Transmission Routes and Vector Potential of the Poultry Red Mite *Dermanyssus gallinae*

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Abstract

The poultry red mite, *Dermanyssus gallinae* is a blood-feeding ectoparasite causing irritation, stress and, in severe infection, anemia and death of its avian host. This parasite not only causes welfare problems in poultry but also financial losses in egg production worldwide.

The aims of this thesis were to elucidate the transmission routes of *D. gallinae* to poultry facilities and to investigate the potential of the parasite to be a vector of *Erysipelothrix rhusiopathiae*, the agent causing poultry erysipelas.

Investigation of the 5.8S ribosomal RNA (rRNA) gene and the two internal transcribed spacers (ITS) of *D. gallinae* from poultry premises and wild birds in Sweden indicates that wild birds are of minor importance in the infection of *D. gallinae* to poultry farms. Instead there are indications that mites from wild birds in Sweden could be a separate species. The transmission of *D. gallinae* to poultry is therefore most likely to follow another path. Population genetic analysis using the mitochondrial cytochrome oxidase c subunit I (COI) gene as a molecular marker revealed that most farms had a homogenous population of *D. gallinae*. This investigation included farms from Norway and Sweden and no common haplotype was found across countries. This implies that transmission is connected to the egg-producing system and most likely one or a few common sources of infection are present. However, the exact nature of these sources could not be identified.

The bacterium *E. rhusiopathiae* could be isolated from *D. gallinae* collected from a farm during an outbreak of poultry erysipelas, and mites and hens were infected by the same bacterial strain. This means that *D. gallinae* is a potent reservoir of this agent. However, under experimental conditions uptake and transmission could not be demonstrated and therefore the vector competence of *D. gallinae* with regard to the erysipelas agent is still uncertain. The mite should, however, not be excluded as a potential vector possibly spreading the infection within and between farms.

Keywords: *Dermanyssus gallinae*, poultry red mite, laying hens, transmission, ITS, CO1, vector, reservoir, poultry erysipelas, *Erysipelothrix rhusiopathiae*

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”Ju mer man tänker ju mer inser man att det inte finns något enkelt svar.”

Nalle Puh, A.A. Milne

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List of Publications

This thesis is based on the work contained in the following papers, referred to by their Roman numerals in the text:

I Brännström, S., Morrison, D.A., Mattsson, J.G. & Chirico, J. (2008). Genetic differences in internal transcribed spacer 1 between *Dermanyssus gallinae* from wild birds and domestic chickens. *Medical and Veterinary Entomology* 22, 152-155.

II Øines, Ø. & Brännström, S. (2010) Molecular investigations of cytocrome oxidase c subunit 1 (CO1) and the internal transcribed spacer (ITS) of the poultry red mite *Dermanyssus gallinae* (DeGeer) in Northern Europe, with implication for its transmission between layer farms (manuscript).

III Eriksson, H., Brännström, S., Skarin, H. & Chirico, J. (2010) Characterization of *Erysipelothrix rhusiopathiae* isolates from laying hens and poultry red mites (*Dermanyssus gallinae*) from an outbreak of erysipelas (Submitted manuscript).

IV Brännström, S., Hansson, I. & Chirico, J. (2010). Experimental study on possible transmission of the bacterium *Erysipelothrix rhusiopathiae* to chickens by the poultry red mite *Dermanyssus gallinae. Experimental and Applied Acarology* 50, 299-307.

1 Introduction

Poultry production, including both meat and egg production is an industry of global importance and the health and welfare of poultry is a topic extensively discussed. In 2005, the global production of hen eggs was estimated to over 65 million tons (Anonymous, 2009a). In Sweden the consumption is about 200 eggs per person and year, and we have almost 6 million egg producing poultry. The number of poultry has been relatively stable over recent decades but the number of commercial poultry farms is decreasing. The egg-producing industry is dominated by farms with 30,000 to 100,000 layers, which require production systems that can stand up to current animal welfare standards as well as being profitable. The previously used battery cages for layers have been banned in Sweden since 1999, and will be so also within the European Union from 2012 (Anonymous, 1999). Laying hens should be kept in systems where they can perform their natural behaviors, such as dust bathing and having the possibility to lay their eggs in nests. They should also have access to perches that provides resting places during night. The newly introduced so-called furnished cages, and the different forms of free range systems, are providing decent environments for the birds (Tauson, 2005). However, unfortunately, these environments are also beneficial for the poultry red mite or chicken mite, *Dermanyssus gallinae* (DeGeer, 1778), a blood-feeding ectoparasite that causes stress, reduced egg production, anemia, and sometimes even death of its host. The poultry red mite is distributed worldwide, and is expected to be an ever increasing problem in egg producing facilities in Europe as a consequence of the approaching ban on conventional battery cages (Sparagano, 2009). The economical losses caused by poultry red mite infections in Europe have been estimated at 130 million GBP annually (De Luna *et al.*, 2008; van Emous, 2005). Since *D. gallinae* has proved very difficult to eradicate once it has established in poultry houses, more knowledge is needed on how the

mite enters the facilities. Moreover, since this mite feeds on blood from its host it could also be a potential vector of other pathogens, possibly spreading infection within and between farms. *Dermanyssus gallinae* can occasionally also bite humans, and hence, it is a working environmental issue for the farmer as well. Thus, for many reasons, it is evident that efficient and durable control strategies must be developed for this parasite.

This thesis is focused on studies of the transmission of *D. gallinae* between poultry facilities, and of the potential of this parasite to act as a vector or reservoir of the blood borne zoonotic bacterium *Erysipelothrix rhusiopathiae*, the cause of erysipelas in several species of domestic birds and mammals.

2 Background

2.1 *Dermanyssus gallinae*

2.1.1 Description

Dermanyssus gallinae is a blood-feeding arthropod parasite belonging to the class Arachnida, sub-class Acari, order Mesostigmata and family Dermanyssidae (Taylor *et al.*, 2007). The unfed adult female is around 0.75 mm long and when engorged up to 1.5 mm, and it is thus easy to observe by the naked eye. The male is slightly smaller than the female, and larvae and nymphs are smaller than adults (Fig. 1) (Baker, 1956; Sikes & Chamberlain, 1954). The mite is pear-shaped, and unfed larvae and nymphs are transparent to grayish-white in color while a newly fed mite is red to black (Fig. 6B) (Evans & Till, 1966). It has a weakly sclerotized dorsal shield that tapers posteriorly (Fig. 1). The ventral shield of the male is broader and longer than that of the females, and the female has a characteristic D-shaped anal shield (Fig 2). The mouth parts (chelicerae) are slender with small scissor like parts at the ends (chelae); and the four pairs of legs (coxa) of the nymphs and adult mites are located at the frontal part of the body. The larvae have three pair of legs (Baker, 1999).

Figure 1. Adult female (left) and male *D. gallinae* mites. (SEM: Johan Höglund, SLU)

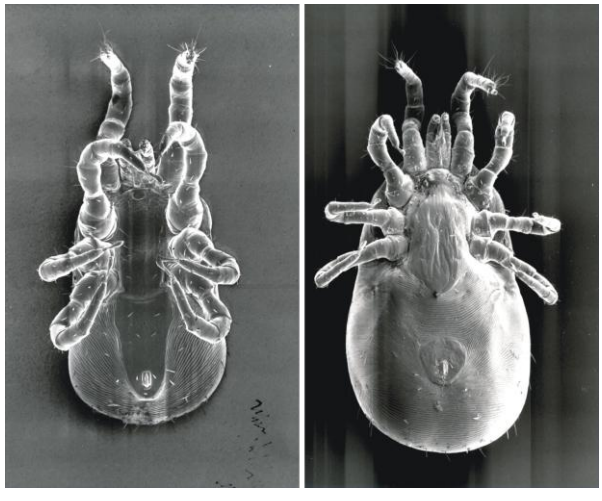

Figure 2. Ventral aspect of male (left) and female adult *D. gallinae.* (SEM: Johan Höglund, SLU)

2.1.2 Life Cycle and Behavior

Dermanyssus gallinae lives close to its hosts in the nests of wild birds, as well as in poultry premises (Kirkwood, 1963). The mite eggs are deposited in cracks or crevices in the poultry house and hatch into larvae in 2-3 days (Collins & Cawthorne, 1976). The larvae molt into protonymphs in 24–48 hours without feeding. The protonymphs have a blood meal, and then molt into deutonymphs in another 24–48 hours, and thereafter a second blood meal is required for the deutonymphs to become adults (Fig. 3) (Baker,

1956). The life cycle can be completed within 7 days under optimal conditions. The female mites feed on blood and lay eggs after each meal repeatedly, about 5-8 times during their life, with an average number of 2-4 eggs per blood meal. This means that one female mite can produce 20-30 eggs in a life time if a host is present (Oliver, 1966). *Dermanyssus gallinae* is haplo-diploid which means that male mites are haploid and female mites are diploid. In other words, male *D. gallinae* have one set of chromosomes and females have two (Oliver, 1977). They are thought to be arrhenotokous, which means that male mites develop from unfertilized eggs whilst females can only develop from fertilized eggs (Cruickshank & Thomas, 1999). The mites feed and reproduce at temperatures between 10 and 37 ˚C with an optimum around 25-30 ˚C. The optimal relative humidity for reproduction is around 65-75 % (Maurer & Baumgartner, 1992). *Dermanyssus gallinae* can survive for up to 9 months without food (Nordenfors *et al.*, 1999), and therefore it has to react to outer stimuli, such as changes in temperature, when a host is returning to the nest, allowing it to feed directly upon the host at its arrival (Kilpinen, 2001).

