

# Tick-borne fever in sheep – production loss and preventive measures

Sjodogg hos sau – produksjonstap og forebyggende tiltak

Philosophiae Doctor (PhD) Thesis

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## **Preface and acknowledgements**

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## List of papers

### Paper I:

Grøva L, Olesen I, Steinshamn H and Stuen S 2011. Prevalence of *Anaplasma phagocytophilum* infection and effect on lamb growth. Acta Veterinaria Scandinavica 2011 53:30.

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### Paper II:

Grøva L, Olesen I, Steinshamn H and Stuen S 2011. The effect of lamb age to a natural *Anaplasma phagocytophilum* infection. (Submitted Small Ruminant Research 2011)

### Paper III:

Stuen S, Grøva L, Granquist E G, Sandstedt K, Olesen I and Steinshamn H 2011. A comparative study of clinical manifestation, haematological and serological responses after experimental infection with *Anaplasma phagocytophilum* in two Norwegian sheep breeds. Acta Veterinaria Scandinavica 2011 53:8.

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### Paper IV:

Grøva L, Olesen I and Ødegård J 2011. Heritability of lamb survival on tick-exposed pastures. (Manuscript)

The contributions of Lise Grøva to the papers included in his thesis were as follows:

Paper I:

Designing the study jointly with the co-authors and responsible for data collection, the processing of the statistical analysis of the data and compiling the draft manuscript and subsequent revisions after co-authors and reviewers comments.

Paper II:

Designing the study jointly with the co-authors and, together with Snorre Stuen, responsible for data collection. Responsible for processing of the statistical analysis of the data and compiling the draft manuscript and subsequent revisions after co-authors comments.

Paper III:

Participating in designing the infection study. Reading, commenting and approving of the final manuscript.

Paper IV:

Planning the research jointly with the co-authors, preparing the dataset for statistical analysis and discussing the statistical analyses. Responsible for compiling the draft manuscript and subsequent revisions after co-authors comments.

## Abstract

A major challenge in sheep farming during the grazing season along the coast of south-western Norway is tick-borne fever (TBF) caused by the bacteria *Anaplasma phagocytophilum*, that is transmitted by the tick *Ixodes ricinus*. The justification for this study is based on our limited knowledge on the effect of *A. phagocytophilum* infection on indirect losses i.e. reduces weight gain and weaning weight in lambs. Further, there is a lack of efficient and sustainable preventive measures to tick-infestation and TBF which implies a need for new knowledge. Knowledge on the effect of age of lamb to a natural *A. phagocytophilum* infection and genetic variation in robustness to *A. phagocytophilum* infection are possible preventive measures that are investigated in this study.

A study (**PAPER I**) was carried out in 2007 and 2008 to examine the prevalence of *A. phagocytophilum* infection and its effect on weaning weight in lambs. The study included 1208 lambs from farms in Sunndal Ram Circle in Møre and Romsdal County in Mid-Norway, where ticks were frequently observed. All lambs were blood sampled and serum was analysed by an indirect fluorescent antibody assay (IFA) to determine the antibody status (positive or negative) to *A. phagocytophilum* infection. The possible effect of *A. phagocytophilum* on autumn live weight and weight gain was analysed using the MIXED procedure in SAS. The overall prevalence of infection with *A. phagocytophilum* was 55 %. A reduction in weaning weight of 3 % (1.34 kg,  $p < 0.01$ ) was estimated in lambs seropositive to an *A. phagocytophilum* infection compared to seronegative lambs at an average age of 137 days. The results indicate that *A. phagocytophilum* infection has a negative but low effect on average lamb weight gain. The study also support previous findings that *A. phagocytophilum* infection is widespread in areas where ticks are prevalent, even in flocks that treat lambs prophylactic with acaricides.

A field trial (**PAPER II**) was carried out in 2008 and 2009 on two sheep farms in tick infested areas where *A. phagocytophilum* is present, to examine if there is an effect of age of lambs to a natural *A. phagocytophilum* infection. Three trial groups per farm and year, including a total of 336 lambs, were established as follows: 3E; lambs  $\geq$  three weeks old when turned out on pasture and early time of birth, 1L; lambs  $\leq$  one week old when turned out on pasture and late time of birth, 3L; lambs  $\geq$  three weeks old when turned out on pasture and late time of birth. Recordings of weight, tick-counts, rectal temperature, other clinical signs of disease and mortality, together with blood serology and blood smears were used to analyse the effect of age of lambs to a natural *A. phagocytophilum* infection. Gompertz weight curve parameters were estimated for all lambs, and the individual lamb parameter estimates and performance traits were further analysed using the MIXED procedure in SAS. The incidence of fever, tick-bites, clinical disease and death of lambs were analysed statistically using the PROC LOGISTIC procedure in SAS. There were incidences of tick-bites, clinical disease (including fever) and mortality in all trial groups indicating no effect of lamb age to a natural *A. phagocytophilum* infection. However, lambs infected in spring with *A. phagocytophilum* in the 1L group had higher ( $P < 0.05$ ) maximum growth rate (358g/day) than infected lambs in 3E (334g/day) and 3L (310/day) groups. Pasturing of  $\leq$  one week old lambs on tick-infested pastures, can therefore be recommended in order to reduce weight losses due to *A. phagocytophilum*. Note should however be taken on annual and seasonal variations in tick activity relative to lambing, variants of *A. phagocytophilum* involved and turnout time as this probably will influence the effect of pasturing young lambs.

An infection study (**PAPER III**) was carried out in 2008 and 2009. Five-month-old lambs of two Norwegian sheep breeds, Norwegian White (NW) sheep and Old Norse (ON) sheep, were experimentally infected with a 16S rRNA genetic variant of *A. phagocytophilum* (similar to GenBank accession number M73220). The experiment was repeated for two subsequent

years, 2008 and 2009, using 16 lambs of each breed annually. Ten lambs of each breed were inoculated intravenously each year with 0.4 ml *A. phagocytophilum*-infected blood containing approximately  $0.5 \times 10^6$  infected neutrophils/ml. Six lambs of each breed were used as uninfected controls. The clinical, haematological and serological responses to *A. phagocytophilum* infection were compared in the two sheep breeds. The present study indicates a difference in fever response and infection rate between the two breeds after experimental infection with *A. phagocytophilum*. The clinical response seems to be less in ON-lambs compared to NW lambs, but further studies are needed to conclude on the possible higher protection against *A. phagocytophilum* infection in the ON-breed than other Norwegian breeds.

Estimation of heritability for survival of lambs on tick-exposed pastures (**PAPER IV**) was conducted using data from the Norwegian Sheep Recording System. Data on lambs of the Norwegian White (NW) sheep breed from flocks participating in ram circles (cross flock organized breeding program) with recordings in the Norwegian Sheep Recording System and registered with cases of TBF or using prophylactic treatment against ectoparasites at any one time in 2000 to 2008 were included, making a total of 126 732 lambs. Analysis of the data was conducted using a linear model in DMU software. The estimated heritability for the direct effect on lamb survival was 0.22. The estimated maternal variance in proportion of phenotypic variance of lamb survival was close to zero. The heritability of direct effects on lamb survival indicates a potential for a selection response to improve survival of lambs on tick-exposed pastures. This heritability cannot, however, be directly attributed to robustness to *A. phagocytophilum* as the lambs in this study are not confirmed infected with *A. phagocytophilum*.

Our findings show that *A. phagocytophilum* does cause a significant but relatively low reduction in live weaning weight in lambs. Furthermore, the bacteria seem to be widespread

in areas with ticks, but its pathogenic effects may be variable. The proposed preventive measures of turning lambs  $\leq$  one week old on pastures in tick infected areas show a potential to reduce indirect losses to TBF in Norwegian sheep farming, bearing in mind that annual and seasonal variations in tick-infestation will influence the effect of this preventive measure. The indications of breed differences as well as an estimated heritability of 0.22 in survival of lambs expected to be exposed to tick-infestation, indicates potential for improving performance on tick-exposed pastures. It is suggested that further studies should be done to identify genetically robust animals.

## Sammendrag

En betydelig utfordring i saueholdet i løpet av beitesesongen langs sør-vest kysten i Norge er sjudogg forårsaket av bakterien *Anaplasma phagocytophilum* som overføres med flåttene *Ixodes ricinus*. Bakgrunnen for denne studien er basert på vår begrensede kunnskap om effekten av *A. phagocytophilum* infeksjon på indirekte tap dvs. på redusert tilvekst og høstvekt hos lam. Det er mangel på effektive og bærekraftige forebyggende tiltak mot flått og sjudogg, noe som tilsier at det er behov for ny kunnskap. Kunnskap om effekten av alder på lam ved naturlig *A. phagocytophilum* infeksjon på beite og genetisk variasjon i robusthet mot infeksjon av *A. phagocytophilum* er aktuelle forebyggende tiltak som er undersøkt i denne studien.

En studie (**PAPER I**) ble gjennomført i 2007 og 2008 for å undersøke forekomsten av *A. phagocytophilum* infeksjon og effekt på høstvekt hos lam. Studien inkluderte 1208 lam fra gårder i Sunndal, Todal og Ålvundeid værering i Møre og Romsdal, hvor flått er observert. Det ble tatt blodprøve av lam og serum ble analysert ved en indirekte fluorescerende antistoff analyse (IFA) for å bestemme antistoff status (positiv eller negativ) til *A. phagocytophilum* infeksjon. Effekten av *A. phagocytophilum* infeksjon på høstvekt og tilvekst ble analysert ved hjelp av MIXED prosedyren i SAS. Prevalensen av infeksjon med *A. phagocytophilum* var 55%. En reduksjon i høstvekt på 3% (1,34 kg,  $p < 0,01$ ) ble estimert hos lammene som var seropositive til en *A. phagocytophilum* infeksjon sammenlignet med seronegative lam ved en gjennomsnittlig alder på 137 dager. Resultatene indikerer at *A. phagocytophilum* infeksjon har en negativ, men lav effekt på gjennomsnittlig tilvekst. Studien støtter også tidligere funn om at *A. phagocytophilum* infeksjon er utbredt i områder der flått er utbredt, selv i flokker som behandler lammene forebyggende mot flått.

Et feltforsøk (**PAPER II**) ble gjennomført i 2008 og 2009 på to sauegårder i flåttinfiserte områder hvor *A. phagocytophilum* var påvist tidligere, for å undersøke om det er en effekt av

alder på lam ved *A. phagocytophilum* infeksjon. Tre forsøksgrupper per gård og år, inkluderte total 336 lam, og ble etablert som følger: 3E; lam  $\geq$  tre uker gammel ved beiteslipp og født tidlig om våren; 1L, lam  $\leq$  én uke gammel ved beiteslipp og født seint om våren, 3L; lam  $\geq$  tre uker gammel ved beiteslipp og født seint om våren. Registrering av vekt, antall flått, temperatur, andre kliniske tegn på sykdom og død, sammen med serologi og blodutstryk ble brukt til å analysere effekten av alder av lam ved en naturlig *A. phagocytophilum* infeksjon. Gompertz vekstkurve parametere ble estimert for alle lam, og disse estimatene i tillegg til tilvekst og høstvekt ble videre analysert ved hjelp av MIXED prosedyren i SAS. Forekomsten av feber, flåttbitt, klinisk sykdom og død ble analysert statistisk ved hjelp av PROC LOGISTIC prosedyren i SAS. Det var forekomst av flått-bitt, klinisk sykdom (inkludert feber) og dødelighet i alle forsøksgrupper, noe som indikerer at det var ingen effekt av alder på lam ved en naturlig *A. phagocytophilum* infeksjon. Imidlertid hadde lam smittet med *A. phagocytophilum* i 1L gruppen høyere ( $P < 0,05$ ) estimert maksimal tilvekst (358g/dag) enn lam i 3E (334g/dag) og 3L (310/dag) grupper. Beiteslipp av lam  $\leq$  en uke gamle på flått-infisert beite, kan derfor anbefales for å redusere vekttap på grunn av infeksjon med *A. phagocytophilum*. Man må imidlertid være oppmerksom på år og sesong variasjon i flått-aktivitet i forhold til lamming, varianter av *A. phagocytophilum* involvert, og tidspunkt for beiteslipp da dette sannsynligvis vil påvirke effekten av beiteslipp av lam som er  $\leq$  en uke gamle.

