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Blue Wing Disease of Chickens and Viruses Involved

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Paper I Page 10	Discussion	Line 12	IBD	IBH
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Acknowledgements			All theses	thesis

Blue wing disease of chickens and viruses involved

Björn Engström

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Abstract

Blue wing disease (BWD) is an acute disease in chickens that causes high mortality in 2-3 week- old chickens.

The major pathological lesions are haemorrhages in skin and muscles especially on the wings, and gangrenous dermatitis and depletion of lymphocytes in lymphoid tissues; the thymuses being most severely damaged. There were, however, no specific lesions in the bone marrow in this study. Atrophy of bone marrow leading to severe anaemia is common in most other countries. BWD is also called chicken infectious anaemia (CIA) in these countries.

The disease was suspected of being transmitted vertically from breeders to their progeny through the egg. BWD was not transmitted horizontally to other chickens kept in proximity.

Chicken anaemia virus (CAV) and avian reovirus were isolated from diseased birds and it was possible to transmit the disease experimentally by parenteral inoculation with both CAV and reovirus. CAV alone did not cause any clinical signs of disease, but a cloned low pathogenic reovirus enhanced the pathogenicity of CAV, leading to clinical symptoms and increased mortality at 2 to 3 weeks after inoculation.

A serological survey of antibody to CAV confirmed that CAV was transmitted from certain breeder flocks which were originally infected during egg production, demonstrated by a late seroconversion to CAV in these flocks.

Prevalence of antibody to CAV in commercial broiler flocks at slaughter was relatively low. Sero-positive flocks were most often found in houses where outbreaks of BWD had occurred a short time before. Satisfactory sanitation of infected houses with standard cleaning and disinfection was difficult, but after 2-3 crops of chickens in these houses no antibody to CAV could be detected in the birds at slaughter.

Key words: Chickens, breeders, broilers, blue wing disease, clinical signs, pathology, transmission, virus isolation, chicken anaemia virus, avian reovirus, experimental infection, dual infection.

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Blue Wing Disease of Chickens and Viruses Involved

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To the memory of my father, the engine driver on my journey from the working class

Abstract

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Contents

Appendix

Papers I-IV

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV.

I. Engström, B.E. and Luthman, M. (1984). Blue wing disease of chickens: signs, pathology and natural transmission. Avian Pathology, 13, 1-12.

II. Engström, B.E. (1988). Blue wing disease of chickens: isolation of avian reovirus and chicken anaemia agent. Avian Pathology, 17, 23-32.

III. Engström, B.E., Fossum, O. and Luthman, M., 1988. Blue wing disease of chickens: experimental infection with a Swedish isolate of chicken anaemia agent and an avian reovirus. Avian Pathology. 17, 33-50.

IV. Engström, B.E. Prevalence of antibody to chicken anaemia virus in Swedish chicken flocks correlated to outbreaks of blue wing disease Submitted for publication.

Copies of the articles are included by kind permission of the publisher of the journal.

Abbreviations

BWD	blue wing disease
C.	Campylobacter
CAA	chicken anaemia agent
CAV	chicken anaemia virus
CEL	chicken embryo liver
CIA	chicken infectious anaemia
CPA	cyto-pathogen effect
ELISA	enzyme linked immunosorbent-assay
GP	grandparents
IAE	infectious avian encephalomyelitis
IBDV	infectious bursal disease virus
IBH	inclusion body hepatitis
MAS	malabsorption syndrome
MDV	Marek´s disease virus
MSB1	MDCC-MSB1
SPSCP	Swedish prophylactic Salmonella control programme
REV	reticulo-endotheliosis virus
SN	serum neutralisation
$TCDI_{50}$	mean tissue culture infective doses

Introduction

Poultry production in Sweden

In Sweden, layers for egg production and broilers for meat production are the major branches of the country's commercial poultry production.

Poultry production is concentrated to southern Sweden. In some areas there are quite short distances between the farms, but as a whole the farms are rather isolated from each other with relatively little risk for trans-mission of contagious diseases. The northern border for broiler production is on the level of Stockholm, but layers are kept even in the far north of Sweden.

As in most European countries poultry production in Sweden today is more strictly organised than other types of animal production.

The poultry industry can be represented as a pyramid (Figure 1). At the apex is a small group of primary breeders, with great-grandparents GGP).

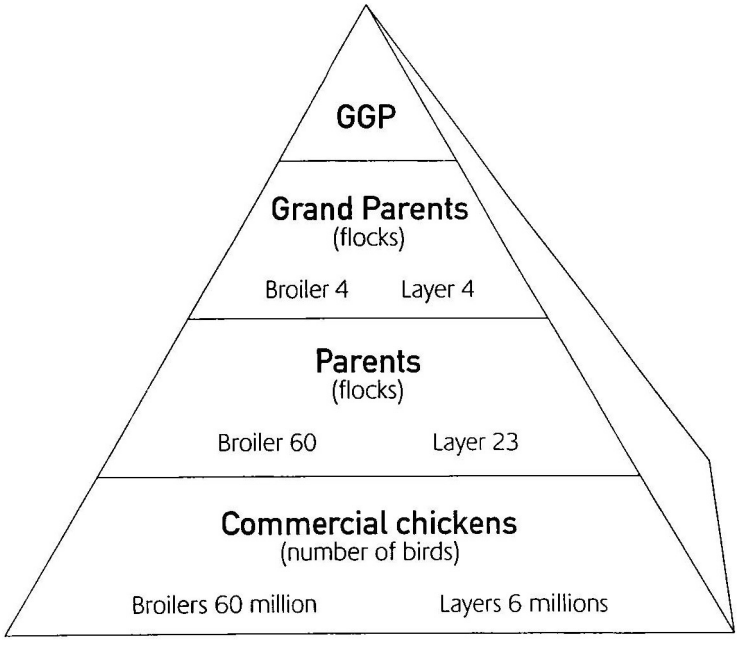

Figure 1. *Hierarchical structure of the poultry industry, with the number of flocks or birds in different generations in Sweden*

There are no commercial *basic breeding* establishments for poultry in Sweden today. All breeders are imported as day-old grandparent (GP) chicks. Every year about eight groups of GP chicks are imported. These GP birds grow up in well-isolated quarantines for 16-18 weeks and are then moved to other houses if they

are found free from contagious diseases. Here they produce eggs, which will be hatched and become the parent generation.

As few as nine breeder establishments with GP- and parent-flocks are able to supply the whole of Sweden with chicks of both types. Some day-old parent chicks are exported as well.

In order to run a breeder company you need to plan the production very carefully, from the importation of GP to the delivery of the final product.
If a disease causes high mortality in a GP-flock there may be both a lack of parents and of the final product for a long time. Therefore, good manage-ment and strict rules of hygiene are required to limit the risks for transmission of diseases and secondary bacterial infections in the intensive poultry industry. Indeed, many improvements have gradually been made during the last decades, especially in the broiler sector.

The *health* of poultry in Sweden is of a very high level viewed in an international perspective. All GP- and parent flocks and all broiler chickens are tested free from mycoplasmas (*M. gallisepticum* and *M. synoviae*) and infectious bronchitis. The whole Swedish poultry population is free from Newcastle disease, infectious laryngotracheitis, fowl pox, avian influenza and avian pneumovirus infection. Only a limited number of flocks of layers and broilers have been found to be infected with infectious bursal disease virus (IBDV). All outbreaks of IBD in Sweden, so far, have only caused low mortality and vaccination has not been employed. The GP and parent stocks have only been vaccinated against infectious avian encephalomyelitis (IAE) and Marek`s disease (MD). Outbreaks of MD are occasionally seen in commercial layers. In broilers, outbreaks of MD occurred for the first time in the 1990s and were not related to outbreaks of blue wing disease (BWD).

Egg production

Egg production has a long history of small-scale production most often in combination with other animal husbandry activities. Small national breeding companies started using artificial hatching in the 1930s and the size of the flocks grew slowly. Since 1960 the situation for egg production has changed dramatically. Some of the old breeding companies started importing new leghorn hybrids in order to improve the production of hens, and at the same time, large egg production units were built after battery cages were introduced to Sweden.

