectieziekten

Opkomende en opnieuw opkomende infectieziekten hebben de afgelopen 20 jaar een grote invloed gehad op menselijke en dierlijke gezondheid, voedselvoorraden, economieën, biodiversiteit, en het milieu. Geschat wordt dat 75% van alle opkomende infectieziekten bij de mens zoönotisch (pathogenen van dierlijke oorsprong die ook de mens kunnen besmetten) en van virale oorsprong zijn. Sinds 1980 zijn er meer dan 35 infectieziekten opgekomen bij de mens, waarvan AIDS, SARS, West Nile virus infectie, Ebola virus infectie, en vogelgriep wel het meest in de publieke belangstelling hebben gestaan. Het recent onderkennen van het opduiken van deze ziekten kan deels worden toegeschreven aan verbeterde diagnostische technieken, maar de voornaamste oorzaak moet toch worden gezocht in onze veranderende wereld. Belangrijke factoren van deze veranderingen in het menselijk gedrag zijn: (1) demografische veranderingen: exponentiële groei van de bevolking, wereldwijde urbanisatie, het indringen in leefgebieden van wilde dieren, (2) sociale veranderingen: globalisatie van de handel, vermeerdering van legale en illegale diertransporten en handel in wilde dieren (levende dieren en "bushmeat"), (3) veranderingen in ecosystemen: ontbossing, eutrophicatie van water en een kleinere populatie van roofdieren met ziektedragers als prooi; en (4) klimaatsveranderingen: het opwarmen van de aarde. Vee en pluimvee hebben zwaar geleden door directe mortaliteit en ruimen van dieren als gevolg van infectieziekten. Zo hebben H5N1 hoog pathogene aviaire influenza (HPAI) virus infecties geresulteerd in de dood van meer dan 220 miljoen vogels in 41 landen in de laatste paar jaar. Het effect van opduikende infecties is van speciaal belang voor bedreigde diersoorten, die door uitbraken aan de rand van uitsterven kunnen komen te staan.

## Serologische onderzoeken naar antilichamen tegen virale infecties

Het vroeg opsporen van uitbraken door uitgebreide surveillance in dieren naar (zoönotische) ziekteverwekkers en ziektepatronen is een kritiek onderdeel van het beheersen of voorkomen van uitbraken van deze infecties. Het verbinden van uitgebreide surveillance studies naar ziekteverwekkers en ziektebeelden in wilde en gedomesticeerde dieren aan soortgelijke surveillances bij de mens kan een belangrijke bijdrage leveren aan het opsporen en bestrijden van opduikende (zoönotische) infecties. Er zijn veel al dan niet wetenschappelijke projecten waarin niet-gedomesticeerde dieren worden gevangen, en het verzamelen van bloed en opslaan van serum kunnen van nut blijken bij het vroeg opsporen van uitbraken, of in retrospectieve studies. Serologische studies naar antilichamen tegen verschillende virussen kunnen een beeld geven van het vóórkomen van virale infecties in wilde populaties. Alhoewel het effect van deze virussen in de verschillende wilde soorten vaak

niet bekend wordt door dit soort onderzoekingen, kunnen ze toch een rol spelen in het vroeg opsporen van uitbraken, en informatie verstrekken over de ziekteverwekkers bij de betreffende diersoorten in het onderzochte gebied, en zo eventuele rehabilitatie- of translocatieprojecten van belangrijke informatie voorzien.

Wij hebben dit soort bloed- of serumbanken meerdere keren kunnen gebruiken. Op verschillende locaties in Canada werden serum- en bloedmonsters bewaard van de een groot aantal niet-gedomesticeerde roofdieren en zeezoogdieren - poema (*Felis concolor*), lynx (*Felis lynx*), Amerikaanse das (*Taxidea taxus*), vismarter (*Martes pennanti*), veelvraat (*Gulo gulo*), wolf (*Canis lupus*), zwarte beer (*Ursus americanus*), grizzly beer (*Ursus arctos*), ijsbeer (*Ursus maritimus*), walrus (*Odobenus rosmarus*), en beloega (*Delphinapterus leucas*) - die tussen 1984 en 2000 verzameld waren. Deze monsters konden gebruikt worden voor de onderzoekingen naar de prevalentie van antilichamen tegen verschillende virussen in deze soorten (Hoofdstuk 2.2.1.). Wij hebben antilichamen tegen verschillende virussen aangetoond in soorten waarin deze of verwante virussen niet eerder zijn beschreven, zoals antilichamen tegen canine adenovirus type 2 (CAV-2) in walrussen; tegen zeehondenziekte virus/phocine distemper virus (PDV) in zwarte beren, grizzly beren, ijsberen, lynxen en wolven, tegen dolfijnen morbilli virus (DMV) in grizzly beren, ijsberen, walrussen en wolven; tegen canine para-influenza virus in zwarte beren en vismarters, en tegen dolfijnen rhabdovirus (DRV) in beloegas en walrussen. Verschillen in het vóórkomen van de verschillende ziekteverwekkers kan gerelateerd zijn aan verschillen in populatiedichtheid, en de verdeling van andere diersoorten (inclusief gedomesticeerde diersoorten) die bronnen van infectie kunnen vormen. Als voorbeeld: de veelvraten in deze studie kwamen uit een afgelegen gebied, waar een verminderde blootstelling de reden zou kunnen zijn voor de afwezigheid van antilichamen tegen alle ziekteverwekkers waarop we hebben getest. In verschillende soorten waren de antilichamen gericht tegen meer dan één van de morbillivirussen (hondeziekte virus/canine distemper virus [CDV], PDV en DMV) waartop werd getest. De aanwezigheid van antilichamen tegen de morbillivirussen welke gewoonlijk in verband worden gebracht met infecties in zeezoogdieren (PDV en DMV) in op het land levende roofdieren was verrassend. Alle morbillivirussen kruisreageren in serologische testen, maar de hoogste antistoftiters worden vrijwel altijd gevonden tegen het homologe virus, waardoor het waarschijnlijk is dat in deze dieren naast een infectie met CDV, infecties met PDV- of DMV-achtige virussen ook voorkomen.

Serum opgeslagen in Frankrijk kon worden gebruikt om het potentiële risico van virale infecties op een kleine populatie van de Europese nerts (*Mustela lutreola*) in een gebied waarin in de toekomst mogelijk gefokte dieren worden vrijgelaten voor het behoud van deze soort. De Europese nerts is een klein roofdier uit de familie der marterachtigen die zowel op het land als in het water actief is. Het is een ernstig bedreigde diersoort die vroeger voorkwam in grote delen van Europa, en nu nog slechts in twee gefragmenteerde