En infeksjons studie (**PAPER III**) ble gjennomført i 2008 og 2009. Fem måneder gamle lam av to norske sauseraser, Norsk kvit sau (NKS) og Gammelnorsk sau, ble smitta med en 16S rRNA genetisk variant av *A. phagocytophilum* (tilsvarende GenBank nummer M73220). Forsøket ble gjentatt i to påfølgende år, 2008 og 2009, med totalt 32 lam av hver rase. Ti lam av hver rase ble inokulert intravenøst hvert år med 0,4 ml *A. phagocytophilum*-infisert blod som inneholder omtrent  $0.5 \times 10^6$  smittet nøytrofiler / ml. Seks lam av hver rase var kontrollam



som ikke ble smitta. Klinisk, hematologisk og serologisk respons til *A. phagocytophilum* infeksjon ble sammenlignet i de to sauerasene. Studien indikerer en forskjell i feber respons og infeksjons rate mellom de to rasene etter eksperimentell infeksjon med *A.*

*phagocytophilum*. Den kliniske responsen synes å være mindre i lam av rasen Gammelnorsk sau i forhold til NKS lam, men videre studier er nødvendig for å konkludere om Gammelnorsk sau er mer beskyttet mot flåttbårne infeksjoner enn andre norske raser.

Estimering av arvegrad for overlevelse av lam på flått-eksponerte beiter (**PAPER IV**) ble utført med data fra Sauekontrollen. Data fra lam av rasen NKS fra flokker som deltar i væreringer, registrert med tilfelle av sjodogg eller bruk av forebyggende behandling mot ektoparasitter i perioden 2000-2008 er inkludert, totalt 126 732 lam. Analyse av data ble utført med en lineær modell i programvaren DMU. Den estimerte arvegraden for direkte effekt av overlevelse hos lam var 0,22. Den estimerte morvariansen i andel av fenotypisk varians av overlevelse hos lam var nær null. Arvegraden på 0,22 på overlevelse av lam indikerer potensiale for å forbedre overlevelse av lam på flått-infisert beite. Denne arvegraden kan imidlertid ikke knyttes direkte til robusthet til *A. phagocytophilum* infeksjon da lam i denne studien ikke er bekreftet smittet med *A. phagocytophilum*.

Våre funn viser at smitte med *A. phagocytophilum* fører til en signifikant, men relativt lav reduksjon i høstvekt hos lam. Bakterien *A. phagocytophilum* ser ut til å være utbredt i områder med flått, men dens sykdomsfremkallende effekt kan variere. Det foreslåtte forebyggende tiltaket med beiteslipp av lam  $\leq$  en uke gamle på flåttinfiserte beiter indikerer at dette kan redusere indirekte tap til sjodogg. År- og sesongvariasjon i flått aktivitet vil imidlertid påvirke effekten av dette forebyggende tiltaket. Indikasjoner på rase-forskjeller samt en estimert arvegrad på av 0,22 for overlevelse av lam på flått-infiserte beiter tyder på at det er potensialet for å forbedre produksjon på flått infiserte beiter gjennom avl. Det anbefales at videre studier bør gjøres for å identifisere genetisk robuste dyr.



## Introduction

Ticks and tick-borne diseases have received increased attention the last years and are of great concern for health and welfare of animals and people. In Norway, tick-borne fever (TBF) is stated as one of the main scourges in sheep farming in coastal areas (Stuen, 2003), being caused by the bacterium *Anaplasma phagocytophilum* and transmitted by the tick *Ixodes ricinus*. There is concern among sheep farmers in areas where *I. ricinus* is abundant on the indirect and direct losses that are caused by TBF (Grønn Forskning, 2010), and the Norwegian Food Safety Authority considers restrictions of grazing on pastures with high losses due to the severe welfare problems (The Norwegian Food Safety Authority, 2011). Norwegian sheep farming is based on grazing unfenced rangeland in mountains and forests, which implies challenges for management of the production system as well as animal welfare dilemmas. Practical and sustainable measures to reduce losses to TBF are important to ensure sheep farming in areas of coastal Norway, and thereby avoid loss of present cultivated grazing landscape which will contribute to increased bush encroachment, and possibly even more favourable conditions for ticks (Wilson, 1986; Bryn, 2002; Steinheim et al., 2002; Vangen et al., 2007; Austrheim et al., 2008; Speed et al., 2011). Also, free range pasture and mountain pastures provide valuable resources as grazing land and contributes to a rich biodiversity (Bryn, 2002).

In practice, ticks are currently mostly controlled by different chemical acaricides (George et al., 2008). The incidence of acaricide resistance in ticks (Thullner et al., 2007) and undesirable environmental consequences (Edwards et al., 2001) has led to a demand for more effective and sustainable control measures of ticks and tick borne diseases. Focusing on solving disease challenges by preventive and other sustainable measures, is also in accordance to the basic principles of organic agriculture (International Federation of Organic Agriculture Movements (IFOAM), 2011).

This thesis investigates the possibility of preventing losses to *A. phagocytophilum* infection on tick-exposed pasture by exposing young lambs to natural tick-infestation and *A. phagocytophilum* infection, as well as exploring possibilities for genetic improvement of robustness to TBF. The study also elucidates the extent of weight loss that can be expected in lambs due to an *A. phagocytophilum* infection.

### **Ticks - habitat and distribution**

Ticks are suggested to be the most harmful ectoparasite of domestic and wild animals (Samish and Rehacek, 1999), and one of the most important vector of human disease in the world, second only to mosquitoes (Goodman et al., 2005). A total of 14 species of ticks have been reported in Norway (Mehl, 1983; Jore et al., 2011). However, the tick species *I. ricinus* is described as the important vector for tick-borne diseases in Norway (Stuen, 2003).

Ticks are obligatory bloodsucking arthropods. The *I. ricinus* tick is a 3-host tick with 4 developmental stages, i.e., egg, larva, nymph and adult. A blood meal as a larva, nymph and adult is necessary to molt from each stage to the next and for the adult female to produce eggs. Larva and nymphs may feed on both mammals and birds of all sizes, but adults require medium- to large-sized mammal hosts for reproduction. The lifecycle of *I. ricinus* is normally between 2-3 years but can be extended to 6 years (Sonenshine, 1992).

*I. ricinus* is an exophilic species found in open or semi open biotopes, most often on the surface of vegetation litter or herbaceous shrubs (Estrada-Pena et al., 2006; Vassallo et al., 2009). *I. ricinus* are sensitive to climatic conditions and they require a microclimate with a relative humidity of at least 80 % to avoid dying from desiccation. They are therefore restricted to areas where the soil surface remains humid through the driest times of the year i.e. in areas with moderate to high precipitation and a certain cover of vegetation (Gray, 1991). Gray (1998) further indicates that the greatest tick infection prevalence occurs in deciduous woodland harbouring a diverse mix of host species.

The normal distribution area of *I. ricinus* ticks in Norway has been described as the coastal areas of Norway as far north as Brønnøysund in Nordland county (N 65°30') (Tambs-Lyche, 1943; Mehl, 1983). Recently, it is suggested that *I. ricinus* has expanded the distribution area further north, up to approximately 69°N, and to higher altitudes than previously described (Jore et al., 2011). These recent findings are based on a multi-source approach, including, amongst others, public registrations from hunters to a webpage ([www.flaattogflue.no](http://www.flaattogflue.no)) established by the Norwegian Veterinary Institute and the Norwegian Institute of Public Health Registrations on the tick burden on cervids. However, cervids commonly migrate and may therefore, however, bring ticks into new areas, not necessarily indicating an extension of the tick distribution area.

Climate change (i.e. warmer winter climate), changes in land use (i.e. bush encroachment) and an increase in the deer population are factors expected to increase the population of ticks (Sonenshine, 1993), giving rise to concern that challenges with *A. phagocytophilum*, and other tick-borne diseases, will increase in Norway in the coming years. Various studies have been conducted on how the *I. ricinus* tick population interact with climate, vegetation and host conditions. A number of studies show that ticks extend their distribution further north and to higher altitudes with warmer winters and increased vegetation and host populations (Lindgren et al., 2000; Daniel et al., 2003; Danielova et al., 2006; Materna et al., 2008; Jore et al., 2011). Cadenas et al. (2009) found differences in questing<sup>1</sup> tick density between north- and south-facing slopes in Switzerland. Gray et al (2009) suggests that climate change and altered agricultural practices, with bush encroachment of extensive areas of previous pastureland contributes to the expansion of the distribution of *I. ricinus*. Kirby et al. (2004) found that the tick burden of red grouse in Scotland has increased significantly from 1985 to 2003, suggesting that this increase in tick abundance is related to higher numbers of deer (both roe

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<sup>1</sup> a behavior exhibited by ticks to increase their chances to get in contact with a suitable host

deer and red deer) and changing climatic conditions. A study from The Netherlands suggests that grazing has a negative effect on small rodents as well as on ticks (Gassner et al., 2008). Tick abundance is also shown to be positively correlated with deer abundance (red deer and roe deer) and negatively correlated with altitude (Gilbert, 2010). In Great Britain, it is further suggested that tick abundance and distribution has increased over recent years (Scharlemann et al., 2008). In Sweden it is shown that the limits of *I. ricinus* distribution corresponds with climate characterized with snow cover of 150 days and a vegetation period averaging 170 days (Jaenson et al., 2009). Bush encroachment caused by less use of grazing land and increased deer population (Milner, 2006), as is observed in coastal areas in Norway, are likely to make these areas more favourable for tick survival and reproduction (Gray et al., 2009).

### **Anaplasma phagocytophilum and tick-borne fever (TBF)**

Ticks can carry a number of pathogens, both bacterial, protozoan and viral that cause diseases in man and animals such as Lyme Borreliosis (caused by *Borrelia burgdorferi* s.l.), TBF (caused by *A. phagocytophilum*), babesiosis (caused by *Babesia divergens*), and tick-borne encephalitis (TBE) (caused by the TBE flavivirus). It is however the bacterium *A. phagocytophilum* that is the tick-borne disease agent associated with losses in sheep farming in Norway. *A. phagocytophilum* can infect not only sheep but is shown to also infect cattle, goats, horses, dogs, cats, moose, roe deer, rodents and humans (causing human granulocytic anaplasmosis) (Woldehiwet, 2006). The tick-borne louping-ill virus causes louping-ill in sheep and has been registered in Norway (Gao et al., 1993; Stuen, 1999), but is not considered to be a major cause of loss in sheep farming in Norway.

#### ***A. phagocytophilum***

*A. phagocytophilum* is a Gram-negative obligatory intracellular bacterium belonging to the family *Rickettsiaceae* that is transmitted by *Ixodes* ticks (Foggie, 1951). *A. phagocytophilum* has been renamed several times; *Rickettsia phagocytophila* in 1949, *Cytoecetes*

*phagocytophila* in 1962, *Ehrlichia phagocytophila* in 1984 and *Anaplasma phagocytophilum* in 2001 (Dumler et al., 2001).

A number of different variants of *A. phagocytophilum* are described (Stuen, 2003; Ladbury, 2008). Different variants are found to circulate within infected sheep and flocks at the same time, as well as between flocks in tick-endemic areas (Stuen et al., 2002b; Ladbury et al., 2008). There is a diversity of variation in virulence and clinical manifestation among these variants (Stuen et al., 2003a), and the different variants may behave and cycle differently in the host (Stuen et al., 2008). There are also indications that variants hold differences in geographical distribution (Massung et al., 2002) and that gene clusters of *A. phagocytophilum* are correlated with distinct host species (Scharf et al., 2011). The genetic differences of different isolates obtained from various host species and their potential to infect and re-infect a different species remains to be understood (Woldehiwet, 2010; Stuen et al., 2009).

*A. phagocytophilum* infects neutrophils and survives for several months by avoiding bactericidal defence mechanisms in immune-competent sheep (Foggie, 1951; Granquist et al., 2008; Woldehiwet, 2010). Recent research has increased our understanding of the biology, epidemiology and pathogenesis of *A. phagocytophilum* but it is not known how it spreads from the site of tick feeding to other sites, where it multiplies before the development of bacteraemia, nor where the sites of persistence are in the animal (Woldehiwet, 2010).