Four different hybrids of leghorn have been imported, some from the North American continent and some from Europe.
The layer chicks, i.e., the replacement pullets, are reared in rearing farms for 16-18 weeks. At that age they are transferred to the egg-producing farms. The vast majority of birds are kept in battery cages during both rearing and egg production. Today 86 percent of the eggs are produced in farms with more than

production. Today 86 percent of the eggs are produced in farms with more than 5 000 hens while only two hundred farmers produce 95% of the eggs.

The egg production sector was not affiliated to the voluntary prophylactic salmonella control programme at the time of these studies. The standard of hygiene there was not as good as in the broiler industry and the isolation of the rearing farms for pullets and parents was not as strict either.

Broiler production

Broiler production started in the 1950s with new kinds of hybrids that were heavy and fast-growing.

All broiler GP stocks are imported to Sweden, from several European countries. Since 1979 six different commercial hybrids have been imported to Sweden, but only three hybrids have been in Sweden at the same time.

There are five different integrations of broilers in Sweden. Each integ-ration consists of several parent flocks, one hatchery, several broiler farms and between one and three processing plants. Eggs from several parent flocks within an integration are usually hatched together in the same hatching machine, but the progeny from each parent flock are delivered to the broiler farms in separate boxes and the number of birds from each parent flock is recorded in the hatchery. There is a small exchange of eggs between the hatcheries, which is recorded as well.

Most broilers are raised in new modern houses, but some in old converted cow sheds. The litter spread on the floor consists of either straw or wood shavings and is discarded and replaced between each batch of birds.

The feed is produced in local feed factories and is characterised by relatively high proportions of locally produced wheat and barley. All broiler feed supplies are steam-pelleted.

Broilers in Sweden have never been vaccinated. Parent flocks on the other hand have been vaccinated against MD and IAE viruses for many years and against chicken anaemia virus (CAV) since 1990. Broilers are slaughtered between 30 and 50 days of age. Blue wing disease (BWD) was the major health problem in broiler flocks during a 10-year period from 1977 to 1987.

Salmonella control programme

Since 1970 all broiler integrations have been affiliated to the Swedish prophylactic Salmonella control programme (SPSCP).
A reinforcement of the programme was carried out in 1987 after a study of the transmission route for Campylobacter (C.) in broiler chickens. C. was frequently found in the surroundings of the chicken house and the most important route of

thus causing colonisation in the chickens (Berndtsson et al., 1996). Footbath with disinfection solution was not sufficiently effective to prevent the transmission of C. Therefore changing footwear at a well-defined hygiene barrier was recommended instead in this report. This change proved to be a great improvement for controlling Salmonella as well.

The SPSCP aims to protect each part of the production chain from being contaminated with Salmonella from the surroundings and to prevent transmission from an infected flock at as early a stage as possible (Engström, 1994).

Strict hygienic measures are applied including cleaning and disinfection of equipment and tools entering the house and the changing of clothes and footwear before a person enters a poultry pen. The all-in all-out procedure with immediate removal of manure from the house and the farm, strict cleaning and disinfection of the houses between each batch of birds has prevented transmission of diseases to a consecutive flock in the same house. The programme has limited the spread of Salmonella (< 1%), as well as viral diseases transmitted by contact throughout the whole broiler production chain. The prevalence of C. in Swedish broiler flocks was reduced from 50% in 1986 to 15% in 1990.

Transmission of diseases by eggs

Transmission of diseases from the mother to the foetus/child is well-known in many species. In mammals the transmission may occur during the whole pregnancy, but in birds and other egg- laying creatures it has to take place during the production of the egg in the female. In the individual birds the transmission can go on for days or weeks depending on the microbe involved. In the flock the transmission can continue for weeks or months depending on the rate of spread within the flock.

In birds some microbes cause both embryo-mortality, leading to poor hatching results, and disease in the hatched chicken. IAE for example is a viral disease that causes depression of egg production, reduced hatchability of the eggs and mortality in the young chicken. Other microbes only cause disease in the offspring. One of these is CAV, the cause of BWD.

To prevent transmission of this type of disease there are two different solutions depending on the pathogenesis of the diseases: *Eradication* of the disease in breeding flocks (as with Salmonella pullorum, avian leucosis and mycoplasmosis) or prophylaxis by *vaccination* (controlled infection) of breeders before start of egg production (as with IAE and CAV).

Bursa of Fabricius and thymus- primary targets for two immunosuppresive virus diseases

The bird's immune system does not differ in principle from that of mammals. But in contrast to mammals, birds have a *bursa of Fabricius,* an organ where the B-lymphocytes mature early in life. The lymphocyte precursor cells start to migrate from the yolk sac into the bursa after day 7 of embryogenesis. In the bursa they immediately start to mature/differen-tiate into B-lymphocytes. The B-cells multiply in the bursa, but as early as day 18 of embryogenesis many of them leave to the peripheral lymphatic organs. Here they finally differentiate into plasma cells in response to stimulation of antigens (Stitz, 1994).

The Thymus has the same role for the maturation of T-lymphocytes as the bursa has for the B-cells. Precursor cells enter the thymus in three waves starting at embryogenesis on day 6, 12 and 18. Each wave lasts for 1.5 to 2 days and is followed by intensive thymocyte proliferation, maturation and migration to the peripherical lymphatic organs, which goes on for a period of approximately 3 weeks. (Göbel, 1996).

Both the bursa and thymus receive precursor cells already in the egg. Immunocompetent B- and T-cells are available in the peripheral lymphoid tissues at the time of hatching. However, complete maturation of the immune-system takes several weeks.

Most viruses infecting chickens have a secondary immunosuppresive effect, but only two viruses, IBDV and CAV, replicate primarily in B- or T-lymphocytes.

IBDV replicates in IgM-bearing B-cells in the bursa and causes total destruction/depletion of the lymphocytes in the follicles. There are lesions in many other lymphatic tissues as well, but not to the same extent. The immunosuppresive effect of IBD gradually decreases when the birds grow older and the significance of the bursa decreases.

CAV attacks the precursor T-cells in the thymus, resulting in a very severe transient depletion of the cortex thymocytes. CAV also depletes haemo-cytoblasts in the bone marrow at an early stage of the infection. CAV-induced lesions in thymus and bone marrow occur mainly when chickens are infected *in ovo* or shortly after hatching. The immuno-suppression caused by CAV is transient, and 2-3 weeks after the infection the immune system has recovered.

Blue wing disease

In 1972 a new acute disease in young chickens appeared in Sweden. The sick chickens died suddenly and had subcutaneous haemorrhagic oedema under the wings. The farmers called it blue wing disease (BWD) due to the lesions on the wings.

The first outbreak appeared on the island of Gotland in the Baltic Sea, but during the following years outbreaks were reported in other parts of Sweden as well.
In 1977 I met my first case of BWD. It was in the 14-day-old broiler chickens that arrived at the National Veterinary Institute and for over a month, birds from other farms arrived with the same lesions. They had all been hatched in the same hatchery and 20-100% of the birds in the flocks were progeny of the same parent flock. The outbreaks started at 11-15 days of age and continued for 1-2 weeks. Mortality varied between 2% and 15% in these flocks. The highest mortality was registered in the first outbreaks of the enzootie, and in farms with poor hygiene.

The birds were generally depressed and died suddenly. The most obvious lesions were gangrenous dermatitis and haemorrhages in the skin most often on the wings and very small thymuses.

BWD was most common in broilers, but there were outbreaks in other categories of chickens as well. Only one outbreak was observed in grandparents (GP) kept in quarantine. This was in 1979 in a group of GP-layers imported from Canada. Occasional outbreaks also occurred in parents of both broilers and layers and in commercial layers.
The disease always appeared in a series of outbreaks and could be traced back to certain parent flocks although each flock often consisted of progeny from several parent flocks. The suspicion of egg-transmission arose early, but could not be confirmed because no etiological agent was found. The parents who were suspected of being the transmitters of BWD did not show any sign of disease at all.