populaties (een kleine in Zuid-west Frankrijk en Noord-west Spanje, en een grotere in Oost Europa) voorkomt. Oorzaken van de achteruitgang zijn onder andere stropen, habitat verlies, competitie met de grotere en sterkere Amerikaanse nerts (*Mustela vison*) die in dezelfde gebieden voorkomt door ontsnappingen van pelsdierboerderijen en zich nu in Europa heeft gevestigd, en ziekten. Fokprogramma's zijn opgezet in Estland en Spanje met als doel het vrijlaten in de gebieden waar ze vroeger voorkwamen, als middel om deze soort te behouden in het wild. Om de mogelijke rol van infectieziekten op de achteruitgang van de Europese nerts te onderzoeken werd een serologisch onderzoek in 480 vrij levende marterachtigen van vijf verschillende soorten uitgevoerd die tussen 1996 en 2003 in Zuid-west Frankrijk waren gevangen. Antilichamen tegen CDV werden gevonden in alle soorten (Europese nerts 8.7%, Amerikaanse nerts 4.5%, bunzing [*Mustela putorius*] 20.4%, steenmarter [*Martes foina*] 33.3%, en boommarter [*Martes martes*] 5%). Antilichamen werden vaker gevonden in steenmarters en bunzingen, mogelijk als gevolg van de leefomgeving van deze soorten, die veel dichter bij de mens (en dus ook niet-gevaccineerde gedomesticeerde dieren) is dan die van de andere soorten. Antilichamen tegen CAV werden gevonden in alle soorten behalve de boommarter. Antilichamen tegen canine para-influenza virus (CPIV) kwamen voor in lage aantallen Europese nertsen, Amerikaanse nertsen en bunzingen, maar in geen van de *Martes* soorten. Het vinden van antilichamen tegen hondsdolheid/rabiës virus (RV) in drie dieren was verrassend, aangezien het onderzoeksgebied vrij van RV is verklaard. Een mogelijke verklaring zou kunnen zijn dat dit antilichamen tegen de nauw verwante vleermuislyssa virussen waren, die met de gebruikte methode niet kunnen worden onderscheiden van antistoffen tegen RV. Een andere mogelijke en misschien wel meer waarschijnlijke verklaring is, dat het hier gaat om niet-specifieke reacties, omdat de gevonden antilichaam hoeveelheden rond de detectiegrens lagen. De hogere prevalentie van antilichamen tegen CDV dan die tegen de overige virussen, en het voorkomen van antilichamen tegen CDV in alle soorten suggereert dat dit virus een belangrijk effect kan hebben in de zeer vatbare soorten van vrij-levende marterachtigen, hetgeen verstrekkende gevolgen kan hebben voor het behoud van de ernstig bedreigde Europese nerts.

Alle families van de taxonomische orde Carnivora (roofdieren) zijn voor zover bekend vatbaar voor infectie met CDV. Infectie met dit morbillivirus is een van de meest belangrijke ziekten die sterfte veroorzaken. Mortaliteit en morbiditeit verschillen per soort, maar sterfte kan tot 100% van geïnfecteerde dieren oplopen. Uitbraken van infectie met CDV en de nauw-gerelateerde PDV hebben grote uitbraken met hoge sterfte veroorzaakt in naïeve populaties op het land levende roofdieren en zeezoogdieren, en hebben met name plaats gevonden in gefragmenteerde populaties.

Antilichamen tegen de verschillende morbillivirussen werden in sera van dieren door heel Canada en door het hele onderzoeksgebied in Frankrijk gevonden, hetgeen nogmaals bevestigt dat CDV wijdverspreid onder vrij-

levende roofdieren voorkomt. Er zijn nooit massale sterftes als gevolg van een morbillivirus infectie gerapporteerd in vrij-levende roofdier populaties in Canada of Frankrijk. Dit zou kunnen komen door de endemiciteit van het virus, een verminderde gevoeligheid, of een lage populatiedichtheid van sommige soorten (zoals beren). Een andere mogelijkheid is dat sterfte van sommige van de meer vatbare soorten, zoals de marterachtigen, niet opvalt doordat de dieren veel kleiner zijn, en daardoor zieke en dode dieren niet gevonden worden.

## Klinische symptomen van PDV infectie in gewone zeehonden

In de zomer van 2002 werd de Noord-Europese gewone zeehond (*Phoca vitulina*) populatie getroffen door een PDV epidemie – zo'n 22 000 zeehonden stierven als gevolg van dit virus. Klinische symptomen werden gedocumenteerd van zeehonden die binnengebracht werden met klinische verschijnselen in de zeehondencrèche in Pieterburen gedurende de epidemie, waarvan bij sectie door middel van RT-PCR werd vastgesteld dat ze geïnfecteerd waren met PDV. De meest prominente klinische verschijnselen waren respiratoire symptomen (in 100%), conjunctivitis (in 70%), en neurologische symptomen ontwikkelden zich in ongeveer de helft van deze zeehonden. Ernstige neurologische verschijnselen waren één van de euthanasie-criteria gedurende de epidemie, en een groot aantal zeehonden die geëuthanaseerd. werden buiten de crèche werden niet in deze studie betrokken, door afwezigheid van een complete dataset. De klinische symptomen die gedurende de epidemie van 1988 werden gedocumenteerd suggereerden een veel lagere incidentie van neurologische symptomen, dus was het veelvuldig voorkomen van neurologische symptomen onverwacht in 2002. Op het moment van binnenkomst had een groter aantal van de zeehonden een imuunglobuline G (IgG) antistof titer, dan een IgM titer. Dit suggereert dat de zeehonden binnen werden gebracht in een later stadium van de ziekte, en dat IgG titers bij een uitbraak in een naïve populatie nuttiger zijn bij het diagnosticeren van PDV infectie van jonge zeehonden (uit een naïeve populatie) bij aankomst in een opvangcentrum dan IgM titers. De hoogte van IgG titers bleek niet gecorreleerd te zijn met het ontbreken van klinische symptomen, of met een langere overlevingsduur. De gerapporteerde uitputting van de lymphoïde organen in dode zeehonden tijdens de epidemie werd niet gereflecteerd in het bloedbeeld bij de zeehonden op de dag van aankomst bij de zeehondencrèche. De hematologische waarden lieten verder een absolute granulocytose, trombocytose, anemie, en een verhoging van de totale witte bloedcellen zien.

Recente technologische ontwikkelingen vergemakkelijken een snelle reactie op uitbraken door middel van snelle identificatie van het betrokken agens en verbeterde diagnostische methoden, maar ook door de beschikbaarheid van specifieke therapieën en vaccinaties. Het gebruik hiervan zal zich doorgaans moeten beperken tot in gevangenschap gehouden

dieren.

## Vaccinatie tegen morbillivirus infecties

Vaccinatie is een van de middelen die gebruikt kan worden voor het beschermen van individuele dieren tegen infectieuze ziekten, maar ook voor het bestrijden van grootschalige uitbraken. In dierentuinen en in opvangcentra voor wilde dieren kunnen infectierisico's geminimaliseerd worden door een verminderde mogelijkheid van contact met niet-gevaccineerde wilde of gedomesticeerde dieren door middel van afscheidingen, en het gebruiken van strenge hygienische maatregelen en quarantaineprotocollen voor dieren voordat zij bij aan de collectie worden toegevoegd. In rehabilitatiecentra is de gezondheids- en immunologische status van dieren veelal gecompromiteerd, en is de populatiedichtheid en daardoor intra- (of inter-) soort contact doorgaans hoger dan die in het wild. Wanneer biologische veiligheidsprotocollen niet goed worden nageleefd, ontstaat dus een hoger infectierisico.