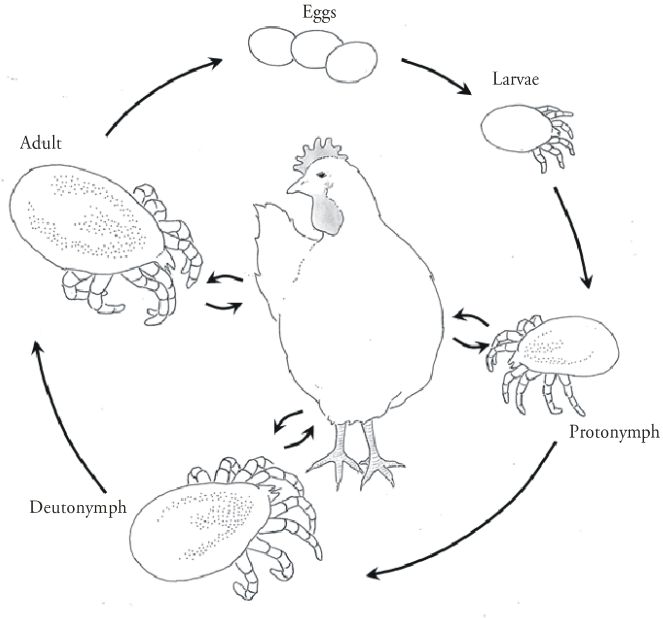

Figure 3. Life cycle of *D. gallinae.* (Drawing by Katarina Näslund, SVA)

2.1.3 Hosts

Dermanyssus gallinae is an avian parasite, and domestic birds such as the chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) are the most common hosts. It is also found on a variety of wild bird species (Baker, 1999). In the absence of an avian host it occasionally attacks mammals such as dogs, cats, rodents, horses and even humans causing irritation, dermatitis and pruritus (De Luna *et al.*, 2008; Mignon & Losson, 2008; Brockis, 1980; Williams, 1958). It reproduces mainly on avian hosts, but it has been shown that *D. gallinae* can produce viable eggs also when fed on mice and rabbits (Sikes & Chamberlain, 1954).

2.2 Housing Systems in Egg Production

2.2.1 Free Range Systems

About 60% of the Swedish egg-producing hens are kept in free-range systems, 8% of these being organic farms where the birds have access to an outdoor area as well. Free-range is when the hens can move unimpeded in a large area and have free access to feed, water, and litter allowing them to perform natural behavior such as laying their eggs in nests and to dust bath. Several systems designed for different stocking rates are available, and they all have some features in common. The floor of the house is partly or entirely covered with litter, and perches are placed above the ground, providing resting places at one or several levels in the system. Nests are also placed above the ground and often at multiple levels. Water and feed are provided at numerous places to facilitate feeding and drinking at any chosen occasion (Tauson, 2005). An example of an aviary free-range system is shown in Fig. 4.

Figure 4. An aviary free-range system with three levels above the ground on the right side. Food and water are provided on the bottom two floors, and perches and water on the top floor. The nests are on three levels on the opposite left side, and wooden shavings are used as litter. (Photo: Lotta Jönsson, SLU)

2.2.2 Battery Cages and Furnished Cages

The traditional battery cages are extensively used worldwide but will be banned in all countries of the European Union from 2012 (Anonymous, 1999). The battery cages have stocking densities of 400-700 cm^2/bird and are made of metal with a wire net as floor, with water nipples inside the cage and a feed trough on the outside. In 1999 the battery cage was banned in Sweden, and nowadays 40% of egg-producing hens in Sweden are kept in so-called furnished cages, also made of metal with wire net floor. Furnished cages are designed to keep smaller groups of hens (8-20 birds) than are free-range systems, and have a stocking density of about 600 cm^2/bird according to the 1999 EU legislation (Tauson, 2005; Anonymous, 1999). A feed trough is often placed in the front of the cage, and water nipples are on the inside. Each cage has a nest with a litter bath on top to save space, and one or two perches in line or crossing each other depending on the design of the cage (Fig. 5). The furnished cages are designed to allow the hens to perform natural behavior such as laying eggs in seclusion, resting

on perches and to some degree dust bathing (Abrahamsson & Tauson, 1997).

Figure 5. Furnished cage for 8 birds. A food trough is in the front of the cage, litter is placed on top of the nest, and the eggs will roll out on the net under the feed trough. (Photo: Lotta Jönsson, SLU)

2.3 Transmission of *D. gallinae*

2.3.1 Occurrence and Population Dynamics of *D. gallinae*

Dermanyssus gallinae is present in poultry premises worldwide and in all kinds of housing systems but the prevalence seems to be higher in back-yard flocks and free-range systems all over the world (Sparagano *et al.*, 2009; Nordenfors & Höglund, 2000). Aviary free-range systems for laying hens provide hiding places for the mite, which contributes to favorable reproduction conditions. The mites gather in the hens' nests, in crevices of the perches and in the litter, these places with close access to the birds (Roy *et al.*, 2009b). In studies of the distribution patterns of *D. gallinae* it has been

shown that mites seem to spread throughout the house from one or few original places. These sites are probably difficult to clean and access with treatment, or could be close to an outside possible source of infection (Nordenfors, 2000).

2.3.2 Possible Transmission Routes

Egg-producing poultry are raised to about 16 weeks of age in rearing farms, and thereafter are transported to the egg-producing facilities, where they will be kept until they are about 80 weeks old. The hens start to produce eggs by 18–20 weeks of age, and continue to do so until slaughtered. During this period, eggs will be transported on trays to a packing facility, often used by several farmers in the region. The trays can be made of plastic, and they are washed and reused and sent back to the farm again. Some packing facilities use cardboard trays that can be heated in microwave ovens before reused, or alternatively, returned to the farm without any treatment. This system of transporting live chickens and eggs provides several pathways for the mites to be transferred between farms. Wild birds building nests on the house or adjacent to it could also be a source of infection, as well as rodents moving in and out of the house (Mul & Koenraadt, 2009).

2.3.3 Molecular Approach and Selection of Target Genes

When studying transmission patterns of *D. gallinae* it is not possible to follow the route of a single mite between farms, and hence such investigations must have another approach. When a population of any given organism reproduces, the genetic variation can be used as a tool for establishing relationships between individuals, populations and species. Molecular sequence data have proven to be useful when investigating both evolution and physical movement of organisms. The genome evolves by mutations and recombination of genes over generations, and the amount of differences between two sequences could indicate how closely related they are, often illustrated as phylogenetic trees (Brown, 2007). Such trees based on morphological characters, which are considered important for the speciation of the organisms studied, have been extensively used for a long time. However, molecular sequencing provides accurate data much faster than does characterization of morphological structures, and has therefore become the principal tool by which phylogenetic trees are constructed. When comparing sequences, it is of great importance to choose suitable

genes for the comparison. If the objective is to study relationships between species, then the target genes can be conserved regions known to be present in related species, and when studying intra-species variation genes that evolve more rapidly need to be chosen (Page & Holmes, 1998).

When studying evolution at the genetic level the ribosomal RNA (rRNA) genes have been extensively used, because of their combined structure of rapidly evolving regions and evolutionarily conserved sequences (Wuyts *et al.*, 2004). The rRNA molecules are essential parts of the ribosome, which is pivotal for the translation of messenger RNA (mRNA) to protein. The small subunit (SSU) rRNA gene and the large subunit (LSU) rRNA gene are evolutionary conserved genes with several features that make them useful for many types of studies. The rRNA genes have, in particular, been used to resolve phylogenetic challenges at several taxonomic levels; and the non-coding regions between those genes, the internal transcribed spacers (ITS) 1 and 2, are used to study relationships between species of the same genus, or even within species (Schultz *et al.*, 2005; Berrilli *et al.*, 2002). Consequently, genes from the rRNA-complex are often among the first to be studied in any organism.

When studying populations within a species, rapidly evolving genes need to be studied, and the mitochondrial genes have been shown to be efficient tools to understand intra-species variation (Cruickshank, 2002). The mitochondrion is an organelle within the cell with its own genome, and nucleotide substitutions occur more frequently in the mitochondrial genes than in the nuclear genome. This is probably because the mitochondria lack some of the DNA repair system that operates on nuclear genes (Brown, 2007). Therefore, evolutionary differences between genes of the mitochondria can link related specimens to each other, by comparing the nucleotide substitutions.

2.3.4 Control of *D. gallinae*

Once established it is difficult to control and eliminate *D. gallinae* from poultry houses. The "all in all out" strategy practiced by commercial famers allows the facility to be cleaned thoroughly, which can greatly reduce the mite population. This strategy is not used in back-yard flocks, which makes it more difficult to clean and treat those houses (Nordenfors, 2000).

Several types of compounds could be used against *D. gallinae*; however, many of them are not suitable due to food safety reasons (Chauve, 1998). Resistance development in *D. gallinae* populations towards pyrethroids has been suspected (Fiddes *et al.*, 2005; Nordenfors *et al.*, 2001; Beugnet *et al.*, 1997), and development of resistance could limit the usability of candidate substances. At present, there is only one pharmaceutical against *D. gallinae* licensed for use with poultry in Sweden (Anonymous, 2009b). The active substance, phoxim, is an organophosophorus compound that inhibits the enzyme cholinesterase at the nerve synapses, causing paralysis and death of the mite (Jokanovic & Prostran, 2009). This substance is efficient but the mite has to come in contact with the formula to be killed (Abdel-Ghaffar *et al.*, 2009; Meyer-Kuhling *et al.*, 2007), and this is true for all compounds used against *D. gallinae.* Moreover, the mite is often hiding in inaccessible places, and can consequently escape treatment (Nordenfors, 2000). Another substance used in the control of *D. gallinae* is silica dust (SiO_2), which is spread at sites were aggregations of mites are recognized. However, silica dust is not harmless to the birds, and therefore one should be careful during its distribution. The farmers themselves can also be affected from inhaling silica dust, which makes it unattractive from both a working environment and an animal welfare point of view (Anonymous, 2009b).