BWD was the most devastating broiler disease in Sweden at the time of the present study. A large number of outbreaks with high mortality occurred. In 1980 one hundred outbreaks were registered and in 1983-1984 more than two hundred outbreaks occurred during a period of 23 weeks.
BWD has occurred in many other countries and has been described under such names as chicken infectious anemia syndrome, anemia-dermatitis syndrome or haemorrhagic syndrome. The features of the disease have been very similar in all countries. However, in Swedish BWD cases, the symptom of anaemia was less severe than in other countries.

Other new acute diseases in broilers

Several new acute chicken diseases had appeared between 1962-77, most of them caused by various viruses. Two of them had features in common with BWD, but all showed other features as well.

Infectious bursal disease (IBD) was first diagnosed in Gumboro, Delaware, USA and therefore given the name Gumboro disease as well. It was first described by Cosgrove (1962).The cause of IBD is a BIRNA-virus, infectious bursal disease virus (IBDV). IBD is an acute disease with general symptoms

similar to those seen in BWD. The main lesion is destruction of bursa of Fabricius, but only minor lesions can be found in the thymus and other lymphoid tissue.

IBDV causes lesions in bursa of Fabricius from the first day of life until the involution of the bursa. Clinical outbreak of IBD is most often seen from 3 to 6 weeks of age but not before the age of 11 days. IBDV is only transmitted horizontally. In Sweden, BWD has only occasionally appeared in combination with IBD. On these occasions the BWD outbreaks occurred later than usual, at 30-35 days of age.

Inclusion body hepatitis (IBH) became a problem in 1970s in the USA (Bickford, 1972), Canada (Petit & Carlson, 1972), Australia (Wells et al., 1977) and Europe (Hoffman et al., 1975; McFerran et al., 1976; McPherson et. al., 1974).

This disease occurs between 4-9 week of age mainly in broiler flocks. It is an acute disease usually with a short duration in the flock (3-4 days). Mortality varies between 2-10 %. The clinical symptoms of IBH are the same as for BWD and IBD and are characterised by a general depression. The pathognomonic lesion is necrotic hepatitis with intra-nuclear inclusion bodies in the hepatocytes. Anaemia, icterus, haemorrhages of various organs, especially the muscles and bone marrow degeneration are usually present, but vary in severity. *Gangrenous dermatitis,* which is one feature that IBH has in common with BWD, is described in outbreaks of IBH in USA (Rosenberger et al., 1975). In some outbreaks lesions in the bone marrow was the most obvious lesion and suggestions have therefore been made to call the disease hepato-myelopoetic disease (Hoffman et al., 1975). Stein (1975) even described the disease as basically an infectious anaemia, with gross liver changes observed only occasionally. In Sweden we have not seen any lesions in the bone marrow in cases of IBH.

In the UK, McFerran et al. (1976) and McPherson et al. (1974) report that the disease may be transmitted vertically from certain parents.

Virtually every serotype of adenovirus has been isolated from naturally infected birds. It has, however, been difficult to reproduce the disease in 4-9-week-old chickens with isolated adenovirus through a natural route of infection. Adenovirus is probably not the sole cause of IBH. The lesions in bursa of Fabricius in IBH cases were often very similar to those found in IBD. The disease IBH disappeared from most countries when IBD was controlled by vaccination. In Sweden where we do not vaccinate against IBD there are occasional outbreaks of IBH in flocks with subclinical outbreaks of IBD even today.

CAV had not yet been discovered in the 1970s, but today it is obvious that CAV was involved in the pathogenesis of IBH with bone marrow lesions, similar to those found in CAV infections. The outbreaks of hepato-myelopoetic disease in Germany (Hoffman et al., 1975) also involved lesions in thymus and other lymphoid tissue typical of CAV infection.

Presentation of avian reovirus and chicken anaemia virus (CAV): two viruses that play an important role in this thesis.

Avian reoviruses belong to the orthoreovirus genus in the Reoviridæ family.
Avian reoviruses have been isolated from chickens with a variety of disease conditions as well as clinically normal chickens. Reovirus is most likely the primary cause of the disease known as tenosynovitis. Reovirus has also been associated with several other conditions such as respiratory disease, enteric disease and runting syndrome, but an etiological relation-ship has not always been firmly established or defined. Avian reoviruses have recently been found to have immunosuppresive properties, which will be discussed below.

The avian reovirus particle is a non-enveloped virus with a double capsid structure of icosahedral symmetry. Estimates on viron size have varied; 70-75 nm is the most common estimate. The estimated number of subunits in the outer capsid varied greatly in different studies, 92-132 (Robertson & Wilcox, 1986).

Avian reoviruses are resistant to ether and chloroform, are stable over a wide pH range and are relatively resistant to heat.

Avian reovirus genome consists of 10 separate double-stranded RNA segments, which can be separated into three size classes: L (large), M (medium) and S (small).
The virus proteins also falls into three size classes: λ (large), μ (medium) and σ (small). Twelve avian reovirus proteins have been identified. The four structural proteins μ 2C, σ2, σ3 and σC are located on the viral outer capsid and are virus-neutralising antigens (Meanger et al., 1995; Ni & Kemp, 1995;).

Avian reoviruses are antigenically heterogeneous and eleven different serotypes have been demonstrated (Robertson & Wilcox, 1986).

Chicken anaemia virus (CAV), formerly called chicken anaemia agent, was first isolated in Japan during an investigation of a vaccine accident which occurred in the field involving reticuloendotheliosis virus (Yuasa et al., 1979). The virus was thus not isolated from birds with symptoms similar to BWD the first time. However, when CAV was inoculated into day-old chickens, they developed lesions in the thymus at the age of 14 days in the same way as chickens with BWD. CAV was then strongly suspected to be a causative agent involved in the pathogenesis of BWD.

CAV is a small non-enveloped virus particle, with an average diameter of 23 - 25 nm. CAV has a buoyant density of 1.33 - 1.34 g/ml (Gelderblom et al., 1989; Todd et al., 1990). The virus capsid is composed of 32 structural subunits arranged in a class P=3 icosahedron with a triangulation number of 3 (McNulty et al., 1990*c*).

CAV particles are resistant to acetone, chloroform, ether and pH 3.0. CAV is partially inactivated after heating at 80°C for 30 min and completely inactivated after 10 min at 100°C (Goryo et al. 1985; Taylor, 1992; Urlings et al., 1993).

Electron-microscopic analysis of the capsid DNA showed that it is a circular single-stranded DNA of approx. 2300 nucleotides (Gelderblom et al., 1989; Todd et al., 1990).

Three CAV proteins have been identified (Noteborn & Koch, 1995): VP1 (51.6 kDa), VP2 (24 kDa) and VP3 (13.6 kDa). VP1 is most likely the capsid protein, VP2 is still of unknown origin and VP3 is called the apoptin and is responsible for the apoptosis of the infected thymocytes.

There are at least two other non-enveloped, icosahedral animal viruses with a circular single-stranded DNA genome, but of a smaller size. They are porcine circovirus (PCV) (Tischer et al., 1982) and psittacine beak-and-feather-disease virus (PBFDV) (Ritchi et al., 1989). A disease in pigeon is also caused by a similar virus (Woods et al: 1993). CAV, however, belongs to a novel virus family separate from PCV and PBFDV (Noteborn & Koch, 1995) and has yet to be classified.

A number of DNA analyses based on cloned CAV-DNA from various strains isolated around the world revealed only minor differences among the CAV isolates (Noteborn & Koch, 1995). This confirms the obser-vations of McNulty et al. (1989, 1990*a*) and Yuasa & Imai (1986) that all known CAV isolates constitute a single serotype.

Aims of the study

The aims of this study were:

1. To describe the features of blue wing disease (BWD): clinical signs, pathological lesions and pattern of transmission.