Hoewel gedomesticeerde honden veilig kunnen worden gevaccineerd met een vaccin dat gemodificeerd (ofwel verzwakt) levend (MLV) CDV bevat, is er een grote variatie in reactie op deze vaccins in niet-gedomesticeerde dieren. Het virus in deze vaccins is gemodificeerd zodat het nog steeds een infectie in honden veroorzaakt, maar zonder ziekteverschijnselen te veroorzaken. Deze modificatie blijkt voor een aantal niet-gedomesticeerde dieren niet voldoende te zijn, waardoor de dieren toch een klinische infectie doormaken, soms met dodelijke afloop. Op dit moment zijn vrijwel alle commercieel beschikbare morbillivirus vaccins gebaseerd op MLV's. Veiligere alternatieven zijn geïnactiveerde virus vaccins, die geen infectie kunnen veroorzaken, maar hun vermogen om een adequate immunologische response te stimuleren is doorgaans evenredig verminderd. Op dit moment zijn er geen geïnactiveerde CDV vaccins commercieel verkrijgbaar, omdat de werking minder is in vergelijking met de MLV vaccins, en de markt voor niet-gedomesticeerde dieren te klein is. Andere veilige alternatieven zijn experimentele sub-unit vaccins, en recombinant vaccins die kanariepokkenvirus als een drager hebben. Recombinant CDV vaccins zijn momenteel geregistreerd in de Verenigde Staten, maar mogen in de EU niet gebruikt worden aangezien het niet-geregistreerde genetisch gemodificeerde organismen zijn.

Vaccins worden in het algemeen niet geproduceerd voor niet-gedomesticeerde dieren, en er is bij het gebruik van deze vaccins in deze soorten dus altijd een extra risicofactor als gevolg van het niet-geregistreerde gebruik. Omdat bedreigde niet-gedomesticeerde diersoorten doorgaans niet in challenge experimenten kunnen worden gebruikt vanuit een natuurbehoud perspectief, worden vaccin-geïnduceerde antistoftiters gebruikt als een surrogaat om vaccin-effectiviteit te meten. In gedomesticeerde dieren is vastgesteld dat er een correlatie is tussen de hoogte van de geïnduceerde virus neutraliserende antistof titer en de mate van bescherming tegen infectie en ziekte.

Een goed voorbeeld van de voordelen van strenge hygiëne- en quarantainemaatregelen in combinatie met veilige en effectieve vaccinatie werd duidelijk in de zeehondencreche tijdens de PDV epidemie van 2002. Zeehonden worden bij binnenkomst gevaccineerd met een CDV-immuunstimulerend complex (CDV-ISCOM) vaccin (oorspronkelijk ontwikkeld tijdens de PDV epidemie van 1988), en daarna gedurende 30 dagen in quarantaine geplaatst van waaruit ze bij de overige dieren worden geplaatst als vaccin-geïnduceerde CDV-specifieke antistoftiters voldoende hoog zijn bevonden. De nauwe verwantschap van CDV en PDV zorgt voor kruisbescherming tussen beide morbillivirussen. Zeehonden die de quarantaine ruimtes verlieten, maar nog wel aanwezig waren in de crèche op het moment dat PDV-geïnfecteerde dieren werden binnengebracht, vertoonden geen PDV-gerelateerde klinische verschijnselen gedurende hun verblijf in de crèche.

## Vaccinatie van Europese nertsen tegen CDV

De ernstig bedreigde Europese nerts is zeer vatbaar voor infectie met CDV, en klinische vaccin-geinduceerde infecties zijn beschreven in deze soort. In een fokprogramma in Estland werden de veiligheid en effectiviteit van een experimenteel CDV-ISCOM vaccin en een in de VS geregistreerd recombinant vaccin (Purevax™, Merial, Duluth, USA) geëvalueerd in Europese nertsen (Hoofdstuk 2.3.1.). Beide vaccins waren veilig, en induceerden virus neutraliserende antilichamen tegen CDV, al deed het ISCOM vaccine dit sneller, en waren de geometrisch gemiddelde titers (GMT) hoger dan die na vaccinatie met het recombinant vaccin. Een jaar na de oorspronkelijke drie vaccinaties waren de antistoftiters gedaald, maar nog steeds aantoonbaar, alhoewel de GMT hoger was in de ISCOM groep, en was het percentage dieren met hoge antistof titers ($\geq 80$) 100%, vergeleken met veel lagere percentages in de recombinant vaccin groep.

Serum antistof titers bepaald door middel van een enzyme-linked immunosorbent assay (ELISA) volgden een vergelijkbaar patroon als die bepaald door middel van een virus neutralisatie (VN) test, maar waren hoger. De VN test wordt beschouwd als de gouden standaard voor het bepalen van immuniteit tegen morbillivirussen, omdat deze de functionele neutraliserende antilichamen gericht tegen de haemagglutinine (H) en fusion (F) eiwitten op het virus oppervlak meet. De ELISA, gebaseerd op in celkweek geproduceerde virus eiwitten, meet niet alleen antilichamen gericht tegen de H en F eiwitten, maar vooral ook antilichamen gericht tegen het nucleoproteïne (NP), en cellulaire componenten. Antilichamenichamen gericht tegen het NP leveren geen bijdrage aan virus neutralisatie, en worden dus niet aangetoond in een VN test. Desalniettemin worden deze antilichamen wel in grote hoeveelheid geproduceerd na infectie of vaccinatie met de klassieke vaccins, maar niet na vaccinatie met de gebruikte ISCOM en recombinant vaccins .

Deze resultaten laten zien dat beide vaccins veilig zijn en een immunologische reactie induceren in Europese nertsen, alhoewel op basis van de antistof titers het CDV-ISCOM vaccin verwacht mag worden dat dit een betere bescherming biedt tegen CDV infectie gedurende minimaal een jaar. Andere beschermende mechanismen die mogelijk worden geïnduceerd door vaccinatie met deze vaccins, zoals inductie van cellulaire immuniteit moeten ook worden overwogen. Uitsluitsel over de effectiviteit van vaccins kan worden verkregen door middel van challenge infecties – wat in deze bedreigde soort onwenselijk is.

## Vaccinatie van Afrikaanse wilde honden tegen CDV

De Afrikaanse wilde hond (*Lycaon pictus*) is een van de meest bedreigde soorten van de taxonomische familie Canidae. Vroeger had deze soort een uitgebreid leefgebied door 39 Afrikaanse landen ten zuiden van de Sahara, maar op dit moment wordt hun aantal geschat op 3000-5500, verdeeld over 15 landen. De dramatische vermindering van deze soort is voornamelijk veroorzaakt door menselijk toedoen, zoals stropen, jacht, en verkeersongevallen, fragmentatie van leefgebieden, competitie met andere roofdieren, en ziekten. Het gefragmenteerde habitat heeft de populatiedichtheid veranderd, en gezorgd voor een grotere kans op inter-species contacten (inclusief niet-gevaccineerde gedomesticeerde honden) en blootstelling aan nieuwe ziekteverwekkers zoals CDV en rabiësvirus.