### *Distribution and prevalence*

*A. phagocytophilum* is widespread in Europe (Stuen, 2007), and the distribution in Norway is mainly along the south, southwest and west coast of Norway (Stuen, 2003). The prevalence of *A. phagocytophilum* in ticks in Norway varies between locations studied; 4.5 % (Radsijevska, 2008), 0-25% (Rosef et al., 2009), and the prevalence in Europe has been found to vary from 0.3 – 34% (Christova et al., 2001; Walker et al., 2001; Smrdel et al., 2010). It is perceived that

*A. phagocytophilum* infected lambs are commonly found in areas with ticks (Øverås, 1972).

The prevalence of *A. phagocytophilum* infection in lambs grazing on tick-infested pastures in Norway is reported to be high, ranging from 55-80% (Stuen and Bergstrom, 2001b; Grøva, 2009) (**PAPER I**). And it is suggested that probably 100% of lambs on tick-infested pastures will acquire an infection with *A. phagocytophilum* (Ogden et al., 1998).

### *Hosts and reservoir*

The understanding of vector and reservoir mechanisms of *A. phagocytophilum* is addressed in various studies but is still not fully understood. The incidence and severity of *A. phagocytophilum* infection in a particular host appears to vary from one region to another; while TBF in sheep and cattle is common in Europe (Woldehiwet, 2006; Stuen, 2007), no cases are reported in ruminants in the United States (Pusterla et al., 2001). It is also shown that there are distinct host species correlated with *A. phagocytophilum ankA* gene clusters and it is suggested that roe deer strains of *A. phagocytophilum* are different from strains found in sheep and cattle, and that strains identified in dogs, humans, horses and cats belong to the same gene cluster (Scharf et al., 2011). It is shown that deer are infected by *A. phagocytophilum*, and it is suggested that red deer have a role as a reservoir for *A. phagocytophilum* (Stuen et al., 2010). Rodents are also shown to harbour *A. phagocytophilum* which suggests that rodents may also be a reservoir of *A. phagocytophilum* bacterium (Bown et al., 2006), but not necessarily important for the variants that cause disease in sheep. The role of birds as potential reservoirs has not been clearly established, but there are observations of *A. phagocytophilum* infection in birds (Hildebrandt et al., 2010). Understanding the reservoir and host mechanisms of *A. phagocytophilum* variants that cause typical signs of TBF in sheep, is of interest in order to implement preventive management strategies.



### *Symptoms of TBF (Norwegian: Sjodogg)*

TBF was first described in Scotland (MacLeod, 1932). However, in Norway, TFB (called sjodogg in Norwegian) has likely been referred to in the literature as far back as in 1780 (Schnabel, 1912).

Even if *A. phagocytophilum* is the causative agent of TBF an infection might not cause clinical TBF. TBF is initially characterised with high fever, inclusions of the *A. phagocytophilum* bacterium in neutrophils and severe neutropenia (Stuen, 2003). Sheep exposed to infected ticks may develop clinical signs of TBF within 14 days. The clinical signs that are commonly observed are an abrupt rise in rectal temperature often above 41° C, a fever period of one to two weeks and occasionally coughing, reduced appetite and dullness (Stuen, 2003). Young lambs and sheep purchased from tick-free areas and placed on tick-infested pastures for the first time are associated with the main disease problems caused by TBF (Woldehiwet and Scott G.R., 1993). TBF is seldom fatal if it is not complicated with other infections, but indirect losses as reduced weight gain are observed in *A. phagocytophilum* infected lambs even when clinical observations of TBF are not registered (Stuen et al., 2002a).

However, the main consequence of an *A. phagocytophilum* infection in sheep is the ensuing immunosuppression resulting in secondary infections that might even be fatal. Tick pyemia, a crippling lameness and paralysis due to infection with *Staphylococcus* spp infections, is commonly observed as a secondary infection to TBF (Foggie, 1951; Brodie et al., 1986). Also septicaemia caused by *Mannheimia haemolytica* (Øverås, 1983; Stuen, 1996) previously described as Pasteurellosis (Gilmour et al., 1982) is associated to be a commonly observed secondary infection to TBF. Abortion in ewes (Stamp et al., 1950) and temporary infertility in rams (Watson, 1964), probably associated with the febrile state of infected animals may

occur. A reduced general condition might further lead to animals being an easy catch for predators.

### *Immunity*

Lambs generally develop immunity after a primary infection with *A. phagocytophilum*. The development of immunity after a primary infection with *A. phagocytophilum* does however vary, and resistance to re-infection is influenced by the variant of *A. phagocytophilum*, the age of the host, the length of time between primary infection and challenge and the frequency of exposure to infection (Woldehiwet, 1982; Foggie, 1951).

### *Direct and indirect losses*

The lamb loss during summer grazing in Norway in 2009 varied between 3 – 15% in the 17 different counties with sheep in Norway, and lamb losses are in general increasing (Norwegian Forest and Landscape Institute, 2011). Predators, blow flies, alveld and TBF are the main causes of death on summer pasture (Vatn et al., 2008). Occurrence of fatal cases to *A. phagocytophilum* infection and secondary infections is described (Øverås, 1983; Øverås et al., 1985; Stuen et al., 2003b; Stuen et al., 2005), and losses of almost one third of the lambs on tick pasture have been observed, most of them due to *A. phagocytophilum* and secondary infections (Stuen and Kjølleberg, 2000). Møre and Romsdal County, with a lamb loss on summer pasture of 12 % (Norwegian Forest and Landscape Institute, 2011), initiated a survey of free range sheep on mountain and forest pastures to identify causes of mortality besides predators. *A. phagocytophilum* infection, was through this study, found to be widespread in Møre and Romsdal (Grøva, 2009) and suggested to be a main suspect in reducing weight gain and increasing lamb mortality and loss in the studied farms (Grøva, 2010).

It is indicated that about 300 000 lambs are affected by ticks and *A. phagocytophilum* every year in Norway (Stuen and Bergstrom, 2001b). In the UK, more than 300 000 lambs have

been found to develop tick pyaemia annually (Brodie et al.1986) and lambs with tick pyaemia commonly die or are of reduced economic value. Sheep flocks on tick pasture may suffer from not only mortality, but also poor growth. Indirect losses expressed as reduced growth in infected lambs compared to non-infected lambs are demonstrated (Stuen et al., 2002a). Awareness of tick infestation is also shown to vary amongst farmers (Stuen and Bergstrom, 2001b).

### **Prevention of TBF**

There are limited preventive measures against tick infestation and TBF available for sheep farmers. Recommended preventive measures against ticks are; clearing vegetation, drainage of land, reduction of host animals, use of acaricides and pasturing of young lambs (Stuen, 1993; Sonenshine, 1993).

### ***Vegetation and hosts***

Clearing vegetation and drainage of land are rarely feasible measures as the grazing areas are often vast unfenced forest and mountain pastures. Habitat modification involving clearing of bush, removal of leaf litter, controlled burning and removal of forest has however shown to be successful to control ticks (Wilson, 1986). Cervid (i.e. deer) population density influences tick abundance and the use of fencing to exclude primary tick hosts such as deer may reduce tick populations (Ruiz-Fons and Gilbert, 2010; Gilbert, 2010). Altering vegetation and the presence of hosts are however often not feasible and associated with high costs and short time effect, as they often must be repeated frequently. Proper maintenance of grazing land probably has potential to reduce the tick population because less vegetated land such as cultivated pastures offers less favourable conditions for tick-survival.

### ***Prophylactic treatment***

In practice, ticks are mostly controlled by acaricides by dipping or ‘pour-on’ application of pyrethroids (Woldehiwet and Scott G.R., 1993; George et al., 2008). Use of acaricides has

also shown to lower the incidence of secondary bacterial infections, such as tick pyaemia (Brodie et al., 1986; Hardeng et al., 1992). Also, Hunt (1986) found that treatment of ewes and lambs with cypermethrin pour-on increased the production with 6 % more lambs at weaning on tick pastures. Furthermore, the mean slaughter weight of treated lambs was 1.5 kg heavier than untreated controls. It is further shown that prophylactic use of long-term acting tetracyclines against *A. phagocytophilum* in the UK has improved weight gain in lambs on pasture (Brodie et al., 1988). Sheep farming in Norway is however based on vast mountain and forest pastures for several months, and there are practical limitations of frequent treatments of lambs. Treatment with pyrethroids on prevalence of *A. phagocytophilum* infection is, however, indicated to have no effect (Hardeng et al., 1992).

There are reports of increased incidence of acaricide resistance in ticks (Nolan et al., 1988; Beugnet and Chardonnet, 1995; Thullner et al., 2007; Morgan et al., 2009). Resistance to acaricides is suspected in *I. ricinus* ticks in UK, but considered difficult to prove (Sargison and Edwards, 2009). The issue of acaricide resistance has however neither been demonstrated nor surveyed in Norway to my knowledge. Globally, issues of acaricide resistance, product withdrawal period after using acaricides, undesirable environmental persistence, and toxicity, are negative issues of acaricides that ask for research to identify new management approaches to control ticks and tick-borne diseases (Samish et al., 2004). Suggested alternative control strategies are habitat modification, use of pheromones, hormones and biological control organisms (e.g. mites and fungi) as well as improvement of host resistance (Samish and Rehacek, 1999).

### *Lamb age at time of infection*

Lambs get colostrum with immunoglobulines (passive immunity) from mother shortly after birth. This passive immunity from the mother helps the lambs to defeat infection and develop a certain level of immunity against infection during the first weeks of life. Eventually the

lambs will develop immunity themselves (active immunity). During the development from passive to active immunity, at about three to six weeks of age, the lambs are especially vulnerable to infection (Tizard, 2004). Maternal immunity may generally alleviate the first infection reaction in lambs, but colostral antibodies are shown to not always protect the lambs completely from *A. phagocytophilum* infection (Stuen et al. 1992, Stuen 1993). It is however shown that the clinical response to *A. phagocytophilum* infection is less severe in young lambs, i.e. lambs less than two weeks old, compared with older lambs (Stuen et al., 1992; Stuen, 1993). Re-infection does occur, but the severity is commonly less than in primary infections (Stuen et al., 2003a). Superinfection<sup>2</sup> can, however, also occur in lambs protected against the first challenged infection (Stuen et al., 2009). Knowledge on the optimal conditions and age of lambs to be pastured on tick infested pastures has the potential to decrease direct and indirect losses to TBF. Such farm management of lamb pasturing is a sustainable preventive measure. One should, however, be aware that lambs may also experience an autumn infection with *A. phagocytophilum*.

### *Natural enemies*

The use of different biological control methods to control ticks is suggested to be a strategy of interest (Samish and Rehacek, 1999). In nature, many bacteria, fungi, spiders, ants, beetles, rodents, birds, and other organisms are suggested to contribute to limiting tick populations, as do, the grooming activities of hosts, i.e. scratching/cleaning activity (Samish and Rehacek, 1999; Jonsson and Piper, 2007). Fungal pathogens, predatory mites and ants are thought to be important tick killers in nature (Chandler et al, 2000; Samish & Rehacek, 1999), and may be used in biological control strategies. Information on mites, as a predator of ticks is very limited. Since mites are known to prey on a large variety of hosts and are used commercially to control various arthropod pests this group may however be an important natural control

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<sup>2</sup> Here: The establishing of a second variant of *A. phagocytophilum* in a host already infected with a primary variant

factor. Hence, it may also contain important candidates for biological control (Samish & Rehacek, 1999). Studies indicate that fungal infections may cause the death of up to 50% of Dermacentor, Ixodes and other ticks (Kalsbeek et al., 1995).

It has also been shown that commercial formulations of the entomopathogenic fungi *Beauveria bassiana* (Balsamo-Crivelli) and *Metarhizium anisopliae* (Metschnikoff) provide a significant reduction in nymphal tick abundance (*Ixodes scapularis*) in residential areas in Connecticut, USA (Stafford and Allan, 2010). Biological control may be done either by conserving and enhancing natural enemies of the tick in the field, by conservation of biological control organisms or by adding biological control agents in field (Eilenberg et al., 2001). It is of great interest to gain knowledge on the potential of tick control with the use of fungi either to the habitat or possibly directly on the host. Application to the host is however likely to face similar challenges as the use of acaricides when it comes to i.e. practical challenges if regular application. Knowledge on natural enemies of *I. ricinus* and development of biological control strategies does however seem to have a potential for reducing tick populations in strategic habitats in Norway, i.e. on fences pastures and recreational areas. The potential for reducing tick-populations with the use of biological control strategy is however criticised for having low potential as tick numbers will be too large for natural enemies to be efficient (Cole, 1965). Also, predators of ticks are commonly generalists and proposed to have limited potential for tick management (Samish et al., 2004). There is further a challenge in creating a sustainable biologic control of ticks in the natural habitat.