2. To investigate the cause of BWD by means of virus isolation from various tissues taken from diseased birds.

3. To perform experimental infection trials in specific pathogen-free chickens in order to verify whether chicken anaemia virus (CAV) or reovirus or both are the etiological agent(s) causing BWD.

4. To find out when parents of chickens with BWD seroconvert to CAV.

5. To study the difference in prevalence of antibody to CAV in grandparents and parents of broilers and layers at 16-20 weeks of age.

6. To study the prevalence of antibody to CAV in commercial broilers at slaughter in relation to outbreaks of BWD.

Comments on materials and methods

A brief description of the Materials and Methods used in the present study is given here. For a more detailed account see papers I - IV.

Field materials

During the first years the field materials consisted mainly of **dead birds** from flocks with suspected outbreaks of BWD submitted to our laboratory for pathological examination and attempts to isolate virus. Formalin-fixed tissues from suspected cases of BWD were also sent to our laboratory from local laboratories for confirmation of the diagnosis.

Visits were made to farms experiencing outbreaks of BWD to study the clinical course of the disease and to collect fresh material for laboratory examination.

For epidemiological investigation, **data** were collected from the parent farms, the hatcheries, the broiler farms and the processing plants to obtain information about egg production, hatchability, rates of mortality, clinical signs, feed consumption and to which farms the progeny of each parent flock had been delivered when an outbreak of BWD had occurred.

Blood samples were collected for serological examination from grand-parents and parents of broilers and layers at different ages and from commercial broilers at the processing plants.

Laboratory methods

Preparation of tissue samples

Tissue samples were taken for histological examination and virus isolation attempts from dead and killed birds both from outbreaks in the field and from experiments. Samples of skin, muscles, heart, liver, kidney, spleen, bursa of Fabricius, thymus, bone marrow and lung were taken from most of the examined birds.

Samples for histological examination were fixed in 10% buffered formalin, processed by standard paraffin techniques and the sections stained with haematoxylin and eosin.

Specimens taken for virus isolation attempts were homogenised, made into a 10% suspension in PBS, frozen, thawed and centrifuged. The superna-tants were stored at - 70°C until used for inoculation into cell cultures.

Cell cultures
Primary chicken embryo liver cells (CEL) prepared from 14 days embryonated SPF-eggs and the T-lymphoblastoid cell line MDCC-MSB1 were used for attempts to isolate virus. MSB1 cells were used in the neutralisation test and immunoflourescent test as well.

Virus isolation
Attempts to isolate viruses both from field cases of BWD and experimental cases were performed in both CEL cell culture and MSB1 cell suspensions. The tissue samples were inoculated in tissue culture tubes and examined daily for CPE (CEL) and inhibition of growth (MSB1).The tissue samples were considered negative after two passages with no CPE in CEL-cells or after seven passages with no morphological changes of the MSB1- cells.

Virus identification
A virus showing CPE in CEL-cells was screened by electron microscopy, applying the criteria of size and morphology. *Reovirus* was then identified by using a concentrated virus preparation as antigen in an agar-gel immunodiffusion test with an antiserum prepared against the reovirus strain S1133 (van der Heide et al., 1974).

Virus isolated in MSB1-cells was examined for physico-chemical properties by treatments with chloroform, low pH, heat; at 56°C for 60 min and at 80°C for 15 min and finally by filtration through filters with an exclusion size of 25 nm. The isolate was also tested by cross-immuno-flourescence using the Gifu-1 strain of CAV(Yuasa et al., 1979) as reference antigen in MSB1-cells.

Serological examinations
Serological examinations were first carried out with a serum neutralisation (SN) test using MSB1 cells. An indirect immunofluorescent test was at one point used in our laboratory, but after a report about the unsatisfactory sensitivity and specificity of the test (Bülow, 1988), the SN-test became the test of choice early in this study. When a commercial ELISA-test with acceptable sensitivity and specificity became available in 1992 it became the chosen test in our laboratory.

Experimental infection trials

Chickens
Embryonated eggs, free from antibody to reovirus and CAV, were purchased from TAD, Cuxhaven (VALO-eggs). The eggs were incubated in a common egg-incubator for 18 days and then moved to a specially-designed hatching machine that was kept in isolation and under positive pressure with air being filtered through absolute-filters. To avoid the transfer of pathogens from the surface of the eggs, the eggs were sanitised with evaporated formalin just after the transfer to the hatching machine. The eggs were hatched in the isolated hatching machine

and the chicks were carefully transferred to the chicken isolators in closed plastic bags to avoid contamination. The chickens were kept in the isolators under positive pressure with air being filtered through absolute-filters during the whole experiment.

Experimental design

We let the design of the experiments develop gradually depending on the results of the previous trials. The experiments were set up with regard to the following questions:

1. Is the disease transmittable using a bacteria-free material from dead birds? - The inoculum material consisted of a suspension of heart from a chicken with BWD , and was inoculated parenterally into day-old chicks.

2. Is an isolate of reovirus obtained from CEL-cells inoculated with the suspension of heart used in (1) causing the disease? - To fulfil Kock´s postulate a non-passaged reovirus was inoculated into day-old chicks.

3. Is it a reovirus strain that is the cause of BWD? - The reovirus isolate from (2), now cloned, was inoculated into day-old chicks. Since the cloned reovirus did not cause any disease, attempts to isolate virus in MSB1-cells from the same suspension of heart as in (1) were carried out.

4. Is an isolate on MSB1-cells the cause of BWD? - An isolate on MSB1 cells, characterised as CAV, was inoculated into day-old chicks.

5. Does a combination of two viruses isolated on different types of cells enhance the symptoms of the disease? - Both the cloned reovirus strain (3) and the CAV-isolate (4) were inoculated together parenterally into day-old chicks.

6. Is a horizontal spread of reovirus and/or of CAV causing the disease? - Groups of birds were inoculated parenterally with either CAV alone or CAV and reovirus. All groups were kept together in the same isolator together with a group of hatchmates that were not inoculated. The floor was covered with a plastic sheet to favour horizontal transmission via faeces.

The same dose of infection of each virus was given in all experiments. Each experiment was repeated at least once. In all six experiments ten hatchmates served as control birds in a separate isolator. All chickens were observed daily for signs of disease. The haematocrite value was deter-mined in all killed birds in the experiments.

Results and discussion

Outbreaks of blue wing disease (BWD) in broilers - course of the disease (paper I)

BWD being a new disease in Sweden, it was necessary to study, describe and systematically compare it to several similar syndromes in other countries. A study of the course of the disease in Sweden was the first step in this task.

Outbreaks of BWD started at the age of 10-16 days when there was a sudden increase in daily mortality and a very acute course of disease. The sick birds became depressed and lay down on their breasts with closed eyes and ruffled feathers. They shivered and died within a few hours. In most countries anaemia with pale heads is an obvious sign of the disease, but in our study, anaemia was not observed as a clinical sign. Most of the dead birds affected with BWD, however, showed different degrees of skin lesions, usually on the wings, with accumulation of a subcutaneous dark blue serosanguinous exudate (blue wing) (Fig. 3, page 26). The peak of mortality occurred at 17 to 21 days of age, and in most outbreaks the mortality rate was down to a normal level at the age of 23 to 26 days.

Typical outbreaks of BWD in broiler chickens have now been described in many countries all over the world under various names like chicken infectious anaemia (CIA), anaemia dermatitis or haemorrhagic syndrome in: **Europe:** Belgium (Froyman et al., 1986), Denmark (Bisgaard, 1983 & Jörgensen, 1991), Germany (Dorn et al., 1981; Vielitz & Landgraf, 1988), France (Picault et al., 1992), Hungary (Farkas et al., 1992), the Netherlands (Braunius, 1988), Poland (Szeleszczuk et al., 1985), the Slovak Republic (Jantosovic et al., 1992), Switzerland (Hoop et al., 1992); the UK (Chettle et al., 1989; McIlroy et al., 1992 and Randall et al., 1984), **Japan** (Goryo et al., 1987; Otaki et al., 1988; Yuasa et al., 1987); the **USA** (Goodwin et al., 1989; Lamichhane et al., 1991; Lucio et al., 1990; McNulty et al., 1989;), **New Zealand** (Stanislawek & Howell, 1994), and **Argentina** (Buscaglia et al., 1994). There is a remarkable similarity in the descriptions of the outbreaks in these reports to our study, with the one exception that in most countries a more pronounced anaemia was recorded.