In een fokgroep Afrikaanse wilde honden in Mkomazi, Tanzania en in Artis Amsterdam Zoo werd de veiligheid en effectiviteit van het CDV-ISCOM vaccin en het recombinante vaccin (Purevax™, Merial, Duluth, USA) geevalueerd, zoals eerder werd gedaan in de Europese nerts. Met beide vaccins waren twee vaccinaties noodzakelijk voor het induceren van lage antistof titers. Na 2 vaccinaties waren de GMT en het percentage dieren met een hoge antistoftiter in de VN test laag voor beide vaccins (ISCOM groep: GMT=8 en 8% ≥ 80; recombinant groep: GMT=28 en 25% ≥ 80). De GMT getest met een ELISA na twee vaccinaties met het recombinante vaccin was vergelijkbaar met die in de Europese nerts, terwijl 2 doses van het ISCOM vaccin lagere GMT induceerden dan in de Europese nerts. Een jaar na vaccinatie was het percentage dieren met een hoge antistoftiter groter in de recombinant groep, in zowel ELISA als VN testen. De GMT in de recombinant groep was vergelijkbaar met die van de ISCOM groep in de ELISA (84 vs 80), maar hoger in de VN test (38 vs 6). De hogere antistoftiters in de dieren 1 jaar na vaccinatie vergeleken met die na 2 vaccinaties toont aan dat een "booster effect" optreedt na de derde vaccinatie, alhoewel dit niet geverifieerd kon worden omdat geen bloed was afgenomen na de derde vaccinatie.

De resultaten geven aan dat CDV-ISCOM vaccinatie CDV-specifieke antilichamen in Afrikaanse wilde honden induceert, maar dat deze niet effectief virus neutraliseren, en de vaccins dus waarschijnlijk weinig effectief zijn. Alhoewel beide vaccins veilig en weinig effectief zijn in het induceren

van VN antilichamen, kan aan de hand van de geïnduceerde antistof titers worden verwacht dat het recombinant vaccin een betere bescherming biedt gedurende minimaal een jaar na vaccinatie. Echter, beide vaccins induceren lage gemiddelde antistoftiters, en slechts een klein percentage van de dieren behaalt een voldoende hoge antistoftiter. Aan de hand van geïnduceerde serum antistoftiters in andere niet-gedomesticeerde soorten met dezelfde batch van dit CDV-ISCOM vaccin, lijkt de reden voor inductie van lage VN antistoftiters gezocht te moeten worden in het intrinsieke onvermogen van de wilde hond om adequaat te reageren op deze vaccins. De fragmentatie van het leefgebied en van de populaties van de Afrikaanse wilde hond kan hebben geresulteerd in een kleine genetische basis, wat negatieve gevolgen voor het immuunsysteem kan hebben gehad.

## Vaccinatie van carnivoren in Diergaarde Blijdorp tegen CDV

In Blijdorp worden een aantal roofdieren gehouden die vatbaar zijn voor infectie met CDV. Ze worden gevaccineerd met CDV-ISCOM volgens een standaard protocol: jonge dieren krijgen drie vaccinaties met drie weken tussentijd vanaf de leeftijd van ongeveer 10 weken, waarna jaarlijks wordt gevaccineerd met één dosis. Bloed wordt afgenomen vóór vaccinatie, en antistof titers worden bepaald door middel van ELISA en VN test. In de evaluatie van de door het CDV-ISCOM geïnduceerde immuniteit werden alleen dieren gebruikt die niet eerder waren gevaccineerd, omdat dit de resultaten zou beïnvloeden. Er kon geen vergelijking worden gemaakt met het recombinant vaccin, omdat gebruik hiervan niet is toegestaan in Nederland.

De kleine panda (*Ailurus fulgens*) behoort tot de taxonomische familie Ailuridae, en is zeer vatbaar voor infectie met CDV. Reeds in 1976 werd bij deze dieren vaccin-geïnduceerde CDV infectie beschreven. In Blijdorp werden panda's al gedurende langere tijd gevaccineerd met CDV-ISCOM, en data van 16 dieren waren beschikbaar. De GMT gemeten met ELISA was laag, en een aantal dieren had helemaal geen antilichamen. Echter, de laatste jaren reageerden de pandas met hogere antistoftiters, zowel in ELISA als VN test. Deze resultaten komen overeen met een verhoging van het totale antigeen gehalte in het toegediende vaccin van 5 µg/ml naar 10 µg/ml in de laatste 5 jaar. Overige soorten die in het hieropvolgende deel worden besproken werden allemaal gevaccineerd met het vaccin met het hogere antigeengehalte.

Manenwolven (*Chrysocyon brachyurus*) behoren tot de taxonomische familie Canidae, zijn zeer vatbaar voor CDV infectie, en zijn beschreven met vaccin-geïnduceerde CDV infectie. De CDV-ISCOM vaccinatie kon worden geëvalueerd in 2 dieren. Meerdere soorten van de Lutrinae subgroep van de Mustelidae zijn beschreven met CDV infectie: Europese otters (*Lutra lutra*), Aziatische kleinklauw otters (*Aonyx cinerea*), en Noord-Amerikaanse otters (*Lutra canadensis*). In Blijdorp konden we de CDV vaccinatie van 3 Aziatische klein-klauw otters, en 2 Europese otters evalueren. De Maleise civetkat (*Viverra tangalunga*), behoort tot de familie Viverridae, waarin CDV infectie is

beschreven. In Blijdorp konden wij 5 civetkatten 3 jaar volgen, en 3 dieren 4 jaar.

De meeste soorten hadden minimaal twee doses CDV-ISCOM nodig voordat VN antilichamen worden geïnduceerd. De twee ottersoorten zijn de enige waarin antistof titers aantoonbaar zijn na één vaccinatie. Antistof titers ≥ 40 zijn aantoonbaar in alle dieren na twee vaccinaties. Alhoewel maar bij weinig dieren bloed is afgenomen na de derde vaccinatie, zijn er aanwijzingen dat antistoftiters verder omhoog gaan na de toediening van de derde dosis. Een jaar na vaccinatie daalt de GMT in alle soorten, maar deze wordt weer omhoog gebracht door (een) boostervaccinatie (s), en blijft op vergelijkbare hoogte met jaarlijkse hervaccinatie met één vaccindosis. GMTs getest met ELISA zijn doorgaans hoger of gelijk aan de GMT getest met VN. Grote verschillen tussen de gemeten ELISA en VN titers, zoals gezien bij de Afrikaanse wilde hond, werden niet gezien in de soorten die in Blijdorp werden gevaccineerd.

## Conclusies: vaccinatie van niet-gedomesticeerde roofdieren tegen morbillivirussen

De aanwezigheid van antilichamen tegen een aantal virale ziekteverwekkers werd aangetoond in populaties van vrij-levende roofdieren in Canada en Frankrijk. Antistof titers tegen CDV hadden de hoogste prevalentie van alle virussen die werden getest, en het is bekend dat dit virus, en het nauwverwante PDV grote epidemieën met hoge mortaliteit hebben veroorzaakt in vrij-levende niet-gedomesticeerde roofdieren en zeezoogdieren. De klinische symptomen gedocumenteerd in jonge gewone zeehonden tijdens een PDV-epidemie lieten een ander beeld zien dan gerapporteerd in geïnfecteerde dieren tijdens een soortgelijke PDV-epidemie 14 jaar eerder, hetgeen het belang van goed gedocumenteerde klinische symptomen benadrukt voor het stellen van een vroege waarschijnlijkheidsdiagnose. Een vroege identificatie van de verantwoordelijke ziekteverwekker is essentieel in het bestrijden van uitbraken. Vaccinaties kunnen deel uitmaken van de bestrijdingsstrategieën tijdens een uitbraak. CDV-ISCOM vaccinatie is veilig en effectief in het induceren van specifieke antistof titers in vrijwel alle soorten waarin het is getest. Verschillen in vatbaarheid voor CDV infectie zijn beschreven voor verschillende soorten van niet-gedomesticeerde dieren, en verschillen in de antistofproductie in respons op vaccinatie is eveneens gezien in deze studies. Antistoftiters worden op peil gehouden door jaarlijkse hervaccinatie met één dosis. Het gebruik van het recombinant vaccin was veilig en effectief in de Europese nerts, terwijl geen van beide vaccins effectief waren in Afrikaanse wilde honden. De mate van bescherming, gebaseerd op antistof titers, was hoger dan dat geïnduceerd door het CDV-ISCOM vaccin in de Afrikaanse wilde hond, maar lager in de Europese nerts. Deze studies geven een beter inzicht in het effect van veilige vaccinaties in niet-gedomesticeerde roofdieren, maar op dit moment is er nog steeds geen veilig en effectief commercieel

verkrijgbaar CDV vaccin dat gebruikt kan worden in niet-gedomesticeerde dieren in de EU.