### *Host genetic resistance*

#### Tick immunity:

Tick immunity was first described by William Trager in 1939 and it refers to the phenomenon in which ticks are unable to feed successfully after several tick infestations in guinea pigs

(Trager, 1939). Also, tick immunity can be expressed by the reduction in tick weight, the inability to molt after feeding, the reduced number of ticks feeding on a host and the time of attachment and egg mass produced. Cattle with thicker hair are shown to be more susceptible to ticks than those with thinner hair (Fraga et al., 2003), females are more resistant than males, pregnant cows are less resistant than non-pregnant ones and younger animals are more resistant than older ones (Utech et al., 1978; Silva et al., 2010).

A significant breed difference in resistance to ticks between the cattle breeds *Bos indicus* and *Bos Taurus* is found, with the first one being several times more resistant (Lemos, 1985).

Genetic variation is also observed within cattle breeds and heritability estimates of tick numbers vary from very low to high (Regitano and Prayaga, 2010). Tick immunity may not only affect tick feeding but can also interfere with transmission of pathogens (Schuijt et al., 2011).

Genetic variation in disease resistance and the ability to tolerate disease in sheep is reported (Bishop and Morris, 2007) and such variation in resistance to internal parasites in sheep is observed (Bishop and Stear 1999, Stear et al.1995). There is indication of individual variance in susceptibility to *A. phagocytophilum* in sheep in Norway (Stuen 2003; Granquist et al., 2010). Further, variation in grazing behaviour is observed between sheep breeds (Steinheim et al. 2005), where the short-tailed Norwegian breeds were found to browse more on bush vegetation than the long-tailed breed Dala. Such breed difference might also affect the risk of tick-infestation of the sheep. In Norway, no studies on tick immunity on sheep have been conducted. It is however proposed that the Old Norse sheep breed is generally more protected against tick-borne infections than other Norwegian breeds as it is commonly on pasture the whole year around and exposed to natural selection in a tick-exposed habitat.

### Tick borne disease immunity:

Tick-borne diseases are complex and the immunological mechanisms of resistance to the various tick borne diseases are neither well understood nor documented. Currently, the main difference among resistant and susceptible breeds to babesiosis in cattle is not related to whether they become infected or not but, rather, to how they overcome babesiosis, i.e. resilience (Oliveira et al., 2008). Breeding for genetic resistance to tick-borne infections is a potential mean to control losses, but due to the complexity of the tick-borne diseases, selection for such resistance is proposed to be a difficult task (Regitano and Prayaga, 2010).

Selection for increased host resistance to ticks is likely to change the host environment for the tick. Theory of co-evolution suggests that this will result in a selective advantage to tolerant parasites and allow them to adapt for successive generations (Bishop and Stear, 2003). It is, however, modelled that worm adaptation to livestock is not expected to adapt to selection for increased resistance to worm infection in livestock (Kemper et al., 2010). Exploiting a possible genetic variation between or within breeds in host resistance and immunity to an *A. phagocytophilum* infection in appropriate breeding schemes makes a sound basis for effective sustainable control of tick-borne diseases.

### *Vaccine*

Vaccines have been proposed for both tick and tick-borne disease control, but still remains a challenge. A vaccine for tick control of the single<sup>3</sup> host cattle tick *R. microplus* was commercialized in 1995, and has shown to reduce the number of engorging female ticks on cattle (Canales, 2009). For the tick-borne encephalitis virus (TBEV) there is a reliable vaccine to prevent humane infection (Heinz, 2007). There are a number of studies suggesting that there are possibilities of vaccine strategies both to control the ticks directly but also towards blocking the pathogen transmission (de la Fuente, 2006). There are however no vaccine

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<sup>3</sup> Single host ticks spend the parasitic stage of its life on the one host and the tick changes from a larva to a nymph and finally an adult after approx.. 21 days (*R. microplus*).



against *A. phagocytophilum* available yet. Using infected blood from carrier animals to immunize susceptible sheep is not recommended as there is a risk for spread of other infectious agents and there is little control of infection (Stuen and Longbottom, 2011). The fact that there are a number of different variants of *A. phagocytophilum* makes it challenging to find antigens that are common among all variants (Schuijt et al., 2011) for vaccine development.



## Objectives

The main objective of the work conducted for this thesis is to quantify losses due to *A. phagocytophilum* infection and improve sheep welfare and productivity through preventive measures against tick-borne fever and production loss.

This main objective was addressed in the four papers presented in this thesis, where the specific objective of each of the four papers is:

Paper I:

To examine the prevalence of *A. phagocytophilum* infection in lambs on tick-exposed pastures and to quantify the extent of weight loss of lambs that can be expected on such pastures.

Paper II:

To reveal the effect of lamb age when turned out on pasture and exposed to a natural *A. phagocytophilum* infection on lamb performance.

Paper III:

To compare the feral Old Norse sheep and the genetically improved and faster growing Norwegian White sheep with respect to resistance to *A. phagocytophilum* infection.

Paper IV:

To study genetic variation in lamb survival on tick-exposed pastures for possible implementation in selection programs.



## Materials and methods

The issues of TBF and *A. phagocytophilum* infection in sheep in this thesis has been addressed with approaches and methods on different scale, ranging from an infection challenge test where individual sheep were closely monitored with i.e. frequent blood sampling and observations (**PAPER III**) to a population study where the ‘rough’ registration of lambs expected to be exposed to ticks on farm level based on registrations from the National Sheep Recording System in Norway was used to estimate heritability of survival of lambs (**PAPER IV**).

This work has included a field survey with farms in an area anticipated to be moderately tick-infested (**PAPER I**), an on-farm trial including two sheep farms in tick and *A. phagocytophilum* endemic areas (**PAPER II**), a controlled infection study including a total of 64 lambs from two sheep breeds (**PAPER III**) and a study based on lamb recordings from the National Sheep Recording System on farms expected to be exposed to ticks (**PAPER IV**). Issues of *A. phagocytophilum* infection in sheep have been approached on different scales; individual, farm and population scale. These different approaches are attended with different challenges; the controlled infection study being different from farm conditions, the on-farm field trial having a number of non-controllable factors and the population study of lambs on tick exposed pastures being imprecise when it comes to determining actual tick exposure and *A. phagocytophilum* infection in the studied lambs.

### Study material

In **PAPER I**, the study included 1208 lambs from 12 farms in Sunndal Ram Circle in Møre and Romsdal County in Mid-Norway, that were expected to be on moderately tick infested areas.

In **PAPER II** the study included 336 lambs on two sheep farms for two years in tick infested areas where *A. phagocytophilum* was prevalent.

In **PAPER III** the study included a total of 64 lambs of two different sheep breeds that were experimentally infected with *A. phagocytophilum*.

In **PAPER IV** the study included 126 732 lambs from farms expected to be exposed to ticks and/or having experienced cases of TBF infection in 2000-2008.

### Parameters

Diagnosis of TBF and detection of *A. phagocytophilum* in sheep can be done in several ways (Stuen, 2003). Clinical observation of high fever ( $>41^{\circ}\text{C}$ ) and examination of neutrophils by light microscopy from blood smears prepared from blood taken during the fever period confirms a TBF diagnosis. Serology of specific antibodies from blood indicates that the animal has experienced an infection with *A. phagocytophilum* (Stuen, 2003). Detection of *A. phagocytophilum* can also be done by PRC on tissue and blood samples. Complications such as joint inflammations, pneumonia and septicaemia are commonly observed in lambs as consequences of secondary infections due to a primary *A. phagocytophilum* infection, but do not confirm an *A. phagocytophilum* infection alone. An enlarged spleen is indicative of TBF in sheep being the only pathologic change described that can be used by a post-mortem examination (Øverås, 1972; Stuen, 2003). Several studies indicate that *A. phagocytophilum* is prevalent in sheep grazing on pastures where ticks are present (Ogden et al., 1998; Stuen and Bergstrom, 2001b; Grøva, 2009), but observations of ticks on lambs is not an accurate indicator of *A. phagocytophilum* infection.

In **PAPER I** the study focused on determining if lambs had been infected with *A. phagocytophilum* or not during the grazing season, and its effect on autumn live weight. Serology of specific antibodies at the end of the grazing season was used to determine an *A. phagocytophilum* infection and weight information was gathered from the National Sheep Recording System.

In **PAPER II** the study focused on determining the effect and time of *A. phagocytophilum* infection on lamb performance. Serology of specific antibodies and quantification of the level of antibodies at birth, day 56 and day 134, together with blood smears and registration of weight, clinical signs of disease (including rectal temperature), and tick counts on lambs were used to describe the effects of age of lamb to a natural *A. phagocytophilum* infection on lamb performance.

In **PAPER III** the study focused on the clinical, haematological and serological response in lambs when experimentally infected with *A. phagocytophilum*. EDTA blood was sampled daily for preparation of blood smears. The serological response was monitored every week, together with daily observations of temperature and weekly weight registrations.

In **PAPER IV** the study included 126 732 lambs from farms that were expected to be exposed to ticks based on registrations in the National Sheep Recording System. Here, lamb survival was the indicator of robustness used that may reflect a trait of being robust on tick exposed pastures.

### **Statistical methods**

The statistical software SAS (SAS, 1999) was used for the General Linear Models, Mixed Models and Logistic regressions in **PAPER I** and **II**. The statistical software Statistix, version 4.0 (Analytical Software) was used for a two-sample *t*-test to analyse clinical, haematological and serological variables (**PAPER III**). The statistical software DMU was used for heritability estimates of survival (**PAPER IV**) (Madsen and Jensen, 2010).





## Main results and discussion

Our findings indicate that *A. phagocytophilum* seems to be widespread in areas with ticks and that an *A. phagocytophilum* infection does cause a significant but on average low reduction in live weight in lambs. Also, *A. phagocytophilum* infections were found at high altitudes (>600 masl) where ticks are not perceived to be prevalent. The proposed preventive measure on tick infested pastures, of exposing lambs when they are young i.e. about one week old, to a natural *A. phagocytophilum* infection indicated a positive effect on weight gain under field conditions. It did not, however, protect the lambs completely. Breed differences in response to *A. phagocytophilum* infection are indicated, but not confirmed. Furthermore, a significant heritability of 0.22 of survival on tick-exposed pastures was estimated indicating possibilities to improve survival on tick-exposed pasture.

## Prevalence of *A. phagocytophilum* infection

Infection with *A. phagocytophilum* was widespread (55%) in the farms studied in Sunndal Ram Circle (**PAPER I**). Observations of antibodies to *A. phagocytophilum* in 93 % of lambs at weaning (unpublished data **PAPER II**) further suggest that *A. phagocytophilum* infection is common in lambs on tick-infested pastures. These findings were not surprising as earlier observations in Norway (Øverås, 1972; Stuen and Bergstrom, 2001b; Stuen, 2003; Ladbury et al., 2008) also suggest that *A. phagocytophilum* infection in lambs is common when lambs graze on tick-infested pastures. It is suggested that all lambs grazing on tick-infested pastures are likely to acquire *A. phagocytophilum* infection (Ogden et al., 1998). A survey conducted in 2008 analysed 511 blood samples from lambs on 35 different farms in the county of Møre and Romsdal for antibodies to *A. phagocytophilum*. The survey showed that *A. phagocytophilum* infection was present on all 35 farms and antibodies were detected in 74% of the analysed blood samples (Grøva, 2009).

When lambs are turned out on pasture, even in tick-endemic areas, they are, however, not necessarily infected with ticks or *A. phagocytophilum* at time of pasturing. Observations presented in **PAPER II**, showed that an actual infection during the spring pasturing period (approximately 38 days) was considered to be experienced by 48 % of the lambs although 78% of the lambs were registered with tick-bites during this period. This suggests that *A. phagocytophilum* infections do not necessarily occur during the spring, but also in the summer and autumn as has been indicated earlier by Stuen and Kjølleberg (2000).