A study performed in Norway concluded that heat treatment in combination with chemical treatment (phoxim) in empty poultry houses reduces the *D. gallinae* population. Heat treatment was conducted over 48 hours with maximum heat around 50-55 ˚C. The facilities were mite-free during the following production cycle in all treated houses. However, some deformation was observed on plastic equipment in the drinking water system, and the elasticity of the egg transport bands was reduced. This method is quite costly and should be performed during the summer since the outdoor temperature strongly affect the cost (Gjevre *et al.*, 2007).

A few attempts have been made to find suitable predatory mites as candidates for a biological control against *D. gallinae*. Predatory mites are used in the control of other pests, for example in green-houses (Mul *et al.*, 2009). If a predatory mite is able to kill all stages of *D. gallinae*, eradication could be efficient. A few candidate mites have been shown to kill *D. gallinae*; however, it has not yet been shown to work in a poultry house (Lesna *et al.*, 2009). Another biological control method is the use of entomopathogenic fungi. Some experiments have been performed where the selected fungi was shown to have an effect on *D. gallinae*; however, the

multiplication rate of the fungi did not exceed the reproduction rate of the mites, and thus was not efficient enough to eliminate the population of *D. gallinae* (Mul *et al.*, 2009).

2.4 Vector Potential of *D. gallinae*

The ability of a hematophagous arthropod to act as a vector for an infectious agent, i.e. its vector competence, is dependent on the capacity of the arthropod to take up, replicate and transmit the pathogen in combination with factors such as feeding rate, amount of blood fed at each occasion and the availability of the pathogen in question. A vector can be highly competent for one pathogen and incompetent for another, implying that each vector-pathogen system needs to be studied separately (Black & Severson, 2005). The poultry red mite has been suggested to be a vector of several pathogens causing disease in poultry, such as Newcastle disease, fowl cholera, chicken pox, encephalitis, fowl spirochaetosis, erysipelas and salmonellosis (Eriksson *et al.*, 2009; Valiente Moro *et al.*, 2007a; Chirico *et al.*, 2003; Chauve, 1998; Durden *et al.*, 1993; Zeman *et al.*, 1982). Some of these pathogens are zoonotic, which should be considered since *D. gallinae* occasionally can attack humans in absence of avian hosts. In several cases bacteria have been isolated from mites collected from farms with outbreaks of erysipelas, but the significance of *D. gallinae* as a vector of this bacterium is not resolved (Eriksson *et al.*, 2009; Chirico *et al.*, 2003). The poultry red mite has been shown to take up and transmit *Salmonella* Enteritidis between chickens under experimental conditions, suggesting it to be capable of spreading salmonellosis also under field conditions (Valiente Moro *et al.*, 2007a; Valiente Moro *et al.*, 2007b).

2.5 *Erysipelothrix rhusiopathiae*

2.5.1 Prevalence and Epidemiology

Erysipelothrix rhusiopathiae is a facultative non-spore-forming, Gram-positive rod. It is present worldwide, and causes disease in a variety of mammals, birds and fish. It is the causative agent of swine erysipelas, which is the most prevalent and economically important disease caused by this bacillus, and the domestic pig is the most important reservoir of *E. rhusiopathiae* worldwide. Poultry erysipelas is manifested by bacteremia and sepsis, and the turkey is the most frequently and seriously affected bird. The birds

become droopy, develop diarrhea, get pale combs and die (Wang *et al.*, 2010). The introduction of *E. rhusiopathiae* into poultry facilities has not been fully understood, but contamination by organic material from the outdoor area can be a possible transmission route (Mutalib *et al.*, 1993). With presence of *E. rhusiopathiae* inside the house, the birds become infected trough skin lesions or via mucous membranes (Bricker & Saif, 2003). Outbreaks of erysipelas in chickens are reported occasionally, and an increase of reports has been seen in Denmark, Germany and Sweden over the past decade (Eriksson *et al.*, 2009; Køpke *et al.*, 2005; Mazaheri *et al.*, 2005).

2.5.2 Control of Poultry Erysipelas

Removal of manure and dirt is crucial before treatment against *E. rhusiopathiae*, because of the ability of the organism to survive in organic material. If disinfection is not preceded by mechanical cleaning then the treatment will be less effective (Wang *et al.*, 2010). When an outbreak of erysipelas is confirmed in a poultry facility in Sweden, all birds are euthanized and the house is cleaned thoroughly and thereafter disinfected. The fixture and equipment of poultry houses can be difficult to clean thoroughly, and therefore vaccination is a possible additional treatment strategy that has proven useful in the control of *E. rhusiopthiae* in both pigs and poultry (Wang *et al.*, 2010). Poultry can be vaccinated with attenuated live *E. rhusiopathiae* strains and the vaccination gives good protection against re-infection (Kugelberg *et al.*, 2001; Wallgren *et al.*, 2000). Farms confirmed with outbreaks of poultry erysipelas in Sweden are advised to vaccinate the subsequent flocks of birds at their arrival to the farm (oral communication)[1].

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3 Aims of the Thesis

The general aims of this thesis were to elucidate the transmission routes of *D. gallinae* to poultry facilities, and to investigate the potential of the parasite to be a vector of the agent causing poultry erysipelas.

More specifically, each study had the following aims:

I. Investigate if wild birds are a potential source of infection of *D. gallinae* into poultry houses.
II. Map the distribution of *D. gallinae* from poultry facilities and identify factors important in the transmission of mites between poultry facilities.
III. Investigate if *D. gallinae* can act as a reservoir of *E. rhusiopathiae* within a poultry house over time.
IV. Investigate if *D. gallinae* can transmit the bacterium *E. rhusiopathiae* from infected to healthy chickens under experimental conditions.

4 Comments on Materials and Methods

A brief description of materials and methods used in this thesis is presented below. More detailed information is given in each paper (I-IV).

4.1 Collection of *D. gallinae* Mites (I- IV)

Dermanyssus gallinae were collected from poultry farms (paper I, II and III) by means of corrugated cardboard or plastic traps (Fig. 6A). Each trap was placed in a poultry house for 7 days and thereafter individually placed in a plastic bag and sent to the laboratory. Mites used in the experimental transmission study (paper IV) were provided from a laboratory population of *D. gallinae* at the Institute of Parasitology, University of Veterinary Medicine in Hannover, Germany. *Dermanyssus gallinae* from wild birds (paper I) were collected from experimental nesting boxes. The nests were recovered from nine bird species: pied fly-catcher (*Ficedula hypoleuca*), collared fly-catcher (*Ficedula albicollis*), spotted fly-catcher (*Muscicapa striata*), starling (*Sturnus vulgaris*), tree sparrow (*Passer montanus*), blue tit (*Parus caeruleus*), swallow (*Hirundo rustica*), great tit (*Parus major*) and wryneck (*Jynx torquilla*). The nests were individually put in plastic bags and sent to the laboratory. Live mites were collected from the plastic bags and the remaining mites recovered by flotation in water. Briefly, the nest was pressed to the bottom of a water-filled bucket and floating mites were collected with a small strainer. Only mites morphologically verified as *D. gallinae* were used in the molecular investigation.

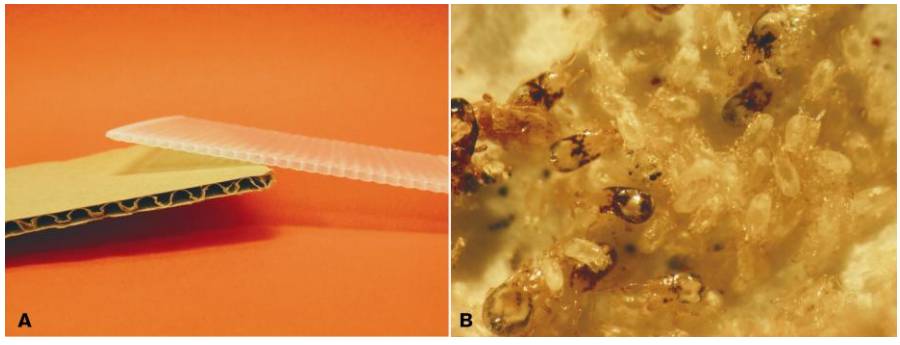

Figure 6. **A**: Corrugated cardboard and semi-transparent plastic traps used to collect *D. gallinae* in poultry premises. **B**: Typical aggregation of mites inside a cardboard trap. Adults and nymphs that have fed on blood are red or grey while unfed larvae and nymphs are transparent to white in color. (Photos: Sofia Holmgren and Sara Brännström, SLU/SVA)

4.2 Preparation of DNA (I & II)

In paper I nymphs and adult mites from the wild bird nests were dead and to some degree desiccated. Some of these mites were therefore difficult to crush, resulting in samples of poor quality or containing insufficient amounts of DNA. Engorged female mites from poultry facilities (paper I) were individually placed in wells of a round-bottomed ELISA plate, and hatched larvae and nymphs were used for preparation of DNA to avoid contamination of host blood. This was not done with the mites in paper II because the contamination was absent in the previous study. Consequently, mites of all life stages were used in the DNA extraction for paper II.

4.3 PCR Amplification and Sequencing (I & II)

To amplify the rRNA SSU gene universal primers targeting the start, central part and the end of the SSU gene were used. A universal primer situated at the 3' end of the SSU gene was used in combination with a primer located at the 5' end of the rRNA LSU gene when amplifying the ITS regions. This was done to ensure that the entire ITS1, 5.8S and ITS2 regions were covered in the PCR product (Fig. 7).