## Vaccinatie van niet-gedomesticeerde vogelsoorten tegen hoogpathogene aviaire influenza virussen

Aviaire Influenza virussen zijn type A influenza virussen, negatief-strengs RNA virussen die behoren tot de familie van *Orthomyxoviridae*. Ze worden onderverdeeld op basis van de oppervlakte eiwitten: haemagglutinine (H, dat zorg draagt voor de binding van het virus aan en het binnendringen van de gastheercel), en neuraminidase (N, een eiwit dat zorgt voor een efficiënte cel tot cel verspreiding na vermenigvuldiging van het virus). Er zijn totaal 16 verschillende H subtypen, en 9 verschillende N subtypen, die theoretisch in allerlei combinaties kunnen voorkomen. Aviaire influenza virussen zijn verder onder te verdelen op grond van hun biologische eigenschappen in laag-pathogene aviaire influenza (LPAI) virussen die geen tot milde ziekteverschijnselen veroorzaken, en hoog-pathogene aviaire influenza (HPAI) virussen die massale sterfte onder pluimvee veroorzaken (ook bekend als klassieke vogelpest). HPAI virussen zijn altijd van de H5 en H7 subtypen, al zijn niet alle H5 en H7 subtypen hoogpathogeen. Tot 2002 werd aangenomen dat HPAI een pluimveeziekte was, en dat wilde (water-) vogel soorten de natuurlijke gastheren waren van LPAI virussen, en dat deze geen ziekteverschijnselen vertoonden na aviaire influenza infectie. In 2002 was er een uitbraak van HPAI virus van het H5N1 subtype in wilde trekvogelsoorten en in gevangenschap gehouden watervogels. Sindsdien heeft dit subtype ten minste 105 verschillende vogelsoorten uit 14 verschillende taxonomische ordes geïnfecteerd, en heeft het zich op een niet eerder vertoonde wijze verspreid door Azië, Europa en Afrika.

In de EU is er momenteel een non-vaccinatiebeleid met betrekking tot routine vaccinatie van pluimvee tegen aviaire influenza. In plaats van vaccinatie worden uitroeïngsmaatregelen gebruikt tijdens een uitbraak in pluimvee, waaronder vallen: (langdurig) ophokken, ruimen van dieren op het geïnfecteerde bedrijf, en afhankelijk van de pluimveedichtheid in het gebied en de epidemiologische situatie preventief ruimen op dichtbijzijnde bedrijven, en eventueel noodvaccinaties. Sinds 2003 zijn wereldwijd meer dan 220 miljoen vogels geruimd om H5N1 influenza-uitbraken de kop in te drukken.

De standaardmaatregelen zoals die voor pluimvee worden gehanteerd (ophokken en ruimen) kunnen zeer nadelig zijn voor het welzijn van dieren in dierentuinen en voor fokprogramma's van bedreigde vogelsoorten. Op grote schaal ruimen van dierentuin-collecties met bedreigde en zeldzame vogelsoorten zou niet wenselijk zijn vanuit een natuurbeschermings oogpunt. Op Europees niveau kan worden afgeweken van grootschalig preventief ruimen als dieren langdurig kunnen worden opgehokt, en uitvoerig getest worden. Echter, de meeste dierentuinen zullen niet de mogelijkheden hebben om hun

gehele vogelpopulatie voor langere tijd op te hokken, en veel soorten zullen zich niet kunnen aanpassen aan het ophokken met toegenomen stress en welzijnsproblemen en verhoogde blootstelling aan andere ziekteverwekkers zoals de verwekkers van aspergillose en "bumblefoot". Om de waardevolle collecties van dierentuinen, onder meer bestaande uit bedreigde en zeldzame vogelsoorten, te kunnen beschermen tegen infectie met HPAI virus, preventieve ruiming en langdurig ophokken, mogen deze onder strenge toezichtsmaatregelen worden gevaccineerd.

Vaccinatie van vogels in dierentuinen als extra toevoeging aan de preventieve beschermende maatregelen tegen HPAI virus infectie (terwijl ophokregels kunnen worden versoepeld) werd voor het eerst in 2003 toegepast toen zich in Nederlands pluimvee een uitbraak van HPAI virus van het H7N7 subtype voldeed. Vogels in dierentuinen werden gevaccineerd met een geïnactiveerd geadjuveerd (H7N1) vaccin, waarvan de gebruikte virusstam een grote homologie bezat ten opzichte van het veldvirus (Hoofdstuk 3.1.). Na vaccinatie had 81.5% een HI antistof titer ≥ 40, een beschreven antistof titer die in pluimvee correleert met bescherming, en die ook bij de gevaccineerde vogels in de dierentuinen als beschermend wordt beschouwd, aangezien infectie experimenten niet in al deze (bedreigde) vogelsoorten zijn gedaan. De totale GMT bij deze vogels was 190. Verschillen tussen de taxonomische ordes werden gezien: vertegenwoordigers van de Anseriformes (watervogels), Galliformes (hoenderachtigen) and Phoenicopteriformes (flamingo's) hadden hogere GMT's en percentages met een titer ≥ 40 dan de andere ordes. Een afname van de geïnduceerde antistoftiter met een toename van het lichaamsgewicht werd ook gezien. Serum antistoftiters werden gemeten door middel van een haemagglutinatie inhibitie (HI) test, en deze kwamen goed overeen met VN titers tegen het veld virus. Dit werd als additionele maat van immunogeniteit gebruikt. De relatief hoge antistoftiters in de verschillende taxonomische ordes onderstreepten de waarde van vaccinatie als een additionele preventieve maatregel tegen HPAI infectie in dierentuinen.