In Sweden a **second peak of mortality** appeared in some flocks around the age of 30- 33 days. The only other reports of such a recurrence of mortality have come from Denmark (Bisgaard, 1983; Jörgensen, 1991). One explanation to these second outbreaks could be that there is horizontal transmission to chicks who lack *maternal antibodies* (Jörgensen, 1991). In several studies maternal antibody has been shown to protect chickens from CAV infection up to the age of three weeks (Goodwin et al., 1993; Otaki et al., 1992; Yuasa et al., 1980*a*). However, in the beginning of a series of outbreaks of BWD many birds are hatched without maternal antibody to CAV. The chickens who die at 30-33 days of age must have been infected horizontally before 14 days of age, because there is an *age*

resistance to CAV. This conclusion is based on the results of several studies (Rosenberger & Cloud, 1989; Yuasa & Imai 1986; Yuasa et al., 1979, 1980*b*) viz. showing that only chickens under 14 days of age are susceptible to infection leading to clinical disease. Age-related resistance could be the result of maturation of humoral immune response or the disappearance of target cells for viral replication (Schat 1994). Jeurissen et al. (1992*a*) suggested that the age resistance may be caused by lack of susceptible thymic precursor cells belonging to the third wave that populate the thymus after hatching. McNeilly et al., however, found in an *in vitro* study that mononuclear cells from chickens were as susceptible to CAV at 28 days of age as at 6 days of age, The difference in immune response at different ages was demonstrated by Yuasa et al. (1983*b*); chickens infected during their first weeks of life do not produce neutralising antibody before three weeks of age, while three-week-old chickens develop neutralising antibody to CAV within 7 days. Further support of the view of developed humoral immuno-competence as the cause of age resistance comes from the results presented by Hu et al., (1993) and Yuasa et al. (1988). They found that they could abrogate the age-related resistance to CAV by embryonal bursectomy, proving that this resistance is antibody-mediated. Thymocytes became infected and severe symptoms were seen in these birds at an older age.

The total *mortality* during an outbreak of BWD varied greatly ranging from 1 to 60 percent.

Many different factors seem to have an influence on the mortality rate in outbreaks of BWD according to our study; 1) the proportion of progeny from certain parent groups which are transmitting the disease vertically; 2) the age of these parents at the time of laying since in most enzootics the mortality was very high in the first outbreaks in a series and then gradually decreased as the parents became older, paralleled with an increasing immunity to CAV in the flock; 3) the presence of other infections in a flock at the same time, often seen in farms with less satisfactory hygiene; dual virus infections and secondary bacterial infections enhance the morbidity and mortality; 4) good management including good habituation toward the keeper (socialisation) generally increases the resistance to infection and decreases mortality (Gross & Siegel, 1982).

In paper I, we found that both *sexes* were equally affected by BWD. Goryo et al. (1987) found, however, that the mortality was higher in male chicks (20%) as compared to females (2.4%). A much higher mortality was found in progeny of GP flocks delivered to broiler farms as compared to progeny delivered to parent farms as described in paper IV. This phenomenon can be explained by a greater susceptibility to BWD in male chickens as compared to females. The deliveries to the broiler farms consisted of 85% males, while the parent flocks only received 15% cocks. During a period of outbreaks of BWD in 1993 (Paper IV) three of them appeared in broiler farms, which reared male and female broilers in separated groups though in the same building and under the same conditions. As seen in table 1, the mortality was higher in the male groups, which supports the theory that male chickens are more susceptible.

Table 1. *Mortality rate in female and male chickens during the third week of age in three broiler farms experiencing outbreaks of BWD*

FARM	MORTALITY (%)	
	FEMALES	MALES
A	1.5	3.0
B	1.1	2.6
C	3.1	5.6

Outbreaks at an older age

In Sweden only four reported outbreaks of BWD, occurred as late as 30 to 35 days of age. These flocks were found to be infected with IBDV as well. As has already been mentioned there is an age- related resistance to CAV up to the age of two weeks. Co-infection with IBDV, however, may break this resistance because of its immunosuppresive effect which makes birds more susceptible to disease at least up to 5 weeks of age (Bülow et al., 1986*a*; Otaki et al., 1989; Rosenberger & Cloud, 1989; Yuasa et al., 1980*b*). Bülow et. al. (1986*a*) showed that MDV and reticuloendotheliosis virus (REV) also had a similar effect.

Diseases probably caused by CAV at an older age (5-9 weeks) have also been described in Japan, (Goryo et al., 1985; Otaki et al.,1987; Takai et. al., 1995; Yuasa et. al., 1983*a*) and Taiwan (Lu et al., 1993). In several of these reports infection with REV or MDV was diagnosed as well.

Pathology (paper I and III)

Autopsies of birds affected with BWD from outbreaks both in the field and the experimental trials were carried out. The main lesions were found in lymphoid tissues, skin, muscles and, in the experimental cases, also in the bone marrow. Apart from the lesions in the bone marrow there was a good agreement between the findings in the field cases and in the experimen-tally- infected birds, although the latter did not catch any secondary infections. The age figures, in days post infection (pi) below, refer to the birds with clinical signs in the experiments.

Lymphoid tissue

The most severe lesions in our study were seen in the thymus of birds between 13 and 18 days pi. The thymus was very small and thin and histologically a complete regression of the cortex was demonstrated with severe depletion of the lymphocytes and proliferation of epithelial reticular cells, though no inflammatory reaction was observed. Bursa of Fabricius was also affected with atrophy of the follicles and depletion of lympho-cytes, although not to the same extent as the thymus. In the spleen the lymphocytic depletion was moderate. Birds older than 22 days pi showed repopulation of lymphoid organs.

In several sequential histopathological (Goryo et al., 1989; Tanigushi et al., 1983) and immunocytochemical studies (Hoop & Reece, 1991; Smyth et al., 1993),

lesions were found during the subclinical initial, phase of the infection as well. All studies were done on one-day-old chickens experimentally inoculated i.m. with a high dose of CAV ($10^{6.5}$ $TCID_{50}$). Depletion of lymphocytes were seen in all lymphoid tissues. The first lesions were seen in the *thymus* at 4 to 6 days pi, with enlargement of lymphoblasts focally in the outer cortex, continuing with depletion of the lymphoblasts a few days later. Intranuclear immunostained inclusion bodies were seen in the thymocytes. Jeurissen et al. (1992*b*) described the depletion of cortical immature thymocytes as apoptosis, i. e. programmed cell death. The dead cells are phagocytosed by the epithelial reticular cells which explains the lack of inflammatory reaction in the thymus, which would otherwise be visible in a necrotic type of lesion. In the *spleen,* minor lesions were seen at 4-5 days pi and in the *bursa* 11-12 days pi. By day 10-20 pi, all lymphoid tissues were involved, showing prominent lesions and only minor variations were recorded in the different studies. The lesions described by others for this period correspond very well with ours. At about 20 days pi repopulation of all lymphoid tissues starts as reported by others and confirmed in our study as well. Our study did not extend beyond 28 days of age but other studies have reported that all lymphoid tissue are fully restored by day 32 pi.

Bone marrow

In the bone marrow of experimentally-infected birds a yellowish discoloration appeared between days 13 and 22 pi. There was an atrophy of the bone marrow with depletion of cells of both the erythrocytic and the granulocytic series. The haematopoetic tissue was replaced by fat- containing cells. At 22 days pi marked proliferation of immature, haema-topoetic cells was observed, indicating a regeneration of the tissue. In the field cases in paper I no lesions were seen in the bone marrow. All birds from outbreaks of BWD were examined between 12 and 20 days of age so lesions in the bone marrow could not have been passed unobserved. In most field outbreaks of BWD in recent years, however, different degrees of atrophy of the bone marrow have been found in Sweden as well.