The prevalence of *A. phagocytophilum* infection on farm level in our study (**PAPER I**) was negatively correlated with altitude (masl). Altitude is suggested as an important habitat factor for tick-survival (Lindgren et al., 2000; Daniel et al., 2003; Danielova et al., 2006; Danielova et al., 2008; Gilbert, 2010), and our observations of reduced prevalence of *A. phagocytophilum* with increasing altitude is therefore in accordance with previous findings. We did however observe *A. phagocytophilum* infection at high altitudes (> 600 masl) where ticks were not expected to be prevalent. Altitudinal and latitudinal shifts in the range of *I. ricinus* have now been suggested in Norway (Jore et al., 2011), and supports our observation of *A. phagocytophilum* infections in grazing areas previously expected to be associated with no tick-infestations.

Prophylactic treatment with acaricides does not necessarily prevent *A. phagocytophilum* infection, as high prevalence of antibodies to *A. phagocytophilum* was observed in flocks where lambs were treated with acaricides (**PAPER I**). This is also observed earlier where lambs treated with acaricides show antibodies to *A. phagocytophilum*, even after only 3 weeks on tick pasture (Hardeng et al., 1992; Stuen and Bergstrom, 2001b). This questions the usefulness of using acaricides to protect lambs against *A. phagocytophilum* infection. The use of acaricides has however shown to reduce losses to *A. phagocytophilum* infection (Mitchell G.B et al., 1986; Hardeng et al., 1992).

## Loss

Sheep farmers may experience both indirect loss (i.e. reduced weight gain) and direct loss (deaths) due to infection with *A. phagocytophilum* (Brodie et al., 1986; Stuen and Kjølleberg, 2000; Stuen et al., 2002a). It is known that there are a number of factors influencing the severity of an infection; the genetic variant of *A. phagocytophilum*, age at infection, immune state of the host and frequency of exposure to infection (Stuen, 2003; Woldehiwet, 2010).

### *Indirect loss*

Weight reduction as a consequence of an *A. phagocytophilum* infection has been indicated in a number of studies (Brodie et al., 1986; Stuen et al., 1992; Stuen et al., 2002a). In **PAPER I** a significant negative effect of *A. phagocytophilum* infection on live weight was detected with 1.34 kg ( $\pm 0.412$ ) lower weight in seropositive lambs compared to seronegative lambs. The average weaning weight and weight gain on the studied farms in **PAPER I** were above county and national average in 2007 and 2008 (Animalia, 2011), indicating that it is possible to maintain growth in spite of high prevalence of ticks and *A. phagocytophilum* infections. Pasture quality and stress levels in general affect performance and robustness to disease. It is expected that the quality of the pastures on the farms involved in this study (**PAPER I**) is high, as weight registrations are above national average. Low average weight gain on summer pasture of less than 150 grams/day (national average is 257g/day (Animalia, 2011)) has however also been observed on farms where *A. phagocytophilum* infection was prevalent (Grøva, 2010).

Different variants of *A. phagocytophilum* exist and they cause different clinical signs (Ladbury, 2008; Stuen, 2003; Stuen, 2009). Infections with variants causing a mild response might therefore be an explanation of the relatively low effect of *A. phagocytophilum* infection on weaning weight observed in **PAPER I**. Seropositive lambs may also have been infected with different variants of *A. phagocytophilum* causing variable response to infection and thus

variable effect on weight gain. The issue of different variants was not addressed in **PAPER I**. The variation (s.e.) of the weight LSMEAN estimates of seropositive and seronegative lambs was  $\pm 0.89$  and  $\pm 0.88$ , respectively (unpublished data). A greater variation in the weight LSMEAN estimate of seropositive vs seronegative lambs might have indicated that an *A. phagocytophilum* infection affects lambs differently, possibly due to different variants. Such difference in variation was, however, not observed. The use of acaricides was not found to be significantly correlated with weaning weight on farm-year level in our study (**PAPER I**).

Even if the modest presumption that 300 000 lambs are infected each year in Norway (Stuen, 2003), a weight loss of 1.34 kg implies a substantial loss of 165 tons of lamb meat per year on the national level. Our study indicates, however, that losses to *A. phagocytophilum* infection does not always cause a substantial loss on farm level as average performance of lambs in the studied farms was above national average (**PAPER I**). Annual variations in time of infection (**PAPER II**) and annual variation in prevalence of infection on farm level (**PAPER I and II**), together with knowledge on different variants of *A. phagocytophilum*, illustrate the challenges of making general conclusions on the extent of indirect loss that can be expected on tick-exposed sheep farms.

### *Direct loss*

Observations of more than 30 % lamb loss related to *A. phagocytophilum* infection are registered (Stuen and Kjølleberg, 2000). The actual causes of deaths on summer pastures are in general unknown for most lamb losses during summer pasturing in Norway (Dahl and Lystad, 1998; Warren et al., 2001). High lamb losses during summer pasturing is a great concern for the sheep industry and finding lost lambs for identifying the cause of death remains a challenge. This is also the case when trying to interpret if *A. phagocytophilum* infection is a possible cause of the lamb losses observed. Direct losses are also proposed to be higher on tick-exposed pastures than on tick-free pastures (Øverås, 1972). No correlation

between prevalence of seropositive lambs and lamb losses on farm-year level were found in this study, and infection of *A. phagocytophilum* as a possible cause of lamb losses was not clear in this study (**PAPER I**).

Even if mortalities occurred in lambs that were turned out on tick-infested pasture  $\leq$  one week old, there was no mortality observed during the spring pasturing period for these lambs, however, only three lambs died on spring pasture altogether (**PAPER II**). Lamb loss on Farm B (**PAPER II**) in 2007, the year before the field trial, was 23 % (Norwegian Forest and Landscape Institute, 2011). The frequent observation of lambs during spring due to participating in the field trial (**PAPER II**), as well as treatment of sick lambs, is thought to be an explanatory factor in reducing lamb losses to 5% in 2008 on this farm.

Observing an average mortality of 3.8% on tick-exposed pasture (**PAPER IV**), besides losses to predators (0.2%), with a range of 0 – 22 % mortality per farm-year combination shows great variation in mortality on such pastures (**PAPER IV**). This illustrates that it is also difficult to make general conclusions on the extent of direct losses that can be expected on tick-exposed pastures.

## Prevention of TBF

### *Pasturing of young lambs*

On tick exposed pastures, turning out lambs when they are young has been recommended as a preventive strategy against losses to *A. phagocytophilum* infection for many years, mainly based on findings in infection studies (Stuen et al., 1992; Stuen, 1993). However, in field studies, many factors are unknown; i.e. the actual tick load on pasture at time of pasturing, prevalence of *A. phagocytophilum* in the tick and the variant of *A. phagocytophilum* present. The effect of turning out young lambs on pasture under field conditions has however not been studied previously.

**PAPER II** showed that there was an effect of pasturing young lambs in tick-endemic areas on weight gain (Figure 1) under field conditions.

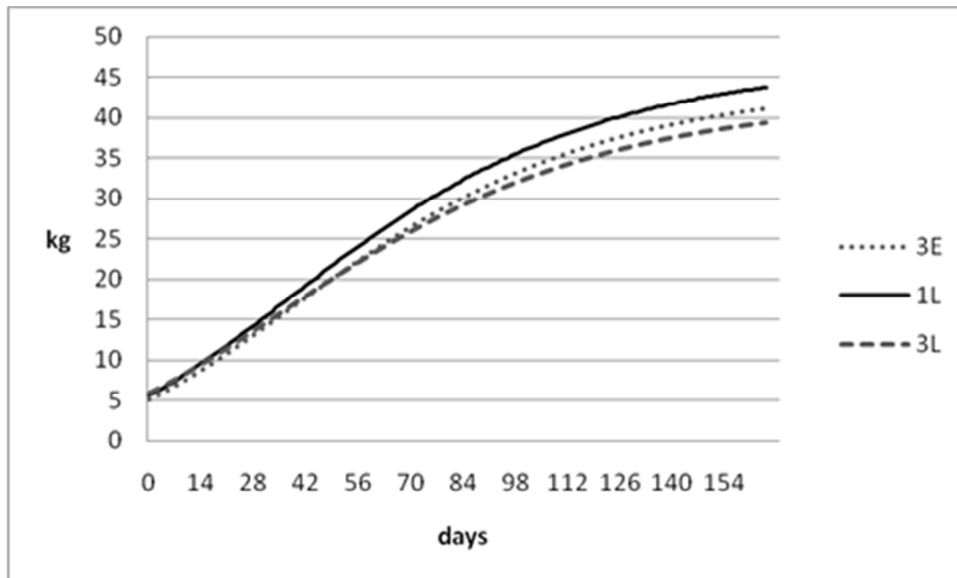


Figure 1. Predicted Gompertz weight curve for spring infected lambs in three trial group: 3E ( $\geq$  three weeks old when turned out and early time of birth), 1L ( $\leq$  one week old when turned out and late time of birth) and 3L ( $\geq$  three weeks old when turned out and late time of birth)  
**PAPER II**

A consequence of turning young lambs earlier out on pasture also implies changes in feeding (shorter period of indoor feeding) and the environmental conditions provided, which may affect weight gain. It is, however, thought to be unlikely that milk production and consequently daily growth rate of lambs is affected substantially due to a difference of two-three weeks longer indoor feeding (Nedkvitne, 1998). The effect of pasturing young lambs is therefore likely to be attributed to *A. phagocytophilum* infection at a young age.

The presence of maternal antibodies in young lambs may generally cause lower active immune response to certain infections and vaccinations by their inhibiting effect on neonatal immunoglobulin synthesis (Tizard, 2004). This inhibiting effect is however considered not to occur for *A. phagocytophilum* infection as young lambs that were experimentally infected with *A. phagocytophilum*, showed a serological response to infection (Stuen, 1993). This indicates that the issue of inhibited neonatal immunoglobulin synthesis is not important to *A.*

*phagocytophilum* infection. Although the level of antibodies has been suggested to influence the effects of *A. phagocytophilum* infection, no significant effect of level of maternal antibodies on weight gain was observed in the field trial presented in **PAPER II** (unpublished results). High level of maternal antibodies does therefore not seem to affect the lambs own immune response.

There were further significant differences between years and trial groups in incidence of spring infection, as well as in incidence of tick-bites (**PAPER II**). This indicates seasonal and annual variations in incidence of tick-bites and *A. phagocytophilum* infection as also observed in other studies (Jaenson and Tälleklint, 1996; Korenberg, 2000). Hence, annual and seasonal variations in tick activity are expected to influence the effect of turning young lambs out on pasture, as also suggested by Stuen and Longbottom (2011).

Previous infection studies have shown that young lambs are not fully protected against *A. phagocytophilum* infection by their maternal antibodies (Stuen et al., 1992; Stuen, 1993). This is also supported by our finding (**PAPER II**) as incidences of clinical signs of disease and mortality were observed in all trial groups and on both farms in 2008 and 2009 although 91% of the lambs had maternal antibodies.

Lambs may also be infected with several variants of *A. phagocytophilum* during the grazing period (Ladbury et al. 2008). It is known that the different variants provide different degrees of protection to re-infection with another strain of *A. phagocytophilum*, which may influence the incidence of clinical disease and mortality (Stuen et al., 2003a). Hence, the success of turning young lambs on pasture may also be dependent on the variants present at the pasture. Also, *A. phagocytophilum* infections may occur throughout the whole grazing season and *A. phagocytophilum* infections during the autumn grazing period after the sheep are gathered from unfenced range pastures and put on autumn pastures are also observed (Stuen and

Kjøllberg, 2000) (Øverås, 1972). The variants involved in autumn infections may therefore cause TBF and secondary infections even if lambs were infected in spring.

### *Genetic variation in robustness*

Genetic variation in robustness is shown in many species to a number of diseases (Bishop et al., 2010), and sheep also show genetic variation in the ability to tolerate infection (robustness) and to resist disease (Bishop and Morris. 2007). For example, genetic differences in resistance to internal parasites in sheep are observed (Bishop and Stear, 1999, Stear et al.1995).