In 2005 besloten de Nederlandse dierentuinen als eerste om, volgens Europees beleid (Decision 2005/744/EC) vogels te vaccineren tegen het steeds dichterbij komende HPAI virus van het H5N1 subtype (Hoofdstuk 3.2.). Vogels werden gevaccineerd met een geïnactiveerd geadjuveerd H5N2 vaccin. De dosis werd aangepast aan het gemiddeld gewicht per soort, op geleide van de twee jaar eerder verkregen data van H7 vaccinatie. De vaccinstam had een grote gelijkenis met de veldstam in Europa op dat moment. Vaccinatie bleek veilig, en induceerde antistof titers in alle verschillende soorten, al werden wel verschillen tussen taxonomische ordes en soorten gezien. Na twee vaccinaties hadden 80% van alle vogels een HI titer tegen de vaccin stam ≥ 40, en was de totale GMT 190. Serum HI titers tegen de veldstam hadden een zelfde verloop, maar waren in het algemeen lager. De breedte van de antistofrespons werd daarna aangetoond door het meten van serum HI titers tegen vier prototype stammen uit verschillende antigene "clades" van H5N1 virussen. De antigene en genetische eigenschappen van virussen

kunnen worden gebruikt om ze in "clades" in te delen op een antigene kaart, waarop de verwantschap tussen de verschillende stammen duidelijk kan worden weergegeven. In combinatie met epidemiologische data wordt deze informatie gebruikt om o.a. virusstammen te selecteren die gebruikt kunnen worden als kandidaten voor humane pandemische vaccins – stammen waarvan het haemagglutinine een hoge antigene verwantschap heeft met veldstammen geven de meeste bescherming. Op basis van de breedte van hun antistofrespons werden de dierentuinvogels in twee groepen verdeeld. Groep 1 (Anseriformes, Galliformes, Phoenicopteriformes, Psittacciformes [papagaaiachtigen] en Struthioniformes [struisvogelachtigen]) lieten een zeer brede respons zien met een voorspelde bescherming tegen toekomstige virus stammen die zich tot op 12 antigene eenheden van het gebruikte vaccin bevinden. Vogels in groep 2 (Ciconiiformes [ooievaarachtigen], Gruiformes [kraanvogelachtigen], Pelecaniformes [pelikaanachtigen] en Sphenisciformes [pinguins]) hadden lagere HI titers tegen de prototype stam van de verst afgelegen antigene clade (A/Indonesia/5/05).

In navolging van de Nederlandse dierentuinen werden vogels in dierentuinen in 13 Europese landen tegen HPAI H5N1 virus gevaccineerd, en werd in 2006 een werkgroep opgezet van de european Food Safety Authority (EFSA) om een wetenschappelijke beoordeling van de effectiviteit van vaccinaties in dierentuinen te maken. In totaal werden 44721 vogels gevaccineerd, en individuele data van 4718 vogels (374 soorten van 19 taxonomische ordes) werden door de lidstaten (MS) beschikbaar gesteld. Verschillen in vaccinatie schema's, doseringen, route van vaccinatie, verschillen in methodologie (er bestaan geen internationale referentie standaarden of standaardisatie), het gebruik van verschillende vaccins[1] in verschillende taxonomische ordes, en het soms incompleet rapporteren van resultaten bemoeilijkten de evaluatie. Alhoewel de meeste landen serum antistoftiters rapporteerden die bij 4 of 8 begonnen, werden de resultaten geëvalueerd voor titers ≥ 16 en 32, wat beschreven beschermende titers zijn in kippen.

Na toediening van twee doses van de H5 en H7 vaccins die geregistreerd zijn voor gebruik in kippen werden verschillen in effectiviteit gezien, op basis van verschillen in serum HI antistof titers. Drie van de vijf vaccins induceerden relatief hoge GMT's en percentage seroconversie in de meerderheid van de vogelsoorten. Duidelijke verschillen werden waargenomen tussen soorten en taxonomische ordes. De route van vaccinatie leek geen invloed te hebben op de resultaten, en voor de meeste soorten was de dosis die is aanbevolen voor pluimvee voldoende. In sommige zwaardere soorten

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[1]

Vaccin A: H5N9 (A/turkey/Wisconsin/68).
Vaccin B: H5N2 (A/duck/Pottsdam/1402/86).
Vaccin C: H5N2 (A/chicken/Mexico/232/94/CPA).
Vaccin D: H5N9 (A/chicken/Italy/22A/98).
Vaccin E: H5N9 (A/chicken/Italy/22A/H5N9/1998).

bleek een hogere dosis noodzakelijk om voldoende hoge antistoftiters te induceren (b.v. in struisvogels 10-voudige dosis voor pluimvee: 10 x 0.25 ml). Echter, een te hoge dosis toegediend op één plek (struisvogels met 10 ml) bleek een negatief effect te hebben op de antistof titer, terwijl ook vaker locale reacties werden waargenomen.

Er waren indicaties dat één vaccinatie voldoende zou kunnen zijn om hoge antistoftiters te induceren in ten minste twee taxonomische ordes. Echter, twee vaccinaties gaven consistent hoge titers te zien in de meeste soorten. Er waren ook aanwijzingen dat antistoftiters zes maanden na vaccinatie aanwezig bleven in verschillende soorten, al waren slechts data van een beperkt aantal dieren beschikbaar. Schadelijke bijwerkingen en mortaliteit in verband met vaccinatie werden zelden gezien, en waren voornamelijk veroorzaakt door stress en trauma tijdens het vangen en hanteren. Er bleken aanmerkelijke verschillen in mortaliteit per dierentuin, hetgeen het belang van zorgvuldig vangen en hanteren van de vogels illustreert.

Een jaar na vaccinatie met het H5N2 vaccin werden vogels in Nederlandse dierentuinen voor de derde maal gevaccineerd met hetzelfde vaccin, en konden antistof titers worden bepaald (Hoofdstuk 3.3.).

Een van de problemen tijdens de evaluatie van alle Europese data was gerelateerd aan het vergelijken van data tussen verschillende laboratoria. Sera verzameld tijdens het hervaccineren en één maand later moest worden getest in het nationaal veterinaire referentie laboratorium (CIDC Lelystad), terwijl voorgaande bepalingen in het Eramus MC weren uitgevoerd. Om te bepalen of vergelijkbare titers werden verkregen, werden 141 bloedmonsters in beide laboratoria getest. De correlatie coëfficient tussen de uitkomsten van de twee laboratoria bleek 0.87 te zijn.

Van 44 vogels in Blijdorp konden titers van alle drie de bloedafname dagen worden beoordeeld. Antistoftiters liepen duidelijk terug in het jaar na vaccinatie: terwijl 80% een positieve titer (≥8), en 68 % een hoog positieve titer (≥ 32) hadden na twee vaccinaties, waren deze getallen een jaar later respectievelijk 61% en 30%. Vier weken na thervaccinatie waren de percentages hoger dan na twee vaccinaties (93% en 77%), al was de GMT lager dan een jaar eerder (88 vs 66).

Zoals ook werd opgemerkt na twee vaccinaties, was er weer een verschil in respons tussen de verschillende taxonomische ordes. Gebruik makend van data van alle vogels getest in Blijdorp (8 ordes) waren er vier die een jaar na vaccinatie geen aantoonbare antistoftiters hadden, en was er maar één orde (Phoenicopteriformes) waarvan de GMT hoger dan 40 was. Vier weken na hervaccinatie hadden totaal 74% van de vogels, en zes van de acht ordes een titer > 40.

Twee jaar na twee vaccinaties met een H7 vaccin, zonder hervaccinatie een jaar later, bleek de antistoftiter nog verder gedaald, zoals werd aangetoond door tevens H7 specifieke antilichamen te meten in sera van dieren die 2 jaar eerder waren gevaccineerd met een H7N1 vaccin.