Sequential studies on lymphoid tissue from experimentally-infected chickens also involved the bone marrow (Goryo et al., 1989; Hoop and Reece, 1991; Smyth et al., 1993; Tanigushi et al., 1983). They found the first lesions 4-6 days pi and a severe, almost complete depletion of the haematoblasts on 10-16 days pi. The same findings were made in our experimental study. The restoration of the bone marrow started at 20 days pi and was completed in all of these other studies by 32 days pi. Anaemia was recorded 12 to 20 days pi.

Skin and muscles

In the *skin* there were ecchymoses and sometimes also small wounds at the sites of bleeding as well. The haemorrhages in the skin often continue a few millimetres out on the shaft of the feathers of the wing and tail (Fig. 2). The skin lesions are most common on the wings, but also appear on the legs, toes, under the chin and on the breast. There was hyperaemia, oedema and bleeding in the

cutis and subcutis, especially in the loose connective tissue and in the fat tissue. Sometimes there are also haemorrhages situated deep in the *muscles* between the muscle cells. Degeneration of muscle cells was also to be seen. In the field cases heavy bacterial infections, subcutaneous exudate, necrosis and degeneration were frequently observed. (Fig. 3)

Figure 2. *Wing from an experimentally infected chicken with haemorrhages in the skin (Exp. 2b)*

Figure 3. *"Blue wing" from a field case with subcutaneous red-blue exudate*

Haemorrhages in the skin and muscles are not regularly described in outbreaks of BWD/CIA, though they have been reported by Bisgaard (1983), Dorn et al. (1981), Randell et al. (1984), Stanislavek & Howell (1994). and Szeleszczuk et al. (1985) Haemorrhages in the muscles were found by Yuasa et al. (1987).

Pope (1991) suggested that the haemorrhages associated with BWD are primarily related to a thrombocytopenia, present in the acute stage of the disease (Tanigushi et al., 1983), that may possibly be complicated by anoxic vascular changes. Thrombocytes in birds like those in mammals are the cellular elements involved in blood coagulation (Zinkl, 1986). In mammals, capillaries and postcapillary venules develop fenestrations and have increased susceptibility to haemorrhages, when abnormally low levels of thrombocytes occur (Kitchens & Weiss 1975).

The haemorrhages were frequently seen on the feather follicles in the parts of the body where the most rapid growth of the feathers takes place at 14 days of age. Because CAV replicates in fast-growing cells, the cells of the feather follicles may be involved in the replication as well. No histopatho-logic lesions in the skin have been reported in any other studies than ours. However, Smyth et al. (1993) found specific immuno-staining revealing CAV in feather pulp cells in a section of skin without any morphological lesions, which is an observation that supports my hypothesis.

In paper III, the highest frequency of ecchymotic haemorrhages was seen in chickens inoculated with both CAV and reovirus. These lesions have been described in only a few studies of experimental infection with CAV, while Tanigushi et al. (1982) and Yuasa et al. (1979) found haemorrhages in the skeletal muscle after infection with CAV alone. Bülow et al. (1986*a*) found an increasing tendency for haemorrhages to appear after dual infection with IBDV and CAV. Pope (1991) found experimentally that in chickens inoculated with both CAV and IBDV, haemorrhages developed rapidly in the subcutaneous tissue of the wings, but not in chickens inoculated with only CAV. Obviously the extent and severity of haemorrhages were increased by dual infections with CAV either together with IBDV as in the above-cited studies or with reovirus as in our study.

Ecchymotic haemorrhages are common in other broiler diseases, for instance IBD (Lukert & Saif, 1991) and inclusion body hepatitis (IBH) (McFerran et al., 1976). In these diseases, however, the haemorrhages are most common in skeletal muscles and not in the skin. In a study of the blood coagulation system in chicks infected with IBD, Skeeles et al. (1980) found an age-related coagulation disorder, which perhaps can explain the haemorrhages associated with that disease. Young 17-day-old chickens not showing any clinical signs had normal blood coagulation, but 42-day-old birds with severe symptoms generally had a prolonged clotting time.

Other tissues

Minor lesions were found in some other tissues as well.

In the **liver** no gross lesions were revealed but mild inflammatory reactions were seen histologically. Inclusion bodies in the cytoplasm of the hepatocytes were sometimes observed in the field cases of BWD, but never appeared in the experimental trials. Goryo et al. (1989) found mild inflammatory reactions in some birds in their experimental CAV infections but they found no inclusion bodies. The inclusion bodies in our study were obviously a secondary finding.

In the **heart** we found a mild myocarditis in the field cases but the more pronounced lesions were found in the experimental cases in birds infected dually with both CAV and reovirus. Goryo et al.. (1989) found granulo-matous lesions in the ventricular wall in only two chickens inoculated with CAV, while Smyth et al. (1993) observed mild inflammatory reactions in the heart in their experimental infection with CAV. As already stated, the most prominent lesions in our experiment were seen in chickens inoculated dually with both CAV and reovirus. It has been suggested that reovirus itself may cause heart lesions in chickens (Robertson & Wilcox, 1986).

Signs of immunosuppression

Several classical signs of immunosuppression have been observed in outbreaks of BWD/CIA. The severe *depletion of thymocytes*, the depletion of lymphocytes in spleen and bursa and destruction of myeloid progenitor cells in bone marrow between 2 and 3 weeks of age are common signs

Several *immunological studies* have confirmed the presence of transient immunosuppression and found that immune defence is fully restored at 30 days post infection. Adair et al. (1991) and Otaki et al. (1988*a*) found that a CAV infection was followed by severe defects in T-cell-mediated functions. Furthermore, McConnell et al. (1983*a,b*) saw a reduction in macrophage function, i.e. lower Interleukin-1 production, Fc receptor expression, phagocytosis and bactericidal activity.

In outbreaks of CAV/CIA there is often an early appearance of *secondary bacterial or fungal infection,* as a sign of immunosuppression. Gangrenous dermatitis often starting from haemorrhages in the skin, has been the most common sign of secondary bacterial infection in Sweden and many other countries as well (Bisgaard, 1983; Braunius, 1988; Dorn et al., 1981; Froyman et al., 1986; Randdell et al., 1984; Stanislavek & Howell 1994; Vielitz & Landgraf, 1988). Occasionally other infections such as pulmo-nary aspergilllosis or coli bacillosis have also been described (Goryo et al., 1985; Hoop et. al., 1992; Randell et al., 1984).

Gangrenous dermatitis has been described in connection with other immunosuppressive diseases such as IBD and IBH at an age of 4 to 9 weeks (Rosenberger et al., 1975).

Cause of BWD - isolation of virus (paper II)

Very early based on the epidemiological and clinical picture and the course of the disease, we suspected that BWD was caused by a virus infection. Virus isolation attempts were carried out on both CEL cell culture and MSB1 cells. Organ suspensions from field cases originating from 13 different outbreaks of BWD were investigated.

Isolates in CEL cultures. As early as 1978 virus was isolated in CEL cell cultures from specimens of liver from cases of BWD. Virus was isolated from many different tissue specimens from natural cases of 12 outbreaks. The CPE induced by 11 of the isolates was of the syncytial type: the cells coalesced and formed large, isolated, irregular, multinucleated cytoplasmic masses. In only one isolate did CPE of the round cell type appear as well.
Electron microscopy showed the presence of virus particles of reovirus size (70 to 80 nm) with two capsids, some of them being empty. In the agar-gel precipitation tests all viruses isolated were identical with the reovirus strain S1133. Thus, the viruses were considered to be avian reoviruses and one of the isolates was then cloned for further studies. As reovirus early in this study was found to be of secondary importance in the aetiology of BWD, no further classification of the reovirus isolates was carried out.