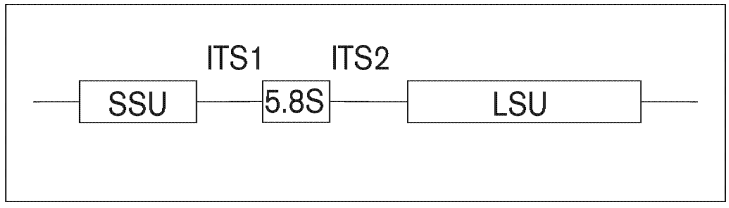

Figure 7. Schematic picture of the rRNA genes that were targeted in paper I.

When the molecular investigation of the mitochondrial gene cytochrome oxidase c subunit I (CO1) was started (study II), little was known about this gene in *D. gallinae*, and hence suitable primers had to be made and tested. Finally, primers were designed by aligning an EST sequence, hypothesized to be *D. gallinae* CO1, with sequences of related organisms. PCR reactions were performed as described in each paper (I & II), and products were purified and sequenced in both directions with standard protocols.

4.4 Phylogeny (I & II)

Sequences were analyzed using the Vector NTI program Suite 10 (Informax Inc., Oxford, U.K.). In paper I an alignment of the groups based on the origin of the mites (domestic or wild birds) was constructed. A model of the secondary structure of ITS1 was then constructed, showing potential stem-loop structures. Phylogenetic analysis was performed on 46 of the samples in paper II, one consensus sequence from each haplogroup and one outgroup: *Dermanyssus hirundinis*. In paper II alignments were transferred to MEGA ver. 4.0 (Kumar *et al.*, 2004) where a neighbor-joining analysis was performed. Population genetic analysis of the relationship between the haplogroups found in the first alignment was undertaken, in an attempt to find evolutionary relationships between the haplogroups. Additional interviews with the farmers who provided us with mites were also conducted, to receive information on the physical connections each farm had with other possible sources of infection.

4.5 Necropsy and Organ Sampling (III & IV)

The hens in the experimental study (IV) were euthanized by dislocation of the spinal cord and thereafter necropsied. Samples of heart blood, liver and spleen were taken and analyzed for the presence of *E. rhusiopathiae*. In study III dead hens were collected from the poultry house and sent to the laboratory, where necropsy and sampling of liver and spleen for bacteriological examination was conducted.

4.6 Bacteriological Examination (III & IV)

Blood samples were taken from the chickens in the experimental study (IV), and put directly in the selective medium crystal-violet sodium-azide (2%) broth at 37 ˚C for 48 hours. The broth was then spread on blood agar plates, and after another incubation *E. rhusiopathiae* was identified, based on morphological characteristics of colonies, microscopic appearance, Gram's staining and biochemical tests. This procedure was followed for all bacteriological examination; however, preparation differed depending on the type of sample investigated. Heart blood was treated as blood samples, whereas liver and spleen were cut into small pieces before being put in selective medium. The mites in study IV were placed in 70% ethanol for 30 sec and thereafter dried, to remove external bacteria, before being crushed and put in selective medium. Mites in study III were crushed directly and put in selective medium without any prior external treatment. This was because the objective of study III was to observe if the mites could be a reservoir of *E. rhusiopathiae*, and hence the external carriage of bacteria was of interest as well.

4.7 Experimental Design of Study IV

The objective of the experimental study (IV) was to investigate whether *D. gallinae* could transmit *E. rhusiopathiae* from infected to uninfected laying hens. One great challenge with this experiment was to find a suitable infection dose of *E. rhusiopathiae*, and therefore a pilot trial was conducted to find a dose high enough to cause bacteremia without being lethal to the birds. The pilot trial was conducted in three parts, evaluating the doses $2.5x10^8$, $2.5x10^9$ and $2.5x10^{10}$ *E. rhusiopathiae* CFU/ml, starting with the lowest dose and evaluating each dose before proceeding to the next. When a suitable dose was found ($2.5x10^{10}$), nine hens were inoculated with *E. rhusiopathiae*, and *D. gallinae* was allowed to feed on the chickens for 5 days. The mites were placed in specially constructed perches providing a suitable hiding place for reproduction and development (Fig. 8). Blood samples were taken from the birds during the whole experimental period, and analyzed for presence of *E. rhusiopathiae*. The chickens were euthanized, and all mites were collected and stored for 20 days at 8 ˚C. Mites were cooled and stored, in an attempt to maximize their feeding rate in the following transmission experiment, and to allow replication of *E. rhusiopathiae* inside the mite. *Dermanyssus gallinae* were then placed on healthy chickens to investigate the capability of the mites to transmit the

bacterium. Presence of *E. rhusiopathiae* in blood samples withdrawn from the chickens was analyzed by bacteriological examination and ELISA.

Figure 8. Specially constructed perches providing hiding and mating place for *D. gallinae* in the experimental study (IV). (Photo: Sofia Holmgren, SLU)

4.8 Preparation of Inocula (IV)

The strain of *E. rhusiopathiae* used for the experimental infection originated from an outbreak of poultry erysipelas in Sweden 2002. The strain had been stored at -70 ˚C in serum broth. The inoculum was prepared as overnight culture, and the total number of viable *E. rhusiopathiae* was counted before inoculating the chickens. The control animals were injected with an equal volume of serum broth to the infected chickens but without *E. rhusiopathiae*.

4.9 ELISA (IV)

By means of standard bacteriological examination it can be difficult to demonstrate the presence of bacteria in blood samples from chickens with bacteremia. Therefore an in-house indirect Enzyme-Linked Immunosorbent Assay (ELISA) modified after Wallgren et al., (2000) was used to detect the presence of serum antibodies specific to *E. rhusiopathiae*.

Crude antigen extract for the ELISA was prepared from the same strain as was used to inoculate the chickens. The serum to be analyzed was separated from the blood samples taken in the experimental study, and then stored at -20 °C until analyzed. The results were expressed as the ratio between the sample optical density (OD) and the OD of the positive control. Statistical analysis was done by comparing the means of OD for control chickens and infected chickens. 95% confidence intervals (CI) were used, and zero overlap of the CIs indicated significant differences between control and infected chickens.

4.10 Sampling of Hens and Mites During Outbreak (III)

In the case study (III) five dead hens were sampled from each section of the house, and the location of the dead hens was noted. Thereafter, corrugated cardboard traps were distributed in the system and in the vicinity of each location of a dead hen. When traps were removed and sent to the laboratory, cleaning of the facility was carried out. When the next sampling of mites was performed, about 15 months later, the traps were placed at the same positions as in the first sampling.

4.11 PFGE (III)

Pulse-Field Gel Electrophoresis (PFGE) is used to investigate whether isolates of bacteria are genetically related. Briefly, one colony of bacteria is embedded in agarose, and lysing *in situ* is performed followed by cleavage with the chromosomal DNA restriction enzyme endonuclease SmaI. The restriction fragments are then run on an agarose gel and the results are illustrated as a DNA restriction pattern for each sample on the gel. The restriction patterns are then compared and determination of the genetic relationship between samples can be made. In paper III a portion of the samples from hens and mites that were positive for *E. rhusiopathiae* was analyzed by PFGE, to investigate the genetic relationship between bacteria isolated from both hens and mites in the poultry house. This was done to investigate whether the mites and hens shared the same strain of *E. rhusiopathiae*, to evaluate the importance of *D. gallinae* as a reservoir of this particular bacterium in poultry houses.

5 Results and discussion

5.1 Transmission Routes (I & II)

The studies of transmission routes of *D. gallinae* were initiated by analyzing the SSU rRNA gene of 19 mites collected from wild birds and domestic chickens. These sequences were all found to be identical.

To increase the resolution we then analyzed the ITS1-5.8S-ITS2 fragment of 10 individual mites collected from nests of wild bird and 23 mites from six different poultry houses. All sequences of mites with wild bird origin were identical, as were the sequences from those of poultry. Mites collected from wild bird nests and morphologically verified as *D. gallinae* differed in 10 base pair (bp) positions in the center of the 207-bp ITS1 region in comparison with *D. gallinae* from poultry houses. The nucleotide differences were not randomly distributed throughout the ITS1 region, but appeared as compensatory base changes (CBC) in the putative stem-loops of the RNA transcript, based on the structure of Morrison (2006) (Fig. 9).

Muller *et al.* (2007) suggested that CBCs can be used as molecular classifiers for speciation, as CBC appears much more frequently when two species are compared, as opposed to within species alignments. The ITS1 region accumulates nucleotide substitutions at a high rate since it is a non-coding region spliced out from the RNA transcript. It has no function in the transcription into protein, and thus it has proven useful in investigations of closely related species within the Acari (Cruickshank, 2002). Moreover, DNA extracted from the wild bird mites was not possible to amplify with primers targeting the mitochondrial CO1 gene used in study II. This was

expected, as this gene is evolving slightly faster than the ITS region and extensive polymorphism within species has been observed in several arthropod species (Cruickshank, 2002).