## Vaccinatie van duiven tegen HPAI H5N1 virus

Van rotsduiven (*Columba livia*) en andere soorten van de taxonomische orde Columbiformes werd beschreven dat zij resistent zijn tegen, of in ieder geval minder gevoelig zijn voor infectie met HPAI virussen. Ook zouden zij lage serum antistoftiters na infectie en vaccinatie vertonen. Een verhoogde gevoeligheid van duiven voor meer recente stammen van het H5N1 virus subtype, en katten die geïnfecteerd werden door het eten van een met H5N1 virus geïnfecteerde duif zijn recent aangetoond, alhoewel er geen vogel-vogel overdracht van het virus lijkt plaats te vinden.

Om te onderzoeken hoe gevoelig duiven zijn voor infectie met HPAI H5N1 virus, en of eventuele uitscheiding van virus verminderd kan worden door vaccinatie werden gevaccineerde en niet gevaccineerde duiven met hoge doses van één van twee stammen HPAI virus van het H5N1 subtype (respectievelijk A/Indonesia/5/05 en A/turkey/Turkey/1/05) uit twee recente antigene clades geïnfecteerd via inoculatie in de luchtpijp en slokdarm (Hoofdstuk 3.4.). De vaccinstam (H5N6) gebruikt in het vaccin had een grote mate van gelijkenis met de twee stammen. Alhoewel duiven inderdaad weinig gevoelig bleken te zijn voor infectie met deze zeer hoge doses HPAI H5N1 virus, trad toch enige morbiditeit en mortaliteit op in de ongevaccineerde groepen, terwijl gevaccineerde groepen beschermd waren tegen het ontwikkelen van klinische verschijnselen. Klinische verschijnselen waren voornamelijk van neurologische aard: ataxie, tremoren, opisthotonus en torticollis. Virus kon worden aangetoond in verschillende organen (hersenen, long, luchtzak, alvleesklier, milt, lever en dunne darm) door middel van RT-PCR op 3 en 27 dagen post-inoculatie (dpi), en kon worden geïsoleerd op 3 dpi (A/Indonesia/5/05: long, luchtzak, milt, dunne darm; A/turkey/Turkey/1/05: hersenen, long, luchtzak, alvleesklier, milt en lever). Door middel van immuunhistochemie kon de aanwezigheid van nucleoproteïne in de long, luchtzak en hersenen worden aangetoond. Infectieus virus werd geïsoleerd uit keel swabs tot 3 (A/turkey/Turkey/1/05) en 4 (A/Indonesia/5/05) dpi. Cloacaswabs bleven negatief tot 27 dpi. Uit gevaccineerde dieren kon alleen op 3 dpi uit de long virus worden geïsoleerd, was er minder virus aantoonbaar door middel van RT-PCR in de verschillende organen, terwijl het virus slechts tot de eerste dag na inoculatie uit de keelswabs kon worden geïsoleerd. Aangezien de toegediende toegediende dosis één dag eerder zeer hoog was, zou de aangetoonde (lagere) virustiters in de pharyngeale swabs een restant van het inoculum kunnen zijn, en bewijst dit niet dat er uitscheiding is opgetreden

## Conclusies: vaccinatie van niet-gedomesticeerde vogels tegen HPAI virussen

De implementatie van biologische veiligheidsmaatregelen is vooralsnog de voornaamste maatregel die dierentuinen tegen de introductie van aviaire influenza virussen kunnen gebruiken. Dit zal ook bijdragen aan het verminderen van het risico van eventuele verdere verspreiding, mocht het virus wel geïntroduceerd worden. Klinische en virologische controles dienen te worden uitgevoerd in het geval van een dreigende dichtbijzijnde uitbraak om te komen tot een vroege opsporing van het virus. Als biologische veiligheidsmaatregelen niet voldoende bescherming kunnen bieden aan vogels in dierentuinen tegen infectie met HPAI virussen afkomstig van wilde vogels – dit mede gebaseerd op een risico-analyse waarin ook welzijn van de vogels wordt betrokken- kunnen H5 of H7 vaccins geautoriseerd voor gebruik in pluimvee worden gebruikt. Recente data van wilde vogelmigratie en prevalentie van HPAI in deze vogels moeten worden meegenomen in de beslissing om al dan niet te gaan vaccineren.

Vaccinatie van dierentuinvogels met de thans in de EU geautoriseerde H5 en H7 vaccins is veilig en effectief gebleken in de meeste taxonomische ordes. Inductie van antistof titers is hierbij als effectiviteitscriterium gebruikt. Vaccins moeten worden toegediend op een manier die hoge antistoftiters induceert in de meerderheid van de gevaccineerde vogels. Alhoewel er aanwijzingen zijn dat één vaccin dosis voldoende is in sommige soorten, geven twee doses zekerheid van hoge titers in de meeste soorten. Behalve als duidelijk is aangetoond dat één vaccinatie voldoet, blijven twee vaccin doses aanbevolen.

Er zijn verschillen waargenomen in de effectiviteit van vaccinatie met de thans in de EU geautoriseerde vaccins tussen soorten en taxonomische ordes. Er is geen verschil tussen de gebruikte route van vaccinatie gebleken. Daarom kan men de keuze van de route van vaccinatie bij verschillende soorten laten afhangen van het gemak van toediening (bijvoorbeeld intramusculair met een blaaspijp bij struisvogels) of de aanwezigheid van grote subcutane luchtzakken hetgeen bij toediening van het vaccin hierin het effect van vaccinatie negatief zou kunnen beïnvloeden. Dit laatste zou bijvoorbeeld bij pelikanen een belangrijke overweging kunnen zijn. Om hoge antistoftiters in vogels op peil te houden lijkt een jaarlijkse hervaccinatie met 1 dosis voor de meeste soorten noodzakelijk te zijn.

Standaard-protocollen voor vaccinatie, bemonstering, uitvoering van laboratoriumtests en rapportage aan de EU moeten worden opgezet om de evaluatie van AI vaccinatie campagnes in vogels te vergemakkelijken. Inter-laboratorium standaardisatie van technieken, en de productie van internationale referentie standaarden zouden moeten worden gerealiseerd, om vergelijkingen tussen verschillende laboratoria en gebruikte vaccins mogelijk te maken.

Mortaliteit en schadelijke bijwerkingen ten gevolge van vaccinatie met de gebruikte vaccins waren laag in de dierentuinen van de EU lidstaten, en voornamelijk het gevolg van stress en trauma tijdens het vangen en hanteren van de vogels. Dierentuinen kunnen deze negatieve effecten dus verminderen door kundig en voorzichtig met de vogels om te gaan tijdens de AI vaccinatiecampagnes. Om indirecte verliezen als gevolg van verminderde broedresultaten tegen te gaan moeten waar mogelijk broedseizoenen worden vermeden. Evaluatie van serologische data kan uiteindelijk zorgen dat dieren minder vaak hoeven te worden gevangen. Als vaccinatieprotocollen gevalideerd zijn voor bepaalde vogelsoorten met bepaalde vaccins, zal een post-vaccinatie antistofbepaling niet meer noodzakelijk zijn. Verder onderzoek moet worden verricht naar de meest effectieve vaccinatie schema's en dosis per soort. Dit zou kunnen leiden tot een vermindering van het aantal vaccinaties in bepaalde vogelsoorten. Daarnaast zou het ontwikkelen van een vaccin dat verneveld kan worden (zoals gebruikt wordt bij het vaccineren van pluimvee tegen Newcastle disease virus) het hanteren van de vogels overbodig kunnen maken in de toekomst.