Reovirus has been isolated from cases of BWD/CIA in several countries (Dorn et al., 1981; Froyman et al., 1986; Randell et al., 1984; Vielitz & Landgraf, 1988). Reovirus may also be isolated from the intestinal tissue of healthy chickens. The fact that reovirus in many cases was isolated from many different tissues indicated the presence of a viraemia typical of virulent reovirus infection. The virus distribution after experimental infection with different reovirus stains has been revealed in several studies. After an oral experimental infection of day-old chickens, with a pathogenic arthrotropic reovirus strain, Kibinger et al. (1985) found that virus had spread widely to many different tissues in the body within 3-5 days. Viruses could be reisolated for up to three weeks in different parts of the intestine and for ten days in mononuclear blood cells and in the bone marrow. In another experiment Ni & Kemp (1995) made a comparison between a high pathogenic and a low pathogenic enterotropic reovirus. The high pathogenic strain behaved as in the previous study i.e. spread widely in the body after inoculation, while the low pathogenic strain virus remained at the site of inoculation-indicating that the distribution of reovirus to various organs is linked to the pathogenisity.

Isolates in MDCC-MSB1 cell cultures. In order to detect a potential infection of CAV, material from the organ suspensions was inoculated on MDCC-MSB1 cells.
Infected cell cultures showed red colour, the cells became swollen and the nuclei contained small vacuoles and accumulation of chromatin as described for CAV by Bülow et al. (1985).
The isolate CAA 1/80 was selected for the further investigations.
From six natural outbreaks of BWD, CAV was isolated from various tissues such as liver, heart, skin, thymus and muscles. The virus was identified as CAV by the physico-chemical properties, i.e. heat resistance, and by cross-immunoflourescence tests with the Gifu-1 strain of CAV (Yuasa et al., 1979).
CAV has been isolated from many different tissues in cases of BWD/CIA. In the period of 10-20 days of age CAV is widely distributed throughout the whole body. (Smyth et al., 1993)

Transmission of BWD (paper I and IV)

Transmission of CAV, the main cause of BWD/CIA, and reovirus isolated in our study can be either vertical or horizontal. In this study both routes of transmission have been elucidated in various ways. Before the cause of the disease was known the connection between the outbreaks and all possible sources of infection was studied. Later when CAV was found to be the cause of BWD/CIA (Paper III); (McNulty, 1991) serological surveillance was carried out to determine the time points for seroconversion, in the suspected breeder flocks. The most important point was to verify that the birds had not been infected before coming into lay. The horizontally-infected breeders transmitted the virus to their progeny vertically. The prevalence of horizontally-infected broilers was studied as well.

Vertical transmission

Field observations (paper I)
Solitary outbreaks of BWD occurred seldom. More often there was a serie of outbreaks during a month.

It was always possible to trace the infection back to one, sometimes two, identified breeder flocks. The parents involved were always young i.e. between 25 and 38 weeks old. Outbreaks started in the first hatch from the suspected parent groups, or at the peak of production at 30 weeks of age, and they usually continued for 2 to 4 weeks, with outbreaks that occasionally went on for 12 weeks.

The suspicion that BWD/CIA might be transmitted vertically from certain young breeder flocks arose in many other European countries as well (Bisgaard, 1983; Chettle et al., 1989; Hoop et al., 1992; McIlroy et al., 1992; Vielitz & Landgraf, 1988) after similar observations.

Serological survey (paper IV)
In order to confirm vertical transmission of the disease from breeders to their progeny a *serological* study on the prevalence of antibody to CAV was carried out in breeder flocks in Sweden. A correlation was made to outbreaks of BWD in progeny flocks.

In the study at hand, differences were found with regard to the prevalence of antibody to CAV in the different categories of birds, when measured at the time of transfer from the rearing houses to the egg production facilities.

GP, kept isolated in quarantine during the rearing period, were usually free from antibody to CAV (21/26), but most of them had seroconverted before commencing laying. Only one of the studied flocks transmitted CAV to the progeny, resulting in outbreaks of BWD. Four out of the five GP-flocks, which had seroconverted during their stay in quarantine, were kept in the same station in a rebuilt chicken house. The quarantine was well-isolated, but might have been CAV-infected from the start and not been decontaminated between each new imported group.

Parents of layers had with one exception seroconverted during rearing and no transmission to progeny was registered. Twenty percent of the ***parents of broilers*** had not seroconverted at the start of egg production (18/94). All these late-infected broiler breeders spread the virus to their offspring leading to outbreaks of BWD.

Previous studies have also shown that outbreaks of BWD/CIA were traceable back to broiler breeders that seroconverted late to CAV (Chettle et al., 1989; Jörgensen, 1991; McIlroy et al., 1992; Vielitz & Landgraf, 1988). Breeders are often infected at an early age, which has been particularly elucidated by McNulty et al. (1988) who showed that broiler parents in the U.K. had often seroconverted at 8-9 weeks of age which explains the rare occurrence of BWD/CIA there and for the same reason in most other countries as well.

The time of introduction of CAV to a flock of birds depends on the degree of isolation from other birds and the standard of the hygienic procedures that are to prevent infections from entering the house.
The strict hygienic procedures enforced in the Swedish prophylactic Salmonella control programme (SPSCP) applied to broiler breeders, but not to layer breeders, is one likely explanation as to why layer breeders are most often infected by CAV during the rearing period. Isolation and strict hygiene have been reported as reasons for late seroconversion to CAV in breeders, from other countries as well as from Sweden. Jörgensen et al. (1995*b*) showed that the percentage of Danish broiler breeders that had seroconverted at 12-15 weeks of age declined significantly in 1991-1993, due to improved standards of housing and hygiene.

Stricter hygiene in attempts to control Salmonella in the UK has also resulted in occasional late seroconversion to CAV and subsequent BWD outbreaks in progeny (McIlroy et al., 1992).Vielitz & Landgraf (1988) also referred to strict hygienic measures as a cause of delayed CAV infection of broiler breeders in Germany.

The theory that transmission of CAV goes from hens to chickens through embryonated eggs has undergone several studies. E.S. Hoop (1992) and Yuasa & Yoshida (1983) found that CAV could be transmitted from the eggs of experimentally-infected dams for a period of up to 14 days though at a rather low frequency (7.5%).

Horizontal transmission

CAV is readily transmitted horizontally. It is excreted by faeces up to 5 weeks after infection (Hoop, 1992). When serum antibody to CAV appears, the excretion of CAV in faeces ceases. It has been shown in several studies that CAV-infected young chickens will transmit the infection to most of the chickens being kept in contact with them. These horizontally-infected chickens will seroconvert without showing any signs of disease (McNulty et al. 1990*a*; Yuasa et al., 1979, 1980*b*) with one exception (Rosenberger & Cloud, 1989), where the chickens were observed for a longer period of time. However, in this latter study, the degree of anaemia in affected birds was modest and no clinical signs were observed.

In our study the *parent* flocks involved in transmission of BWD did not show any signs of disease (Paper I) which was confirmed by many reports. Nor have any signs of disease, recorded as loss of production, been observed. For instance, McIlroy et al. (1992) studied one commercial parent flock transmitting CAV and found no disturbances in egg production, fertility or hatchability. Hoop (1992) found no differences between infected groups and control groups in body weight or egg production in an experimental transmission trial with hens. Most other vertically-transmitted chicken diseases, such as IAE, cause disturbances in egg production in the breeders and poor hatchability of their eggs. No studies of immunosuppression in birds older than one month have been carried out, but a report of Box et al. (1988) claimed impaired response to a killed NDV-vaccine in connection with seroconversion to CAV.