Based on a British study, it has been suggested that there may be a difference in breed susceptibility to *A. phagocytophilum* infection in sheep (Scott, 1983). It has also been reported that TBF was more common in some breeds of Finnish cattle than others (Tuomi, 1966). The observations of substantial individual variation in amplitude of bacteraemia, number of bacteraemia periods, time of serological conversion and response after *A. phagocytophilum* infection (Granquist et al., 2010) may suggest a genetic variation in response to infection in sheep. Indications of breed differences between the Norwegian White sheep and the Old Norse sheep are shown in **PAPER III**. However it was concluded that further studies are needed to confirm such breed difference. The Old Norse sheep are hypothesised to be more protected against tick-borne infections than other Norwegian breeds, due to a continuous high selection pressure (natural selection) and possibly also due to breed differences. The Old Norse sheep are commonly kept on pasture the whole year around in coastal areas where ticks are prevalent. All lambs included in the infection study (**PAPER III**) were, however, born and reared indoors. Knowing that the breeds are commonly kept under different management systems, the hypothesis and observation by farmers that Old Norse sheep is more robust than Norwegian White sheep might be attributed to management factors, rather than actual breed differences. Regular exposure to *A. phagocytophilum* infection is proposed to improve the



lamb's ability to face reinfection. Also, repeated infection by ewes during pregnancy (Brodie, 1985) has shown to affect the level of antibodies in the lamb at birth.

Previous studies of heritability of lamb survival show that lamb survival on summer pasture range from low to moderate, and some studies concluded that survival cannot be improved by selection on this trait (Burfening, 1993; Hatcher et al., 2010), while other studies suggest that survival can be improved by selection (Sawalha et al., 2007; Brien et al., 2010). The estimated heritability of 0.22 for lamb survival in Norwegian White sheep expected to be exposed to ticks (**PAPER IV**) indicates a potential for genetic selection to improve lamb survival in the studied population. However, one should be careful to attribute this heritability directly to an actual robustness to *A. phagocytophilum* infection as the actual infection status of the lambs was unknown.



## Main conclusions

### PAPER I:

In summary, the present study supports previous findings that *A. phagocytophilum* infection is widespread in lambs on tick-infested pastures, and also present on pastures not perceived to be tick-infested, i.e. at high altitudes. It also shows that an *A. phagocytophilum* infection reduces live weight with 1.34 kg on average, but do not always cause substantial direct or indirect losses.

### PAPER II:

The results show that exposing young lambs of one week to a natural *A. phagocytophilum* infection has a positive effect on daily weight gain of spring infected lambs on tick infested pastures. Pasturing of young lambs and natural *A. phagocytophilum* infection during the first days on pasture can therefore be recommended as a preventive measure in order to reduce weight losses due to *A. phagocytophilum* infection. Annual and seasonal variations of tick activity relative to lambing, variants of *A. phagocytophilum* involved and turnout time should however be pointed out as this will probably influence the effect of pasturing young lambs.

### PAPER III:

There are indications of breed differences in response to infection but further studies are needed to confirm if the ON-breed is more robust to *A. phagocytophilum* infection than other Norwegian breeds.

### PAPER IV:

The estimated heritability of lamb survival on pastures expected to be exposed to ticks is 0.22 and indicates potential for genetic selection to improve survival by selection. The heritability cannot, however, be accurately attributed to robustness to *A. phagocytophilum* and TBF infection as the infection status of the lambs is unknown.



## Recommendations and future perspectives

Challenges with ticks and tick-borne diseases are likely to increase in the coming years, and knowledge on the distribution and prevalence of ticks and tick-borne diseases as well as possibilities of preventing losses to *A. phagocytophilum* infection are important for sheep farmers.

Improving our knowledge and understanding about robustness in sheep on tick-infested pastures will probably make an important basis to improve the performance of lambs on such pastures. Pasturing of young lambs in areas where ticks are prevalent is recommended. Our findings of a possible breed difference in response to *A. phagocytophilum*, although not confirmed, together with a heritability estimate of 0.22 on lamb survival on tick-exposed pastures indicate that there are likely genetic factors affecting the lambs response to an *A. phagocytophilum* infection and how this perform in tick-exposed environments.

Identification of genetically robust sheep on tick-infested pastures may be a first step towards implementing robustness as a trait in the breeding scheme. A second step may be to identify genetic markers that can be implemented in a breeding program towards improving resistance to TBF in sheep. Identification of robust sheep on tick infested pastures for selection or finding genetic markers for TBF resistance has not been done yet, and this should be addressed in future research.

Identifying robust animals could be done by using existing data from the Norwegian Sheep Recording System and the National Organization of Pasture Management (OBB) on performance of lambs i.e. weight, growth on summer pasture, losses on pasture as well as pedigree information. These could be extended with additional recording of TBF and *A. phagocytophilum* infection in tick-infested areas. Studying flocks that either experience high direct losses (i.e. >20%), low average but high variance of weight gain, or high prevalence of TBF and/or *A. phagocytophilum* infection in tick-infested areas may provide information that

allow for more accurate genetic analysis. Data on performance of lambs in such flocks of the Norwegian White sheep (NW) breed sired by rams in flocks that are genetically connected as in ram circles or by AI rams, may be utilized to rank rams and ewes with respect to growth and survival on tick infested pastures. Prediction of breeding values may be carried out by BLUP and an animal model using information on additive genetic relationship between animals (also in different flocks), making it possible to account for environmental flock effects. Assortative mating between high performing (high breeding value) ewes and rams as well as between low performing rams and ewes (low breeding value) will produce robust and susceptible offspring that may be used in an infection study.

The board of the Agricultural Agreement Research Fund has granted funding for to the TICKLESS project (2010 – 2014) where the issue of identifying robust animals as described above is proposed to be conducted. The TICKLESS project will also look into biological control of ticks and reveal interactions between the tick population, tick-borne disease prevalence and land management.

An infection study of robust and susceptible lambs plus controls may further be conducted. Lambs can be tested for clinical manifestation and serological response after experimental infection and reinfection with *A. phagocytophilum*. Regular blood sampling from each lamb after challenge and storing of mRNA may allow for further immunological analyses and facilitate future gene expression studies to gain insight into the genetic basis of resistance. Genome-wide screening of single nucleotide polymorphism (SNP's) is available for sheep ([www.sheepmap.org](http://www.sheepmap.org)), and opens for genome-wide selection (GWS) which, i.e., has been applied for mastitis resistance in dairy cattle (Raadsma et al., 2007).

Tick counts have been used to identify robust cattle to the single-host cattle tick *Rhipicephalus microplus* (formerly *Bophilus microplus*) in tropical areas. So far, no study of variation in tick-counts has been conducted on the *I. ricinus* tick to my knowledge. Knowing

that only one cell of *A. phagocytophilum* may be enough to transmit infection (Stuen, 2000), tick count may not be an appropriate measure or indicator to identify robust animals to *A. phagocytophilum* infection. However, a number of factors are involved in the transfer of disease from the tick to the host and tick-count cannot, to my opinion, be out ruled as a trait of interest. If tick-counts on lambs in Norway are to be exploited as an indicator of robustness to an *A. phagocytophilum* infection, a field study involving a minimum of 1200 lambs from several flocks can be carried out. Here, counting ticks on ears or other relevant parts of the body on lambs for two to three consecutive years with 2-4 observation per grazing season while lambs are on tick-infested pastures can make a basis for a study of genetic parameters. Further, the management of sheep flocks may vary considerably; i.e. the Norwegian White sheep (NW) is commonly housed indoors and selected for performance, while the Old Norwegian sheep (ON) is commonly on pasture the whole year and is therefore likely to be regularly exposed to both tick infestation and natural selection to a higher degree than the NW breed. A study focusing on revealing consequence of such management factors on lamb performance in tick exposed areas, where farms with both breeds and management schemes (as described above) are included, has potential to reveal effects of management system, breed and GxE on performance on tick-infested pastures. However, there are practical challenges in setting up such a trial, particularly when it comes to finding appropriate farms.

Even if there is knowledge on different variants of *A. phagocytophilum*, age of the animal and immunologic status of the animal causing differences in response to infection, we do not fully comprehend why the severity of an *A. phagocytophilum* infection seems to vary; i.e. some flocks cope well with a high prevalence of *A. phagocytophilum* infection in the flock, while other flocks may suffer from heavy losses. Understanding what factors are involved with fatal cases of TBF is also likely to improve our understanding of the differences in severity to an *A. phagocytophilum* infection observed between farms and individuals.

Finally, biological control of ticks and vaccination against *A. phagocytophilum* infection are issues that have not been a focus in this study, but are addressed briefly in the introductory section 'Prevention of TBF'. The approach of biological control to control ticks is attractive, but there are a number of challenges that must be faced before this approach is likely to contribute to improve performance in sheep farming on tick-infested pastures. Vaccination against *A. phagocytophilum* seems to have considerable potential in preventing TBF and losses on tick-exposed pastures in sheep farming.



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# Paper I



RESEARCH

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# Prevalence of *Anaplasma phagocytophilum* infection and effect on lamb growth

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## Abstract

**Background:** A major challenge in sheep farming during the grazing season along the coast of south-western Norway is tick-borne fever (TBF) caused by the bacteria *Anaplasma phagocytophilum* that is transmitted by the tick *Ixodes ricinus*.

**Methods:** A study was carried out in 2007 and 2008 to examine the prevalence of *A. phagocytophilum* infection and effect on weaning weight in lambs. The study included 1208 lambs from farms in Sunndal Ram Circle in Møre and Romsdal County in Mid-Norway, where ticks are frequently observed. All lambs were blood sampled and serum was analyzed by an indirect fluorescent antibody assay (IFA) to determine an antibody status (positive or negative) to *A. phagocytophilum* infection. Weight and weight gain and possible effect of infection were analyzed using ANOVA and the MIXED procedure in SAS.

**Results:** The overall prevalence of infection with *A. phagocytophilum* was 55%. A lower weaning weight of 3% (1.34 kg,  $p < 0.01$ ) was estimated in lambs seropositive to an *A. phagocytophilum* infection compared to seronegative lambs at an average age of 137 days.

**Conclusions:** The results show that *A. phagocytophilum* infection has an effect on lamb weight gain. The study also support previous findings that *A. phagocytophilum* infection is widespread in areas where ticks are prevalent, even in flocks treated prophylactic with acaricides.

## Background

Tick-borne fever (TBF) is one of the main challenges in Norwegian sheep farming during the grazing season [1]. TBF is caused by the bacteria *Anaplasma phagocytophilum*, transmitted by the tick *Ixodes ricinus*, and may cause direct (lamb deaths) and indirect loss (reduced growth) in sheep farming. The normal distribution area of *I. ricinus* ticks in Norway is the coastal areas of Norway as far north as Brønnøysund in Nordland county (N 65°30'), Norway [2-4]. *A. phagocytophilum* infected lambs are commonly found in areas with ticks [2,5]. Climate change (i.e. warmer winter climate), changes in land use (i.e. bush encroachment) and an increase in the deer population are factors expected to increase the populations of ticks. An extension of the northern margin of the population distribution of *I. ricinus* and to higher altitudes has been observed [6,7], and has given rise to concerns

that challenges with TBF will increase in Norway in the coming years.

The main consequence of an *A. phagocytophilum* infection in sheep is the ensuing immunosuppression that may lead to secondary infections and cause both direct and indirect losses. Direct losses of ca 30% lamb mortality in a flock due to *A. phagocytophilum* infection have been observed [8,9]. The exact causes of deaths of lambs on pasture have however seldom been determined, because most lambs have been grazing on free range forest and mountain pastures with only weekly attention. Hence only a few lost lambs have been found [10-12]. The extent of indirect production loss due to TBF was 3.8 kg body weight per lamb in a study of a flock with 50 lambs [13] and experimental infection with *A. phagocytophilum* has shown to affect weight for several months after the primary infection [14]. It is also shown that prophylactic use of long-acting tetracycline against *A. phagocytophilum* has improved weight gain in lambs on pasture [15].

Several genetic variants of *A. phagocytophilum* are observed and it is shown that these cause different

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clinical signs with varying haematological and serological response; i.e. differences in duration of fever, maximum temperature, level of antibody titre, and weight reduction [16-18].