De vaccinatiecampagnes in de dierentuinen hebben zich toegespitst op het beschermen van vogels. Naast vogels zijn ook meerdere zoogdieren, waaronder katachtigen en honden, gedocumenteerd met HPAI H5N1 virus infectie. Alhoewel er op dit moment geen commercieel verkrijgbaar vaccin is dat zoogdieren beschermt, heeft een experimenteel recombinant vaccin hoge antistoftiters geïnduceerd in katten. Dit vaccin zou in de toekomst misschien ook in dierentuindieren gebruikt kunnen worden, al zijn de richtlijnen van de EU voor het gebruik van genetisch gemodificeerde organismen streng (zie ook CDV vaccinatie). Tot dan moeten deze dieren worden beschermd door het uitsluiten van infectie door middel van rauw pluimveevlees (gekookt vlees vormt geen gevaar voor infectie).

Infectiestudies hebben laten zien dat alhoewel duiven niet erg gevoelig zijn voor infectie met één van twee recente HPAI H5N1 stammen, zij na vaccinatie toch beschermd zijn tegen het ontwikkelen van klinische ziekteverschijnselen, verminderde virus verspreiding in de verschillende organen laten zien, en in mindere mate of geen virus uitscheiden.

De brede vaccin-effectiviteit in de vele verschillende soorten in dierentuinen illustreert het belang van vaccinatie als een van de maatregelen die gebruikt kunnen worden om niet-gedomesticeerde dieren te beschermen tegen de gevolgen van HPAI virus infectie, zoals preventief ruimen, en langdurig ophokken. Echter, verhoogde biologische veiligheids maatregelen in combinatie met virologisch onderzoek blijven onmisbaar in het bestrijden van uitbraken van HPAI.

Vaccinatie van vogels in dierentuinen moet worden gezien als een extra maatregel om ze te beschermen tegen HPAI virus infecties, terwijl het welzijn van deze vogels niet wordt benadeeld. Daarnaast voorkomt het dat dierentuinen een bron van virus kunnen worden. Desalniettemin blijven pluimvee bedrijven de grootste bron van HPAI virus, aangezien deze

vogels een aangetoonde hoge vatbaarheid hebben, grote hoeveelheden virus uitscheiden, en de populatiedichtheid groot is. Verhoogde biologische veiligheidsmaatregelen op pluimveebedrijven, verminderd contact tussen pluimvee en wilde vogels (zonder hierdoor het welzijn te benadelen), ruimen, en vaccinatie van pluimvee zullen belangrijkere stappen zijn in het beheersen van grootschalige uitbraken zoals de huidige uitbraak van het H5N1 virus.

## ALGEMENE CONCLUSIES

Het vroeg opsporen en de identificatie van uitbraken door uitgebreide surveillance in dieren naar (zoönotische) ziekteverwekkers en ziektepatronen is een kritiek onderdeel van het beheersen of voorkomen van uitbraken van deze infecties, en kan gebruikt worden voor her-introductie of translocatie programma's van bedreigde diersoorten. Vaccinatie kan een toegevoegde waarde hebben voor de biologische veiligheidsmaatregelen die gebruikt worden bij het voorkomen en bestrijden van virusinfecties van niet-gedomesticeerde dieren in gevangenschap. Twee groepen van virussen die verantwoordelijk zijn geweest voor grote uitbraken in niet-gedomesticeerde diersoorten, morbillivirussen en HPAI virussen, kunnen ook bestreden worden door middel van vaccinatie. Op dit moment is er echter geen geautoriseerd veilig en effectief CDV vaccin verkrijgbaar in de EU voor gebruik in deze dieren. Een commercieel recombinant vaccin dat geautoriseerd is in de USA lijkt redelijk te werken in niet-gedomesticeerde diersoorten. Een experimenteel CDV-ISCOM vaccin werkt goed in meerdere niet-gedomesticeerde soorten. Een uitzondering is de Afrikaanse wilde hond, waarin het ISCOM vaccin geen VN antistoftiters induceert, en waarin het recombinant vaccin lage VN antistoftiters induceert.

Vaccinatie van dierentuinvogels tegen HPAI virus van de H5 en H7 subtypes met de in de EU geautoriseerde pluimvee vaccins induceert voldoende hoge antistoftiters in de meeste soorten en taxonomische ordes, die op peil gehouden kunnen worden met een jaarlijkse éénmalige hervaccinatie.

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De auteur van dit proefschrift werd op 20 Januari 1973 in Nieuwveen geboren. In 1985 werd begonnen met de middelbare school op St George's College in Harare, Zimbabwe, en in 1991 behaalde hij zijn A-levels op Campion School, Athene, Griekenland. In 1991 werd begonnen met de studie Diergeneeskunde aan de Universiteit van Utrecht. Tijdens deze studie werden twee onderzoek stages gedaan onder begeleiding van prof. Osterhaus (naar de prevalentie van hepatitis en herpes virussen bij gibbons, en het opzetten van hygiene/quarantaine protocollen in Krabok Koo wildlife breeding centre, Thailand; en naar de prevalentie van verschillende virussen in Canadese wilde carnivoren en zeezoogdieren, in samenwerking met de Canadian Cooperative Wildlife health Centre, Saskatoon, Canada). Na het behalen van het dierenarts diploma in 2002 werd begonnen als AIO aan de afdeling virologie van de Erasmus Universiteit Rotterdam en in Diergaarde Blijdorp – Rotterdam Zoo, resulterend in dit proefschrift. Gedurende het eerste jaar van de promotie was hij tevens dierenarts van de zeehondencreche in Pieterburen.

The author of this thesis was born in Nieuwveen, the Netherlands, on the 20th of January, 1973. He started secondary school at St George's College in Harare, Zimbabwe, graduated in 1991 at Campion International School, Athens, Greece, and started his study in Veterinary Medicine at the Faculty of Veterinary Medicine, Utrecht University. Two research projects (on the prevalence of hepatitis and herpes viruses in gibbons, and setting up hygiene/quarantine protocols in Krabok Koo Wildlife Breeding Centre, Thailand; and on the prevalence of viruses in Canadian terrestrial carnivores and marine mammals, in collaboration with the Canadian Cooperative Wildlife Health Centre, Saskatoon, Canada) were conducted under supervision of prof. Osterhaus. After graduating as a veterinarian in 2002 he started his PhD at the department of virology of the Erasmus University Rotterdam and at Rotterdam Zoo, resulting in this thesis. During the first year of this PhD he was also the consulting veterinarian of the Seal Rehabilitation and Research Centre in Pieterburen, the Netherlands.

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**Book Chapters**

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**J.D.W. Philippa**. 2005. Vaccination of non-domestic carnivores: a review. *European Association of Zoo and Wildlife Veterinarians (EAZWV) Transmissible Diseases handbook, third edition.*

**J.D.W. Philippa**. 2004. Vaccination of non-domestic carnivores: a review. *European Association of Zoo and Wildlife Veterinarians (EAZWV) Transmissible Diseases handbook, second edition: 173-202.*

# PHOTOGRAPHIC CREDITS

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