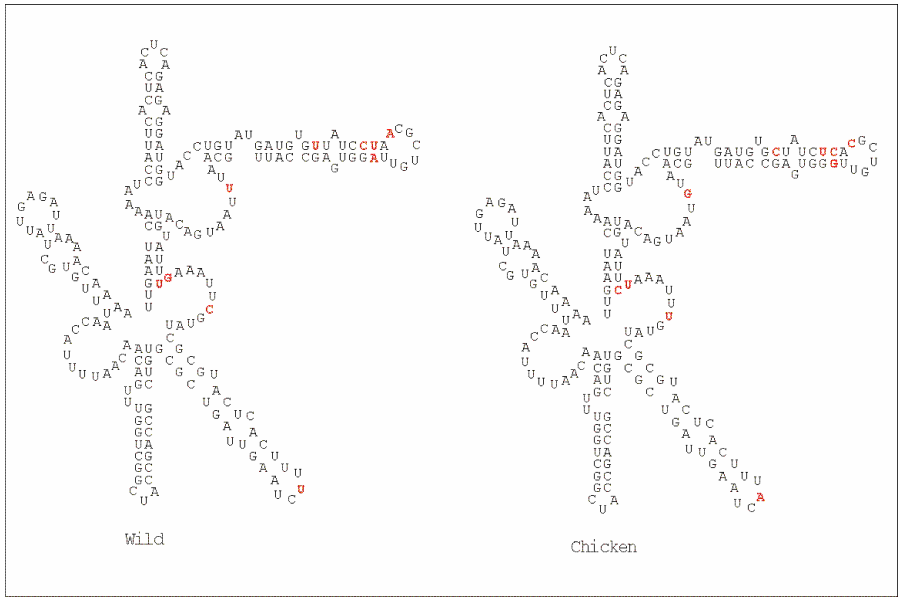

Figure 9. Putative stem-loop structures of the ITS1 region of the rRNA in *D. gallinae* from wild birds and chickens. Nucleotide differences are in red.

When study I was performed *D. gallinae* had not been particularly well studied, and hence there were no GenBank data available to compare our sequence with. However, when a BLAST search was conducted in January 2010 on the consensus sequence from wild bird mites in study I, it was shown to be closer to the *Dermanyssus hirundinis* and *Dermanyssus longipes* entries in GenBank than to that of *D. gallinae*. In a recent investigation on the species boundaries of *D. gallinae*, it was concluded that several lineages of this species are likely to be present, and that *D. hirundinis* and *D. gallinae* are molecularly divergent (Roy *et al.*, 2009a).

Roy and colleagues (2009a) collected mites from wild bird nests in France and found *D. gallinae*, *D. hirundinis*, *D. longipes* and *Dermanyssus carpaticus* from nests of wild birds of the same species as were investigated in study I. The climate and behavior of the wild bird species examined are of importance when comparing these data. Some of the actual bird species migrate from Sweden during winter, whereas the same species

remain as residents when found in e.g. France, Spain and Italy. In study I, the only non-migrating bird species sampled was the great tit (*Parus major*), from which we could only successfully sequence one sample, and this sequence differed in 10 nucleotide positions compared to *D. gallinae* from poultry. Since only one specimen was present in the nest, we cannot exclude that *P. major* could harbor *D. gallinae* in addition to the mite sequenced.

In a study by Lesna *et al.* (2009) it was shown that *D. gallinae* from starlings (*S. vulgaris*) and chickens in poultry houses in the Netherlands were conspecific when using ITS and CO1 as molecular markers. This indicates that *D. gallinae* can in fact parasitize starling hosts in Western Europe. In study I we included seven sequences from mites found in nests of starlings, and all sequences were identical and different from that of *D. gallinae* from poultry houses (Fig. 9). Starlings migrate from Sweden to North-West and Central Europe during winter (Svensson & Grant, 1999). In the Netherlands the mean temperature is above 0 ˚C during the winter months (Anonymous, 2010b) as compared to mean temperatures of about -5 ˚C for several months during winter in Scandinavia (Anonymous, 2010a). Thus, starlings are probably not a good host for *D. gallinae* in Sweden while they seem to be so in the Netherlands.

In study I, we also found arthropods such as *Ixodes* spp., *Hypoaspis* sp. and *Parasitus* sp. in the nests of several wild birds. Some of these arthropods have connection with the nesting material or to the birds living in that particular nest. However, *Hypoaspis aculeifer* is a predatory mite shown to feed on *D. gallinae* (Lesna *et al.*, 2009), and the presence of competing and predatory mites in a wild bird nest should therefore also be taken into account when studying the prevalence of *D. gallinae*. It should be noted that in the study by Lesna *et al.* (2009) *H. aculeifer* was found in nests of starlings, and the presence of this mite was related to the number of *D. gallinae* present in the nest.

Wild birds appear not to be an important source of infection of *D. gallinae* for domestic chickens in Sweden whereas they could be so in other parts of Europe, due to the behavior of migrating birds and the warmer climate. Wild birds could, however, be a reservoir of *D. gallinae* if the mite could migrate to a wild bird nest in the absence of chicken hosts, e.g. if a poultry facility is kept empty for a long period of time. Under normal egg production conditions such a situation would be unusual,

however. When a production cycle is ended, the birds are euthanized, and thereafter the house is cleaned and new pullets arrive within a few weeks. This short period is probably not long enough for the parasite to start searching for new hosts, since it can survive for up to nine months without food (Nordenfors *et al.*, 1999). In a study on host range of *Dermanyssus* species by Roy *et al.* (2009b), it was shown that *D. gallinae* is unique in harboring synanthropic populations, since it is mainly found with bird species living close to humans, such as doves, canaries and other cage-birds. This may indicate that *D. gallinae* has been selected and adapted to an environment of a poultry house, and therefore is unlikely to migrate to nearby bird nests in absence of chicken hosts (Roy *et al.*, 2009b).

If *D. gallinae* should find resident wild bird species attractive as hosts all year around in Scandinavia, it is essential for the mites to survive in the birds' nests during winter. In most parts of Sweden the temperature is below 0 °C for several months during winter, and temperatures of -10 °C and below are not unusual, at least in the middle and northern parts of the country (Anonymous, 2010a). In our laboratory, we have observed that *D. gallinae* can survive in -20 °C for more than 24 h, while 48 h in the freezer has been lethal. Hence, it is not likely that *D. gallinae* could survive in nests of wild birds in Sweden, even if they could feed and reproduce during the winter months. In contrast, a poultry house provides a good environment for *D. gallinae* to feed and reproduce all year around in Sweden, and consequently is a more attractive environment for this parasite.

In study II a large number of *D. gallinae* mites from more than 50 farms in Sweden and Norway (Fig. 10), and some additional samples from Finland, Denmark, Scotland and the Netherlands were analyzed with regards to the mitochondrial CO1 protein coding gene and the ITS region. A total of 283 samples were successfully sequenced and used in the phylogenetic analysis. The investigated CO1 region was 514 bp long, and nucleotide differences between the sequences were found in 86 positions. The samples grouped into 42 haplotypes, as shown in Fig. 11. The nucleotide substitutions were most frequent in the third coding position (75.6%). *Dermanyssus gallinae* from both Norway and Sweden had two major haplotypes (A16 & B9 and A1 & B7, respectively) found in several farms, but no common haplotype was found across both countries. Sweden and Norway are separated by several legislations regarding transportation of animals between the countries, and Norway is not a member of the European Union, which also regulates some of these legislations. Since

movement of poultry between those countries is limited, these results are not surprising. Besides, absence of a common haplotype in farms from Norway and Sweden strongly supports our conclusion that wild birds are of minor importance as sources of transmission of *D. gallinae* to chickens.

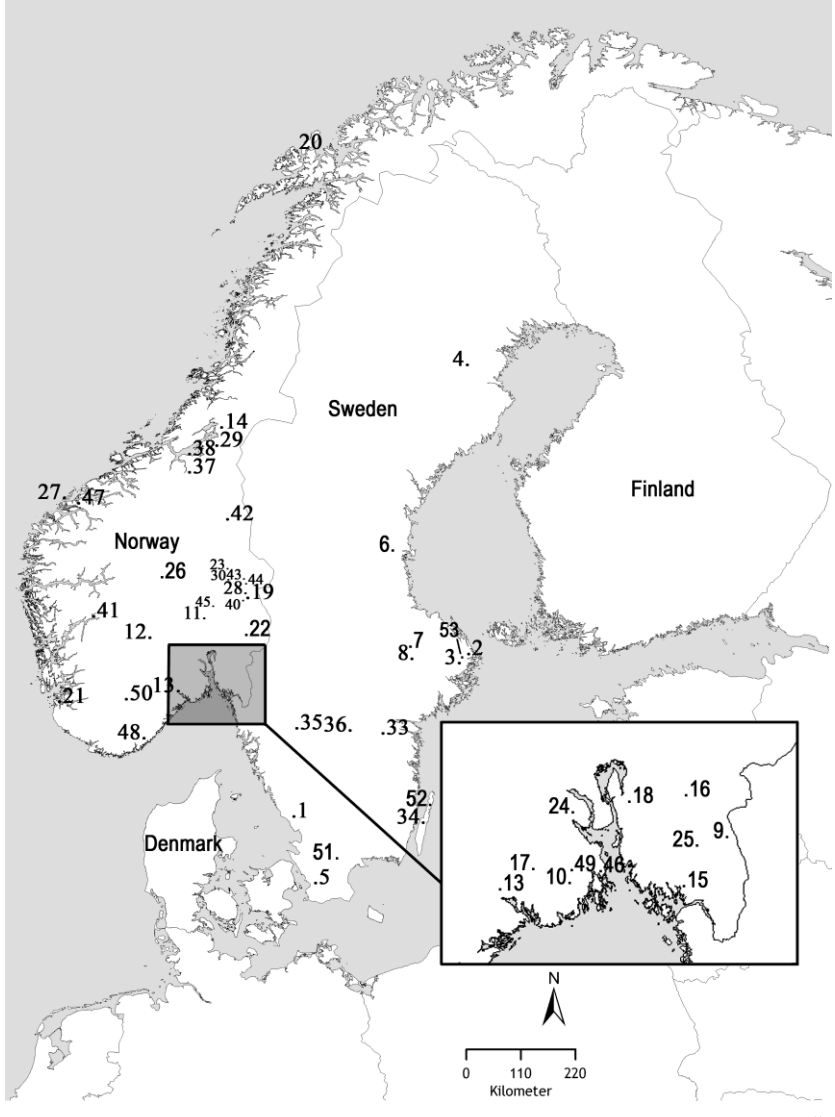

Figure 10. Map of Sweden and Norway showing the location of farms where *D. gallinae* was sampled for study II.