There is great concern about indirect and direct losses to TBF among sheep farmers in areas where *I. ricinus* is abundant. The objective of the present work was to examine the prevalence of TBF in lambs on tick-infested pastures, and to quantify the extent of weight loss of lambs that can be expected on tick-infested pastures.

## Methods

### Study population

Lambs from Sunndal Ram Circle [19] in the county of Møre and Romsdal (Mid Norway) were selected for this study (62°N, 9°E). Sunndal Ram Circle is a ram circle for the Norwegian White Sheep breed and consisted of 21 sheep farmers in 2007 and 2008 who cooperated with progeny testing of 28 ram lambs (868 matings) and elite matings by mating with a total of 280 ewes in 2007 [20]. The studied population of lambs were presumed to be grazing in tick-infested areas as *A. phagocytophilum* infection was confirmed on six farms in Sunndal Ram Circle in 2006.

The study sample included lambs from 12 of the farms in Sunndal Ram Circle that were turned out onto pasture together with their mothers in 2007 and 2008 with spring and weaning weight recordings. Data on spring and weaning weight, age at weighing, sex, rearing rank and mother were collected and obtained through The Norwegian National Sheep Recording Scheme [21]. Table 1 shows mean lamb weights and SD of the sampled lambs in 2007 and 2008. Information on lamb losses on summer pasture was collected from recordings done by the by the Norwegian Forest and Landscape Institute [22]. Cause of direct lamb losses was not determined in this study. Blood samples were collected in 2007 (n = 968) and 2008 (n = 240) during the event of collection and weighing of lambs at the farms in autumn prior to slaughter or selection for breeding. Weight scales were calibrated on the actual day of weighing.

### Farm characteristics and management

A questionnaire was sent to the 12 selected farmers in Sunndal ram circle to gather information on farm

characteristics and management. Information on prophylactic treatment of sheep against ticks and farmers' perception of having ticks on their pastures (yes/no) is presented in table 2. The altitude in meters above sea level (masl) of the spring pastures was 0-200 (masl) for ten of the twelve farms. The remaining two farms; farm D and I, had spring pastures at 100-400 and 700 masl respectively. Altitude of summer pastures varied between 150 and 1300 masl. Spring and autumn pastures were cultivated pastures with bush vegetation. Summer pastures were mountain and valley range land with variable degree of bush and forest vegetation. Considerable between and within farm variation in bush vegetation is typical. Dominant bush vegetation species were not mapped in this study. The production system was in general similar on all farms; lambs were born indoors and then they were let onto spring pasture at the age of 0 - 4 weeks, and lambs were let onto summer pastures after a short period of grazing on spring pasture. During the autumn, lambs were gathered from summer pastures and kept on pastures close to the farm for a short period before slaughter. All sheep and lambs were treated with anthelmintics before they were let onto summer pastures. Prophylactic treatment against ticks was conducted in spring on 9 out of 12 flocks using Coopersect<sup>®</sup> vet 1-2 times before lambs were let on to summer pastures. Prophylactic treatment against ticks was not conducted on three of the farms (farm B, F, I).

### Serology

Blood samples were collected during autumn at an average age ( $\pm$  SD) of  $137 \pm 8$  days. Blood samples were centrifuged for 10 min at 3200 ppm within 24 hours of sampling. Serum was extracted, frozen and later analysed by an indirect fluorescent antibody assay (IFA) to determine the antibody titre to a heterologous horse variant of *A. phagocytophilum* (formerly *Ehrlichia equi*) [5,23]. No antigen from a sheep variant of *A. phagocytophilum* was available. Briefly, a two-fold dilution of sera was added to slides pre-coated with *E. equi* antigen (Protatek International and Organon Teknika). Bound antibodies were visualized by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-sheep immunoglobulin (Cappel, Oranon Teknika). Sera were screened for antibodies at dilution 1:40, and a titre of 1:40 and higher

**Table 1 Mean (SD) of weight parameters of the study population, and county and national average**

	Study population		Møre & Romsdal <sup>1</sup>		Norway <sup>1</sup>	
	2007 (n = 968)	2008 (n = 240)	2007	2008	2007	2008
Age at weaning weight (days)	137 (9.7)	139 (7.8)	145	145	145	145
Weaning weight (kg)	45.7 (8.2)	47.6 (7.7)	42.3	44.6	44.5	45.5
Weight gain spring-weaning (g/day)	285 (54.3)	296 (59.5)	237	260	255	262

<sup>1</sup> [21,39]

**Table 2 Prevalence of seropositive lambs, weaning weight, altitude of pastures, and lamb loss per farm and year**

Farm	Number of samples (2008) n		Prevalence of seropositive lambs %		Minimum altitude of pastures masl	Average weaning weight kg		Lamb loss %	
	2007	2008	2007	2008		2007	2008	2007	2008
A	30		73		0	47.9		5	
B <sup>2</sup>	122	79	67	14	200	44.3	48.7	8	6
C	86		78		0	48.6		0	
D	44		2		100	48.0		36 <sup>3</sup>	
E	72		0		100	47.6		4	
F <sup>1 2</sup>	49		96		0	46.9		17	
G	173	71	58	65	0	44.8	48.2	11 <sup>3</sup>	25 <sup>3</sup>
H	88	90	90	81	0	43.0	46.1	17	11
I <sup>1 2</sup>	123		10		600	41.5		9	
J	58		55		50	50.5		1	
K	101		84		175	46.8		12	
L	22		36		150	50.0		7	
All	968	240	55	54		45.7	47.6		

<sup>1</sup> The farmer perceived that there were no ticks on their pastures

<sup>2</sup> No prophylactic treatment against ticks

<sup>3</sup> There were documented loss to wolverine (*Gulo gulo*) on these farms

was regarded as positive whereas titres below 1:40 were regarded as negative [5].

### Statistical analysis

#### Flock performance

Possible effects of prophylactic treatment, farmer's perception of ticks on pastures and masl of pastures (as regression effect) on the flock's prevalence of infection, direct losses on summer pasture and weaning weight was analyzed using the General Linear Model method of the GLM procedure in SAS [24]. The effect of prevalence of infection on direct loss was also estimated. The initial statistical model included all explanatory effects listed above according to the degrees of freedom available, before non-significant effects were removed by a stepwise procedure. Neither prophylactic treatment nor farmer's perception of ticks on pastures were included in the final regression model as their effect was not significant in this limited dataset. The final regression model used was:

$$\text{Model 1 : } Y1_i = B_0 + B_i x_i + e_i$$

Where Y1 is the prevalence of infection on the farm i (i = 1-12), B<sub>0</sub> is the intercept, B<sub>i</sub> is the regression effect on masl of farm pastures i (x = 0-600) and e<sub>i</sub> is the random residual error.

#### Individual lamb performance

Individual lamb data on weight were analyzed using the Restricted Maximum Likelihood method of the MIXED procedure in SAS [24]. Initial statistical model included the effects of age at weighing (as regression effect), serology, age of mother, sex and rearing rank as fixed

effects and farm, year, father and mother as random effects. The final models used were:

$$\text{Model 2 : } Y2_{ijklmnoq} = \mu + A(x_{ijklmnoq} - \bar{x}) + \text{Ser}_i + \text{AM}_j + S_k + R_l + f * \gamma_{mn} + m_o + S * f * \gamma_{mn} + R * f * \gamma_{mn} + e_{ijklmnoq}$$

$$\text{Model 3 : } Y3_{ijklmnoq} = \mu + \text{Ser}_i + \text{AM}_j + S_k + R_l + f * \gamma_{mn} + m_o + S * f * \gamma_{mn} + R * f * \gamma_{mn} + e_{ijklmnoq}$$

Where Y2 is the weaning weight and Y3 is the weight gain on summerpasture (spring to weaning) of the individual q (q = 1-1208); μ is the overall mean, A is the regression of the fixed effect of age at recording of weaning weight (days); Ser is the fixed effect of the serology result (i = 0, 1; where 0 = seronegative to *A. phagocytophilum* and 1 = seropositive to *A. phagocytophilum*); AM is the fixed effect of age-group of mother (j = 1, 2, 3, 4; where age group 1 = one year old, 2 = two year old, 3 = three year old, 4 = four years and older); S is the fixed effect of sex (k = 1, 2; where 1 = male and 2 = female); R is the fixed effect of rearing rank (l = 11, 21, 22, 31, 32, 33, 41, 42, 43, 44; where the first digit is birth rank and the second digit is rank when let on to pasture); f\*y is the random effect of farm-year (m = 2007, 2008) (n = 1 - 12); m is the random effect of mother (o = 1-618); e is the random residual error. All interactions with fixed effects were included in the initial analyses, but were removed subsequently if they did not show significant effect on weaning weight. Heterogeneous variance for male and female lambs was taken into account.

An analysis of variance for the explanatory effects on weaning weight was done using the GLM procedure in SAS [24].

## Results

### Serology and farm characteristics

Infection with *A. phagocytophilum* was widespread in Sunndal Ram Circle (Table 2). Positive samples were shown on 11 of the 12 farms and the proportion of antibody positive samples on these farms varied between 2 and 96%. On eight farms, 55% or more of the samples were antibody positive. Overall, 55% of the samples were positive for antibodies to *A. phagocytophilum*.

Prophylactic treatment against ticks was not conducted on three of the farms (farm B, F, I) of which two (farms F, I) perceived that there were no ticks on their pastures. On farm E no seropositive lambs were observed, but the farmer perceived that there were ticks on the pastures and used prophylactic treatment. Seroprevalence on farm F and I was 96% and 10%, respectively, and on farm I all pastures were above 600 masl. Infected lambs with *A. phagocytophilum* were observed on farms in spite of prophylactic treatment against ticks, farmers' perception of no ticks on pasture and high altitude of pasturing. The statistical model 1, however, showed that masl had a significant ( $p = 0.038$ ) effect on prevalence of *A. phagocytophilum* (Table 3). There was no significant effect of prophylactic treatment and farmer's perception on prevalence of infection, lamb loss and weaning weight.

### Production loss

The analysis of variance for weaning weight presented in Table 3 shows that effect of the mother explained most variation of weaning weight (32.6%). Here, both additive genetic and maternal effects are included. Antibody results only explained a small but significant proportion of the variance of weaning weight (0.3%).

There was a significant difference ( $\pm$  SE) between Least Square Means (LSM) of antibody positive and antibody negative lambs of  $1.34 \pm 0.412$  kg weaning weight ( $p < 0.01$ ) and  $10.4 \pm 3.3$  g daily weight gain ( $p < 0.01$ ) (Table 4). The weight difference amounts to 3% of

average weaning live weight of lambs in Norway. There was no significant difference of spring weight between antibody positive and antibody negative lambs.

Lamb direct loss during the summer grazing period on the 12 farms varied from 0 to 36%. Predators caused lamb losses in these grazing areas, and lamb losses to wolverine (*Gulo gulo*) were documented in two flocks (Table 2). Losses on farms with no documented losses to predators, varied between 0 - 17%, and four of the farms had losses above country average in 2007. The actual causes of deaths in general were unknown in this study, which is the general case for most lamb losses during summer pasturing [25,12].

## Discussion

### Prevalence

The overall seroprevalence of *A. phagocytophilum* of 55% among lambs in this study is lower than earlier observations of 80% seroprevalence of lambs grazing on *I. ricinus* infested pastures [5]. It is indicated in a UK study that probably 100% of lambs grazing on tick-infested pastures will acquire *A. phagocytophilum* infection [26]. Some of the flocks in the present study were, however, grazing in mountain range land with presumably low tick density [3]. This may explain the relatively lower seroprevalence of *A. phagocytophilum* on some farms. On one farm (farm M), all sheep were grazing at 600 masl and higher, where ticks earlier have not commonly been found in Norway [3]. On this farm 10% ( $n = 12$ ) of the lambs were seropositive. Our finding that prevalence of *A. phagocytophilum* infection is negatively associated with altitude (masl) is in accordance with previous findings [27]. It is also shown that ticks are found at altitudes up to 1100 masl in Central Europe [7]. For farm B the prevalence of seropositive lambs varied from 67% in 2007 to 14% in 2008, indicating considerable variation between years in *A. phagocytophilum* infection.