In *broilers* we found no evidence of horizontal spread of BWD to chicks in adjacent rooms in the same building or to birds kept on premises where an outbreak had occurred in a previous batch of birds (Paper I).CAV is, however, transmitted horizontally, but usually without leading to any signs of disease in the birds.
Horizontal infection with CAV is common in broilers all over the world (McNulty, 1991). In our study the prevalence of antibody to CAV was low when no outbreak of BWD was reported, but higher after outbreaks. It was not possible

to sanitise a contaminated house with a single routine cleaning and disinfection procedure, but after a second or third time the decontami-nation succeeded, as evidenced in seronegative birds at slaughter (Paper IV). House contamination with CAV is probably a common source of infection in broilers because of the resistant character of the virus (McNulty, 1991; Yuasa et al., 1992). Another route could be the introduction of the infection from the surroundings as suggested by Jörgensen et al. (1995*a*), who found a seasonal variation in prevalence of antibody to CAV in broiler flocks with a maximum prevalence in November to April. Our own study in 1993-1994 was performed in October-April.

Campylobacter(C.) is a common contaminating bacteria in broiler flocks all over the world. As C. is not transmitted to the chickens vertically and is very easy to sanitise in an empty chicken house it must be transmitted from the surroundings of the chicken house in order to reach the chickens. The presence of C. in broiler flocks is therefore a good measure of how biosecurity measures are working in practice. In all European countries the prevalence of C. has a seasonal variation with a different time span than that of the variation of CAV found by Jörgensen et al. (1995*a*). Most flocks are contaminated with C. during summer - autumn.

Experimental infection (paper III)

In six experimental studies the chickens were inoculated on the day of hatch with either an organ suspension or isolates of reovirus and/or CAV from an outbreak of BWD to explore whether the clinical signs and post-mortem lesions of BWD could be reproduced. Inoculation was performed intraperitoneally (ip) or intramuscularly (im) to mimic the vertical trans-mission as closely as possible. An alternative would have been in ovo inoculation, which was tried by Lamichhane et al. (1991).

The results showed that the combination of CAV and reovirus causes clinical signs and pathological lesions similar to BWD. The organ suspension and the primary reovirus isolate obviously both contained enough of CAV, apart from the reovirus, to cause the same degree of infection as the dual inoculation with the cloned reovirus combined with a CAV-isolate. CAV alone did not cause any clinical signs in the birds, only anaemia and tissue lesions although much less severe. The cloned reovirus strain by itself did not cause any lesions. However, together with CAV, this reovirus caused mortality during the first week. The dead birds had severe necrotic liver lesions. This type of lesion leading to mortality is also caused by virulent reovirus strains after *parenteral* inoculations without the aid of dual infection with other viruses (Hieronymus et al., 1983; Mandelli et al., 1978). These results clearly show that the pathogenicity of the reovirus was enhanced by CAV.
The size of the dose of CAV is also of importance for the effect of the inoculated CAV on the one-day-old chickens (McNulty et al., 1990*a*; Rosenberger &

Cloud, 1989). A higher dose gives more severe signs of disease. In the experiments carried out in our study an equal dose of the CAV- isolate was always given; hence the results should not have been influenced after either single or dual infections.

In our experiments a low pathogenic reovirus isolate had a synergistic effect on our isolate of CAV with respect to the pathogenicity, i. e. the reovirus enhanced the pathogenicity of CAV and vice versa. The clinical signs, mortality and pathologic lesions became more severe after a dual parenteral infection with CAV and reovirus. In one experiment a group of chickens, inoculated with only CAV, became infected by contact with reovirus from infected birds in the same isolator. Even these chickens, infected with reovirus by contact, developed the same severe lesions as chickens with dual parenteral inoculation, confirming the enhanced pathogenicity of dual infection. However, since the number of chickens was small in this experiment, it ought to be repeated. In such a new experiment the reovirus should be introduced at different ages in order to find out if age at infection is of importance. The role of maternal antibody to reovirus in protection of reovirus infection is also a subject that ought to be studied further.

Classical immunosuppressive viruses like MDV, IBDV and REV have previously been shown in experimental infection studies to enhance the pathogenicity of CAV (Bülow et al., 1986*a*). Another experimental study by Bülow et al. (1986*b*) with dual parenteral inoculation of reovirus together with CAV did not, however, give a conclusive result. They used the very pathogenic reovirus strain S1133 causing 100% mortality within day 5 pi, with the typical necrotic hepatitis lesions, that appear when inoculated alone. The dual infection with CAV in this case surprisingly postponed the mortality in the chickens for 2 days. Another study, however, showed a synergistic effect by enhancing pathogenicity through co-administration of reovirus and CAV (McNeilly et al., 1995). They only used oral infection on one-day-old chickens with CAV or/and two different strains of reovirus. The less pathogenic reovirus strain (Uschida) did not enhance the effect of CAV; in contrast the pathogenic strain (S1133) did enhance the severity of anaemia and tissue damage in the bone marrow, thymus and bursa. The difference between the result when using a low-pathogenic reovirus in this study and ours was most likely due to the mode of administration of CAV i.e. orally vs. parenterally. Rosenberger & Cloud (1989) showed experimentally that chickens were more susceptible to CAV-infection when they were inoculated by the parenteral route, as compared with the oral. Possibly the conclusion can be drawn that a low pathogenic reovirus will enhance the pathogenicity of CAV if CAV is inoculated by the parenteral route but not the oral one. In BWD the parenteral/vertical route of infection is the natural route of transmission. Oral / horizontal infection with CAV, which does not usually lead to disease, would probably only do so when dual infection with a highly pathogenic reovirus strain occurs.

The mechanism of the synergism between CAV and reovirus in enhancing pathogenicity is not clear. Avian reoviruses on their own have been shown to be able to cause immunosuppression with impaired antibody responses in young chickens (Montgomory et al., 1985, 1986; Sharma et al.,1994). Reoviruses cause moderate depletion of lymphocytes in the bursa. Although thymus is not damaged by reovirus infection, T-cell functions are impaired through inhibitory lymphokines probably produced by macrophages (Sharma et al.,1994). Mills & Wilcox (1993) showed in an *in vitro* test that reovirus infected and killed both bone marrow and peripheral monocytes and macrophages, which is suggested enhances the patho-genicity of other pathogens. They also noticed strain differences between reoviruses with regard to the ability to kill mononuclear cells. In a study employing dual infection with IBDV and an intestinal isolate of reovirus. Moradian et al. (1990) found that a combination of both IBDV and reovirus inoculated in young chickens by both intraocular and oral routes resulted in more prominent lesions in the bursa and spleen than the inoculation of IBDV alone. Avian reovirus can obviously enhance the pathogenicity of both CAV and IBDV.

During recent years many studies have revealed various immuno-suppressive effects of avian reovirus infections on chickens. These findings are contributing to an understanding of the enhancing effect of the pathogenicity of avian reoviruses on CAV which also turned up in our experiments unexpectedly and quite surprisingly.

Concluding remarks

Blue wing disease (BWD) is an acute disease in young chickens, which at the start of this study had not been described and was of an unknown aetiology. BWD is the same disease as chicken infectious anaemia (CIA) as described in other countries, although in BWD as it appeared in Sweden, the skin lesions were more prominent and bone marrow lesions were usually absent.

The virus identified as the cause of BWD is chicken anaemia virus (CAV). The disease occurs when it is transmitted vertically from dam to chicken through the egg. Thus horizontal infection with CAV does not cause BWD in immunocompetent chickens.

A low pathogenic avian reovirus isolated from field cases of BWD had an enhancing effect on CAV with regard to development of disease.
The high mortality in many outbreaks of BWD can be explained by co-infection with avian reovirus or other immunosuppressive viruses and secondary bacterial infection due to immunosuppression.

The large number of outbreaks of BWD in Sweden can be explained by the improved standard of hygiene enforced by the Swedish prophylactic Salmonella control programme (SPSCP). These restrictions have post-poned the inevitable introduction of the infection of CAV into breeder flocks from the rearing period to the egg-laying period, usually up to the commencing of lay. Vaccination of the birds with a live vaccine before leaving the rearing house, if they have not been naturally infected, makes the breeders immune and protects the progeny from BWD.

The main goals at the start of this study were to reveal the cause of BWD and to find a prophylactic measure to prevent the transmission of this devastating disease. Both of these goals have now been reached. My contribution was limited to adding a few pieces to the big jigsaw puzzle.

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