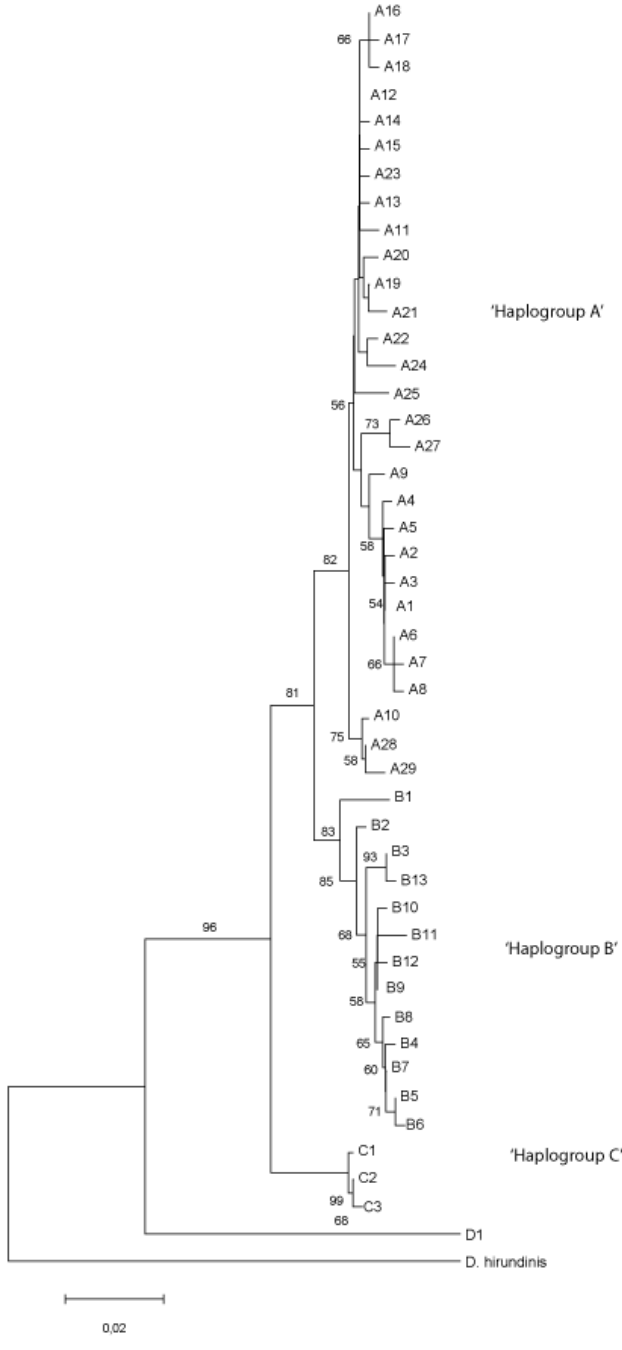

Figure 11. Phylogenetic tree showing the haplotypes of the CO1 sequences analyzed in study II, with *Dermanyssus hirundinis* as outgroup.

Another factor limiting the genetic variation in the *D. gallinae* populations could be the geographic isolation of each farm. The infection of a farm could be caused by only a few individual mites, which would facilitate genetic fixation in a population, so that geographically distant isolates would show larger genetic variance. In study II there was no evidence of geographical grouping of haplotypes within each country. The most common haplotype found in Sweden (A1) was represented in farms from all parts of the country, and the two most common haplotypes in Norway (A16 and B9) were distributed in the same way. *Dermanyssus gallinae* does not have wings and cannot spread on its own. Additionally, it lives most of its life away from the host hidden, in the poultry house; consequently this parasite must hitch-hike on something that can offer a physical path to the poultry house. The results from study II indicate that mite populations are not restricted by geographic distances, and hence, *D. gallinae* is more likely to be transmitted by some common carrier.

The intra-farm genetic variation was relatively low: the majority of farms showed no variations, while 13 farms had two haplogroups present and two farms had more than two haplogroups. The intra-farm variation was larger in Sweden than in Norway, with 56 and 18% of the farms showing variation in each country, respectively. Three Swedish farms were sampled at two occasions, 2004 and 2009, and one of these farms showed variation between years, having only one haplotype represented in 2004 (A1) and two haplotypes in 2009 (A1 and B1). Since *D. gallinae* is thought to be haplo-diploid and arrhenotokous (Cruickshank & Thomas, 1999), one single female can be enough to infect a poultry house. The female will lay eggs that hatch into males with which she can mate, and thereafter produce female mites. The re-establishment of a *D. gallinae* population in a house after cleaning and chemical treatment is also facilitated by this mechanism.

According to coalescent theory, the ancestral haplotype may be identified as the most common one (Posada & Crandall, 2001). In study II the most common haplotypes were A1 and B9 for Sweden and Norway, respectively. These results indicate that a common source of infection, independent of geographic location, is rather likely to transmit *D. gallinae* than mites being repeatedly introduced to the house during a production cycle. For example, if there was a constant movement of mites in and out of a poultry house, there would probably be more than one haplotype present. In study II, we sampled 10 individual mites from each house and we aimed to sample mites originating from several traps, located at different positions

in the poultry house. This was not always possible, since some traps arriving to the laboratory were empty. On the other hand, in those cases when the traps were empty, the mite population was relatively low and perhaps just about to establish. These farms are more likely to have fewer haplotypes present than do farms with dense mite populations, if mites are continuously being transmitted into the house.

The results from interviews with farmers indicated that most of the farms in Norway used Lohmann hybrids for egg production. Two of the Norwegian farms were rearing Lohmann hens, and supplying other farms with birds. The *D. gallinae* haplotypes found in those two farms were also present in the egg-producing farms. However, only a small proportion of rearing farms was sampled in this study and further investigation is needed to test if such farms could be a source of infection. Two of the Swedish farms with only one haplotype present reared their own hens on the farm, and thus did not have any incoming birds to their premises. A majority of the Norwegian farms had for years acquired their animals from the same rearing farm, whereas some of the Swedish farms with multiple haplotypes had changed supplier of birds over the past few years. We could not, however, point out one common source of infection, as one haplotype was present in several farms regardless of bird supplier.

Interviews also revealed that the egg-producing industry in Sweden has taken preventive measures in the fight against *D. gallinae*. Several egg-packing facilities in Sweden have changed their egg-trays from cardboard to plastic ones that can be washed before distributed to the production facility. The cardboard egg trays offer a good environment for *D. gallinae* to hide and reproduce, and several farmers have observed mites in these egg trays. When cardboard egg trays are circulated between farms they are evident physical pathways for *D. gallinae*. Even if the same trays are reused at one farm, the unpacking of eggs at the packing facility could enable cross transmission of *D. gallinae* between farms, allowing mites from one farm to hitch-hike on egg trays to another one. The farms sampled in Sweden delivered their eggs to five different packing facilities, and there were no clear patterns of haplotypes between farms indicating a packing facility as common source. Our results imply that transmission of *D. gallinae* between farms during a production cycle is limited, since the intra-farm variation was low. Moreover, farms sharing an egg-packing facility were found to have separate haplotypes, supporting the conception that exchange of mites between farms during a production cycle is limited.

Several farmers had the experience that thorough cleaning followed by chemical treatment was a successful strategy to reduce the mite population, and also to keep it low for a long period of time. In a few farms, regular vacuum cleaning of identified mite aggregation sites was used as a control strategy that was efficient over shorter periods. However, the mite population was observed to increase again after a few months. Since the intra-farm genetic variation is low, it is evident that small numbers of *D. gallinae* are enough to cause widespread infection of a poultry house. This conception is supported by the farmers' observations that mite populations always seems to recover even if thorough cleaning and chemical treatment is done. Consequently, for the control of *D. gallinae* it is of utmost importance to prevent the parasite from ever entering the poultry house in the first place.

5.2 Vector Potential (III & IV)

In study III, we could isolate *E. rhusiopathiae* from 8 out of 10 pools of *D. gallinae* collected in the poultry house with a confirmed outbreak of erysipelas. The outbreak was noticed by the farmer as high mortality and a drop in egg production. That was also the case in the report by Chirico *et al.* (2003), where *E. rhusiopathiae* could be isolated both from the interior and exterior of *D. gallinae* collected in connection with another outbreak of poultry erysipelas. The isolation of *E. rhusiopathiae* from *D. gallinae* in study III was done after 4 months of storage at 4 °C, but it was not possible directly when the samples arrived at the laboratory. The most likely explanation for this result is that competing bacteria initially present in the samples on arrival at the laboratory could influence the outcome of the bacterial isolation by overgrowth. Furthermore, *E. rhusiopathiae* is a facultative anaerobe and known to survive at low temperatures, and should thus not be killed by storage in at 4 °C (Wood, 1999).

The PFGE results showed homogenous banding patterns of isolates from hens and mites confirming that *D. gallinae* and hens were infected with the same strain of *E. rhusiopathiae.* However, it could not be determined whether mites were the source of infection or if it was brought into the house trough some other path.

The hens in the subsequent flock in the house with erysipelas (study III) were vaccinated against *E. rhusiopathiae*, and clinical signs of disease were

absent during the entire production period. *Dermanyssus gallinae* mites collected from this house 15 months after the outbreak were negative for *E. rhusiopathiae*. When the poultry house was cleaned and disinfected after the outbreak, no additional treatment specifically directed against *D. gallinae* was made. A majority of mites were probably killed and removed as a consequence of physical cleaning. However, it is known that they can easily escape such treatment if hidden in crevices in the furnishing and in nests, and consequently mites were expected to be found in the subsequent flock. Environmental samples with regard to *E. rhusiopathiae* were not taken, and therefore it is unknown if the bacterium was present in the stable. It would have been interesting to sample *D. gallinae* in connection with the stocking of new birds into this house, to investigate whether the surviving mites still harbored bacteria, but unfortunately such sampling was not done. Nevertheless, the fact that we were able to isolate *E. rhusiopathiae* from *D. gallinae* stored at 4 °C for 4 months indicates that the bacteria can survive in or on the poultry red mite for some time; however, the maximum time is unknown.

In study III we did not undertake any molecular investigation of the mites sampled, and therefore it is unknown whether the mite populations present before and after the outbreak was the same, or if new mites had entered the house during the 15 months between the sampling occasions. It is, however, unlikely that *D. gallinae* would be represented by two separate populations in this poultry house before and after the outbreak, given the results from study II. If *E. rhusiopathiae* is present in a poultry house that holds *D. gallinae,* the bacterium could persist in the mite population and accumulate over production cycles. The replication and transmission of *E. rhusiopathiae* would be dependent on the number of mites surviving the physical cleaning between production cycles. If only a few mites survive, they either have to be capable of transmitting the bacterium to their offspring, or the bacteria must replicate within and on the surviving mites, to allow them to spread the bacteria in the poultry house.

Dermanyssus gallinae has been shown to aggregate in the litter and droppings in the poultry house (Roy *et al.*, 2009b), and therefore *E. rhusiopathiae* could theoretically be transmitted to pastures when the birds' manure is spread on the fields. In pigs, it has been suggested that *E. rhusiopathiae* can be transmitted by straw harvested from fields fertilized with manure from pigs affected by severe erysipelas. Herds continuously affected by severe erysipelas became healthy when given straw harvested

from fields fertilized by other sources of nitrogen (oral communication)[2]. Additionally, outdoor pigs are often found to have signs of erysipelas at slaughter (Kugelberg *et al.*, 2001). In poultry production it is most common to use wood shavings as litter and the food is heat treated which should minimize the transfer of *E. rhusiopathiae* into the house. As this bacterium can persist in organic material for many years (Wang *et al.*, 2010), its entry into a poultry facility implies some gap in the biosecurity standards of the farm.

If *D. gallinae* can carry *E. rhusiopathiae* an attendant question is whether they can also transmit the bacterium to poultry causing erysipelas. To further investigate the role of *D. gallinae* as a vector of *E. rhusiopathiae*, an experimental transmission study was undertaken (study IV). This study was designed to mimic natural conditions including both chickens and arthropods, which was a great challenge. Specific Pathogen Free (SPF) chickens are preferably used in experimental studies, to eliminate the possibility that the animals have already been exposed to the pathogen under investigation. However, to create as natural conditions as possible we chose to use layer chickens that were 20 weeks old, and at arrival to the experimental facility they were sampled for presence of antibodies against *E. rhusiopathiae*. For practical reasons, to be in control of the *D. gallinae* mites used, these chickens had to be kept in isolators. However, the mites appeared not to appreciate the high air flow in the isolator, and escaped the specially designed perches and moved towards the bottom of the isolator. Mites were recovered in the perches one week after the airflow was turned off, but they had not fed on the chickens. Additionally, the temperature and humidity in the isolator were adjusted by the ventilation system, and were not optimal for *D. gallinae* to feed and reproduce. Thus, the climate in the isolator was suitable for the hens but less suitable for the mites, and did not fully reflect the climate in a poultry house.

The hens in study IV had clinical signs of erysipelas, such as pale combs, fatigue and anorexia between day 3 and 5 after inoculation, and bacteria could be isolated from blood samples taken on these days. Nevertheless, *E. rhusiopathiae* could not be isolated from heart blood of any of the birds necropsied, whilst one liver sample and four spleens were positive for *E. rhusiopathiae* (Table 1). These results are probably due to the birds' quick

[2] Per Wallgren, Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute, Uppsala, Sweden

recovery from erysipelas during day 6-9 after inoculation. Isolation of bacteria from internal organs has also been shown to be more successful in birds that have died from erysipelas, as compared to sick birds that are killed and tested (Bricker & Saif, 2003).

The results from the ELISA showed that all eight birds positive for *E. rhusiopathiae* were seropositive 7 or 9 days after inoculation. The mites were recovered from the perches after the euthanization of the birds, and the majority were engorged. Nevertheless, uptake of bacteria by the mites could not be demonstrated in the bacteriological examination, and neither could transmission to the healthy birds. The results from this experimental study (IV) should, however, not exclude *D. gallinae* as a vector of the erysipelas agent. There could be several reasons for the outcome of the study (see below), and the results from study III and Chirico *et al.* (2003) have shown that this parasite is capable of harboring *E. rhusiopathiae* externally as well as internally.

Table 1. *Results from the bacteriological examination of chickens in the experimentally infected group (study IV). The control birds are represented by only one individual, as all of them were negative for E. rhusiopathiae throughout the whole experimental period.*

Day	0	3	5	7	9	Post mortem day 11		
Chicken	Blood	Blood	Blood	Blood	Blood	Heart	Liver	Spleen
Infected 1	-	+	-	-	-	-	-	-
Infected 2	-	+	+	-	-	-	-	+
Infected 3	-	+	-	-	-	-	-	+
Infected 4	-	+	+	-	-	-	-	-
Infected 5	-	+	-	-	-	-	-	+
Infected 6	-	+	+	-	-	-	-	-
Infected 7	-	+	-	-	-	-	-	-
Infected 8	-	+	+	-	-	-	+	+
Infected 9	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-

One aspect to take into consideration is that the chickens used in this experiment were young, in good condition and kept in a clean environment. Outbreaks of erysipelas most often occur in the middle or late part of the production cycle, when hens have lower physical fitness. Nevertheless, it has been shown that *E. rhusiopathiae* is pathogenic to 2 day old chickens (Hollifield *et al.*, 2000) and under experimental conditions, to adult laying hens between 17 and 37 weeks of age (Mazaheri *et al.*, 2005).

Relating the time course of bacteremia can also be difficult since acute bacteremia develops over 12-24 hours while sub-acute bacteremia could persist for weeks. Blood samples for bacteriological examination need to be taken at accurate time points for the presence of bacteria to be demonstrated. Similarly, the mites need to feed on the chickens at an accurate time point for them to acquire *E. rhusiopathiae*. Further, transmission of an infectious agent to a host by an arthropod vector is associated with several biochemical and structural factors that must be present in the vector for the pathogen to complete its life cycle inside the arthropod. The pathogen has to penetrate the mid-gut cells of the mite, replicate and finally end up in the salivary glands, from where they can be transmitted during the next blood meal (Black & Severson, 2005).

The vector-pathogen system of *D. gallinae* and *E. rhusiopathiae* is not well understood, but isolation of *E. rhusiopathiae* from the interior of *D. gallinae* has been done on several occasions (III) (Chirico *et al.*, 2003), which demonstrates that the mites can take up this bacterium. Blood-feeding arthropods can stimulate immune responses in the host that can affect the development of the arthropod, reduce its feeding and even kill it. Such defense mechanisms have been shown to affect the transmission of pathogens as well (Wikel *et al.*, 2005). Hosts exposed to bites from pathogen-free arthropods are more resistant to transmission of pathogens when later exposed to infected arthropods of the same species (Wikel & Bergman, 1997).

It is possible that *D. gallinae* needs to feed on hens with sepsis, alternatively, immediately after their death in order to acquire *E. rhusiopathiae* in detectable amounts and of sufficient concentration to enable transmission. Such circumstances may be provided in a poultry house during an outbreak of erysipelas, whereas they are not possible to create in an experiment due to animal welfare considerations. The number of *E. rhusiopathiae* infected *D. gallinae* in study IV is not known, and if only a few individuals were infected then the host immune response towards the mite could have prevented the transmission of *E. rhusiopathiae*, even if replication was taking place inside the mite. Furthermore, if only a few mites were successfully taking up the bacteria then the possibility to miss them in the bacteriological examination was evident, due to the low sensitivity of the test. The transmitted *E. rhusiopathiae* also needs to be of high concentration in order to cause disease or result in production of antibodies detectable by the ELISA. An adult female *D. gallinae* ingests

about 0.2 mg blood per meal (Sikes & Chamberlain, 1954), which means that probably several mites would be required to transmit the pathogen to cause disease. As mentioned earlier, the hens were in good physical condition and were most likely less susceptible to infection by *E. rhusiopathiae* than were the hens in study III, which had been producing eggs for over 40 weeks. A vector-pathogen system of *D. gallinae* and *E. rhusiopathiae* could be determined not only by the amount of bacteria in the mite but also by the population size of *D. gallinae* in the stable. If a sufficient number of *D. gallinae* infected with *E. rhusiopathiae* are present in a house, then the risk of successful transmission leading to outbreak of poultry erysipelas would be increased. However, poultry in a farm with severe *D. gallinae* infection would in general be more susceptible to any infection, because of the stress and influence on the fitness of the hens by this parasite. If it is assumed that *D. gallinae* is not a potent vector of the erysipelas agent, then after all the results from study III and those of Chirico *et al.* (2003) cannot be disregarded, implying that *D. gallinae* can most definitely have a role in the pathobiology of *E. rhusiopathiae*. *Dermanyssus gallinae* could act as a reservoir of *E. rhusiopathiae* inside a poultry house over time, as well as contribute to the re-infection of *E. rhusiopathiae* in a farm, and should definitely not be disregarded as a risk factor in the spread of the erysipelas agent also between poultry facilities.

6 General Conclusions and Future Research

The main objective of the present series of investigations was to elucidate the transmission routes of *D. gallinae* to poultry facilities and to investigate the potential of this parasite to be a vector of *E. rhusiopathiae*, the agent of poultry erysipelas. The following general conclusions can be drawn from the studies.

Wild birds seem to play a minor role in the transmission of *D. gallinae* into poultry facilities in Sweden, since the sampled mites from wild birds were genetically different from *D. gallinae* infecting poultry. The mites sampled from wild birds in Sweden probably belong to a separate species, as based on the GenBank data available in January 2010. The transportation of eggs from the farms was, at the start of study II, considered an important physical pathway for mites between farms. However, transmission of *D. gallinae* between farms during a production cycle seems to be low. Precautions taken by the farmers and egg-packing facilities have probably limited the circulation of mites, and transmission is likely to follow some other pathway. The genetic variation of *D. gallinae* within a farm was found to be very limited. This could be explained by the fact that the mite is haplo-diploid, which means that one single female can be the ancestor of a population of *D. gallinae* within a poultry house. The limited intra-farm variation also implies that infection of *D. gallinae* in poultry facilities in Sweden and Norway is most likely caused by one or only few common sources, rather than a constant exchange of this parasite between farms.

To better understand the complexity of transmission of *D. gallinae* further molecular studies would be necessary to investigate the nature of the probably few common sources of infection of *D. gallinae* in the egg-

production system. Studies on the host specificity of *D. gallinae*, as well as investigations of the survival of *D. gallinae* in wild bird nests during winter in Scandinavia, could be of use in this attempt.

Hens and *D. gallinae* were demonstrated to be infected by the same strain of *E. rhusiopathiae* present in a poultry house during an outbreak of poultry erysipelas. This indicates that *D. gallinae* is a potential reservoir of this agent. Since *D. gallinae* is capable of harboring *E. rhusiopathiae* for several months at low temperatures without access to a host, *D. gallinae* could also be involved in the spread of erysipelas between houses in a farm, as well as between farms. The role of *D. gallinae* as a vector transmitting *E. rhusiopathiae* to poultry is still uncertain. The mite has been shown to acquire bacteria from naturally infected birds, but this could not be verified under experimental conditions. Detailed investigations of the survival and replication of *E. rhusiopathiae* inside *D. gallinae* would be necessary to determine the vector competence of this parasite.

The importance of efficient elimination of *D. gallinae* within a poultry house is emphasized by several results of this work. To avoid re-infection of *D. gallinae* between production cycles within a farm, efficient treatment is needed to kill all of the mites present. If one female survives the treatment, the population of mites will increase as soon as new birds arrive at the facility. *Dermanyssus gallinae* can harbor *E. rhusiopathiae* for several months and, as shown by other authors, the mite should not be ruled out as a reservoir of other infectious agents as well. Elimination of *D. gallinae* between production cycles is therefore important, not only because reproduction and transmission of the parasite should be avoided, but also to reduce the possible spreading of other infectious agents of poultry. Thus, as this mite is predicted to become an increasing problem within the egg-production industry of Europe, as a consequence of the forthcoming ban on battery cages in all EU countries, it is essential to develop strategies for the sustainable control of *D. gallinae*.

7 Svensk Sammanfattning

7.1 Bakgrund

Röda hönskvalster (*Dermanyssus gallinae*) är leddjur som tillhör gruppen spindeldjur. Den är en redesparasit som har fåglar som huvudvärd. Röda hönskvalster är blodsugande och är ett stort problem i framförallt värphönsbesättningar världen över. Angrepp av parasiten kan ge upphov till irritation, stress och blodbrist och vid kraftiga angrepp kan hönsen till och med dö. Kvalstret besöker bara hönan tillfälligt för att suga blod och håller sig sedan gömd i skrymslen och vrår i fågelns omgivning, där de förökar sig och utvecklas från ägg, via larv och nymf till vuxna kvalster. Utvecklingen kräver flera blodmål och livscykeln (Fig. 3) kan fullbordas på ca en vecka under gynnsamma förhållanden. Dagens värphönsstallar erbjuder flera attraktiva gömställen i sprickor i t.ex. sittpinnar och träkonstruktioner. Röda hönskvalster är relativt stora (ca 1-1,5mm långa) och därför ganska lätta att upptäcka. När de är blodfyllda är de röda eller bruna i färgen och ansamlingar av kvalster ser ut som svart-vit prickiga högar som rör sig om man petar på dem. I avsaknad av en fågelvärd kan det röda hönskvalstret söka sig till däggdjur som hästar, hundar, katter och gnagare. Parasiten kan även bita människor vilket kan vara ett problem för de som arbetar i fjäderfäanläggningar med kraftiga kvalsterangrepp. Eftersom röda hönskvalster håller sig gömda stora delar av tiden är det svårt att bli av med dem. De undkommer ofta fysisk rengöring och tvätt och ska man behandla med bekämpningsmedel måste man hitta kvalstrens gömställen.

7.2 Frågeställning

Studierna i denna avhandling har varit inriktade på två huvudfrågor: parasitens spridningsvägar och dess förmåga att sprida andra infektioner d.v.s. parasitens vektorpotential. Det är oklart hur röda hönskvalster introduceras och sprids mellan värphönsbesättningar. Vilda fåglar har misstänkts kunna vara spridare av kvalster men även fysiska smittvägar kopplade till äggproduktionen som t.ex. äggbrickor och levande höns, har diskuterats som möjliga vägar.

Kvalstrets förmåga att sprida blodburen smitta, i form av en bakterie (*Erysipelothrix rhusiopathiae*) som orsakar rödsjuka hos höns, har också studerats. Rödsjuka yttrar sig oftast som en akut blodförgiftning med minskad äggproduktion och hög dödlighet i flocken som följd. I tidigare studier har man lyckats isolera denna bakterie från kvalster som samlats in från gårdar med utbrott av rödsjuka vilket kan tyda på att de skulle kunna vara vektorer för smittan.

7.3 Hur Sprids Röda Hönskvalster till Värphönsanläggningar?

Eftersom det är okänt hur det röda hönskvalstret tar sig in i värphönsanläggningar har man diskuterat om vilda fåglar som bygger bon på eller i närheten av hönshuset skulle kunna vara överförare av kvalstersmittan. För att undersöka detta användes genetiska metoder och resultaten visar att så troligen inte är fallet. Kvalster som hittas i vilda fåglars bon har visat sig vara genetiskt skilda från de kvalster som samlats in från värphönsbesättningar. Mycket tyder alltså på att de vilda fåglarnas kvalster är av en annan art än de som infekterar värphöns. Vidare studerades kvalster från olika gårdar i framförallt Sverige och Norge men även från sydligare europeiska länder. Detta gjordes för att se om gårdar som har samma population av kvalster också var kopplade till varandra genom någon fysisk väg som möjliggör att parasiterna sprids mellan dem. Resultaten från dessa studier visade att många gårdar hade en genetiskt enhetlig population röda hönskvalster vilket betyder att nya kvalster sällan kommer in i en besättning. Det innebär att ett konstant flöde av kvalster mellan besättningar inte är troligt. Flera gårdar hade samma genetiska typ av kvalster vilket pekar mot att det finns enstaka gemensamma källor till kvalstersmittan. Tyvärr fanns det inga tydliga fysiska kopplingar mellan gårdarna i denna studie och därför kunde inte någon gemensam smittokälla påvisas.

7.4 Kan Röda Hönskvalster Sprida Blodburen Smitta?

I en studie undersöktes om röda hönskvalster kan vara bärare eller s.k. reservoar, av rödsjukebakterier i en värphönsbesättning. Kvalster samlades in från en besättning under ett pågående utbrott av rödsjuka och de undersöktes med avseende på förekomst av bakterier. Det visade sig då att de var bärare av just rödsjukebakterien och de kunde bära på bakterien i över fyra månader utan tillgång till höns. Ungefär ett år efter utbrottet samlades sedan kvalster in från samma gård men då var dessa kvalster inte bärare av rödsjukebakterier. Hönorna som fanns i stallet vid tillfället var friska och hade vaccinerats mot rödsjuka. Sammantaget visar denna forskning att kvalster kan bära på rödsjukebakterier, men att det är oklart under hur lång tid de klarar detta.

Vidare undersöktes om kvalster som bär på rödsjukebakterier kan smitta friska hönor och orsaka rödsjuka. Först infekterades en grupp hönor med en dos bakterier som gav symptom på rödsjuka. Fåglarna hade samtidigt kvalster i sin omgivning som hade möjlighet att suga blod från dem. Sådana kvalster flyttades sedan till friska hönor där de kunde suga blod men det visade sig att ingen rödsjukeinfektion kunde påvisas hos dessa hönor. Uppenbarligen är röda hönskvalster möjliga bärare av bakterien men de är kanske inte så effektiva som vektorer, d.v.s. överförare. Vektorburna sjukdomar är ofta svåra att studera på grund av att det finns flera faktorer som kan påverka hur effektiv en vektor är. Dels måste kvalstret kunna ta upp bakterien, men bakterien måste också kunna föröka sig inne i kvalstret och nå salivkörtlarna för att kunna spridas vidare vid parasitens nästa blodmål. Detta komplicerade förhållande mellan kvalster och smittämne måste studeras mer i detalj för att kunna fastställa hur effektivt det röda hönskvalstret är vad gäller att sprida rödsjuka bland värphöns.

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