**Table 3 Results for the analysis of variance on weaning weight of lambs**

Effect	Degrees of freedom	Marginal sum of squares	Marginal increase in $R^2 \times 100$
Mother (farm)	560	25210.22	31.57***
Sex	1	2202.21	2.76***
Rearing rank	8	1135.34	1.42***
Rearing rank (farm year)	21	840.89	1.05
Sex (farm year)	14	839.12	1.05**
Age at recording of weaning weight	1	520.70	0.65***
Age of mother	3	315.92	0.40*
Antibody result	1	264.19	0.33**
Farm (year)	3	168.90	0.21
Error	538	11679.95	-
Model	669	68174.59	85.37

Level of significance different from zero for Marginal SS (type III SS) \*\*\* $p < 0.0001$  \*\* $p < 0.001$  \* $p < 0.01$ .

**Table 4 Least Square Means of weight recordings of lambs, with S.E. and p-value of the LSM difference**

	Antibody negative	Antibody positive	LSM difference	s.e.	p-value
Weaning weight (kg)	45.10	43.77	1.34	0.412	0.0012*
Spring body <sup>1</sup> weight (kg)	13.87	13.74	0.14	0.162	0.4045
Daily weight gain summer pastures <sup>2</sup> (g/day)	278.4	268.0	10.4	3.31	0.0018*

\* Statistically significant.

<sup>1</sup> Spring body weight: Age at spring body weight is used in the model. 18 observations are not used due to missing values.

<sup>2</sup> Daily weight gain summer: 18 observations are not used due to missing values.

### Serology

No antigen from a sheep variant of *A. phagocytophilum* was available. The sensitivity of the serology test may have been improved using a more proper antigen than the heterologous horse variant (*E. equi*) of *A. phagocytophilum*. Earlier studies indicate frequent cross-reactions between different variants of *A. phagocytophilum* [28,29]. However, antibody titre to heterologous strains of *Anaplasma* may be lower than to a homologous strain [30] and this might also affect the risk of false negative titres. Unfortunately, titre values were not obtained in the present study.

The time of infection during grazing period is not known and infection may have occurred on spring, summer and/or autumn pastures. It has, however, been shown that antibody titres can persist for at least 6 months in sheep after the primary infection [31,32]. Although different variants may cause different serological responses [17,33] and a spring infection might give reduced titre values in the autumn, it is expected that serology at the age of 137 days is a reliable indicator of infection or no infection if lambs have been infected during the grazing season [5].

### Weight gain

A difference of 1.34 kg between seropositive and seronegative lambs to *A. phagocytophilum* infection is less than reported from a previous study showing 3.8 kg weight difference [13]. Other studies have also shown relatively higher losses to TBF [8,9,13,14]. Still, if the modest presumption that 300 000 [2] lambs are infected by *A. phagocytophilum* each year in Norway, a 1.34 kg weight loss implies a reduction of 165 tons of lamb meat per year. Also, a reduced carcass weight may cause a reduced carcass quality (muscling), grade and lower price per kg.

No significant difference of spring weight between lambs that were seropositive and seronegative to *A. phagocytophilum* infection in autumn was observed. Average age at spring weight recordings vary between 3 - 63 days (mean = 26, S.D. = 13). This together with the fact that *A. phagocytophilum* infection might affect the live weight for several months after infection [14] implies that weight differences are likely to accumulate with increasing age i.e. at weaning weight. Also, lambs that

show seroresponse to *A. phagocytophilum* infection in autumn, are not necessarily infected in spring, but possibly later in the grazing period.

It is known that there are several genetic variants of *A. phagocytophilum* and that these cause different clinical signs with varying haematological and serological response [16-18]. A genetic variant of *A. phagocytophilum* (GenBank acc. no. U02521) showed no fever, weight reduction or other signs of clinical illness after experimental inoculation [34]. Different variants of the bacterium may show significantly different clinical reaction and cross-immunity [18]. The variants of *A. phagocytophilum* involved in this study are unknown. The variants involved may partly explain the variation in direct and indirect losses to the *A. phagocytophilum* infections observed. However, additional stress factors as individual condition, management and other infections are also important for the outcome of an infection with *A. phagocytophilum*.

Overall, mean weaning weight and daily weight gain of the lambs in this study population were higher than the county and national average (Table 1). Pasture quality and stress levels in general affect performance and robustness to disease. High quality pastures, shown by average weight gain and autumn live weight above national and county average, and possibly low stress levels may explain a relatively low weight difference between seropositive and seronegative lambs.

The analysis of variance for weaning weight showed that the effect of age at weight recording, age of mother, sex, rearing rank and mother explained much more of the variation in weight gain than the antibody result (*A. phagocytophilum* infection), indicating that infection with *A. phagocytophilum* does not necessarily affect the weight substantially.

### Farm characteristics

The results of this study supports previous findings that ticks and *A. phagocytophilum* infected lambs can be found even if farmers perceive that there are no ticks on their pastures and no observed cases of TBF in their flock [13]. It also indicates that prophylactic treatment with acaricides does not prevent infection, as high seroprevalence of *A. phagocytophilum* was observed in flocks

where lambs were treated with acaricides. It is previously shown that lambs treated with acaricides seroconvert after only 3 weeks on tick pasture [5,35]. Routine use of acaricides is not a sustainable measure due to the possibility of developing acaricide resistance [36-38]. The use of acaricides also has practical limitations as regular treatment of free ranging lambs on forest and mountain pastures is not feasible during the grazing season. Use of acaricides has however shown reduced incidence of secondary infections to TBF [37].

The direct losses of lambs on pasture in 2007 and 2008 were in Norway 8.4 and 7.7% respectively. Corresponding losses were 12.0 and 10.4% in the county of Møre and Romsdal [22]. In this study population lamb losses to the predator wolverine (*Gulo gulo*) were documented in two flocks. The actual causes of deaths in general were unknown in this study, which is the general case for most lamb losses during summer pasturing [25,12]. High lamb losses during summer pasturing is a great worry for the sheep industry and TBF is shown to give high losses in some flocks [8]. This study does however not show any correlation between seroprevalence and lamb losses, and the interpretation of TBF as a possible cause of lamb losses in this study is not clear.

## Conclusion

In summary, the present study supports previous findings that *A. phagocytophilum* infection is widespread. It also shows that an *A. phagocytophilum* infection affects live weight. However, *A. phagocytophilum* infections do not always cause substantial direct or indirect losses.

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## Authors' contributions

All authors contributed in designing the study and supervising the writing of the manuscript. LG was responsible for data collection, the statistical analysis and writing the draft manuscript. IO contributed particularly with input on statistical analysis. SS contributed particularly with input into the discussion of the results. All authors approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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# Paper II



# The effect of lamb age to a natural *Anaplasma phagocytophilum* infection

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## Abstract

Tick-borne fever (TBF) caused by the bacterium *Anaplasma phagocytophilum* and transmitted by the tick *Ixodes ricinus*, is a major challenge to sheep farming along the coast of south-western Norway. Few efficient and sustainable preventive measures are available, but older lambs seem to be more susceptible than younger lambs to an *A. phagocytophilum* infection. A field experiment was carried out in 2008 and 2009 on two farms including a total of 336 lambs of the Norwegian White Sheep breed. Three trial groups of lambs on each farm and year were established: 3E; lambs  $\geq$  three weeks old when turned out to pasture and early time of birth, 1L; lambs  $\leq$  one week old when turned out to pasture and late time of birth, 3L; lambs  $\geq$  three weeks old when turned out to pasture and late time of birth. Recordings of tick-counts, rectal temperature, clinical symptoms and mortality together with weight, blood serology and blood smears were used to analyze the effect of age of lambs to natural *A. phagocytophilum* infection. Gompertz weight curve parameters were estimated for all lambs and used to compare weight gain in lambs between the trial groups. There were incidences of tick-bites, clinical disease (including fever) and mortality, but no significant effect of lamb age to a natural *A. phagocytophilum* infection. However, lambs infected with *A. phagocytophilum* in the 1L group had higher ( $P < 0.05$ ) maximum spring growth rate (358g/day) than infected lambs in 3E (334g/day) and 3L (310/day) groups, respectively. Pasturing of  $\leq$  one week old lambs on tick-infested pastures in tick endemic areas, can therefore be recommended in order to reduce weight losses due to *A. phagocytophilum*. Note should however be taken on annual and seasonal variations in tick activity relative to lambing, variants of *A. phagocytophilum* involved and turnout time as this probably will influence the effect of pasturing young lambs.

Keywords: *Ixodes ricinus*, tick, weight gain, production loss, sheep, Gompertz

## Introduction

A main scourge in Norwegian sheep farming is tick-borne fever (TBF) caused by the bacterium *Anaplasma phagocytophilum* and transmitted by the tick *Ixodes ricinus* (Stuen, 2003). The normal distribution area of *I. ricinus* ticks is the coastal areas of Norway as far north as Brønnøysund in Nordland county (N 65°30') (Mehl, 1983). *A. phagocytophilum* infected lambs are commonly found in areas with ticks (Stuen and Bergstrom, 2001a). Climate change (i.e. warmer winter climate), changes in land use (i.e. bush encroachment) and an increase in the deer population are factors expected to increase the populations of ticks. An extension of the northern margin of the population distribution of *I. ricinus* and their spread to higher altitudes has been observed in Europe (Lindgren et al., 2000; Daniel et al., 2003; Danielova et al., 2006; Materna et al., 2008; Jore et al., 2011). This may cause increased challenges with TBF in Norway in the future.

The main consequence of an *A. phagocytophilum* infection in sheep is the ensuing immunosuppression that may lead to secondary infections. Direct loss of around 30 % of lambs in one flock due to *A. phagocytophilum* and secondary infections has been observed (Stuen and Kjølleberg, 2000). Indirect losses as reduced weight gain in infected lambs compared to non-infected lambs have also been observed (Stuen et al., 2002; Grøva et al., 2011). Impaired growth as a consequence of experimental infection with *A. phagocytophilum* has shown to last for several months after the primary infection (Stuen et al., 1992). Several genetic variants of *A. phagocytophilum* have been observed and these variants may show different clinical symptoms with varying hematological and serological responses (Stuen et

al., 2003; Granquist et al., 2010). However, it is mainly the primary *A. phagocytophilum* infection in sheep that is associated with disease problems (Øverås, 1962; Woldehiwet, 1983).

There are few efficient preventive measures against tick infestation and TBF. General advice is to clear vegetation, drain land, treat lambs regularly with acaricides and infect lambs early (Stuen et al., 1992; Sonenshine, 1993; Vatn et al., 2008). However, there is concern about lack of efficient and feasible preventive measures against TBF among sheep farmers in areas where *I. ricinus* is abundant. Clearing vegetation and drainage of land are rarely feasible due to vast grazing areas and high costs. It is shown that prophylactic treatment with long-term acting tetracycline against *A. phagocytophilum* improved weight gain in lambs on pasture (Brodie et al., 1988). Use of acaricides has also shown to lower the incidence of secondary bacterial infections, such as tick pyaemia (Brodie et al. 1986). Routine use of acaricides and antibiotics (i.e. tetracycline) is, however, not a sustainable measure and development of resistance may occur (Nolan et al., 1988; Beugnet and Chardonnet, 1995; Morgan et al., 2009). Practical obstacles and hence costs of regularly treating free ranged lambs with acaricides in vast forest or mountain areas throughout the grazing season also limits the facility and success of such treatment.

Experimental infection studies have shown that clinical response to *A. phagocytophilum* is less severe in young lambs compared with older lambs (Stuen et al., 1992; Stuen, 1993). This difference in response is explained by innate resistance to *A. phagocytophilum* infection (Stuen et al., 1992). When re-infected, the clinical response and symptoms are normally less severe than after primary infections (Øverås, 1962; Stuen et al., 2003). However, this may depends of the variant of *A. phagocytophilum* involved (Stuen et al., 2003). Thus, it may be hypothesized that lambs turned out to tick-infested pastures within the first week after birth will perform better than lambs being more than three weeks old at turn out.



The objective of the present study was to reveal possible effects on performance when turning young lambs, i.e.  $\leq$  one week old compared to older lambs i.e.  $\geq$  three weeks old, on to tick-infested pastures.

## Methods

### Study groups and treatment

A field study was conducted in 2008 and 2009 on two sheep farms in tick endemic areas where ticks and losses to TBF have been observed earlier. The farms were located on the south west coast of Norway in Sandnes municipality (58°53' N, 6°0'E) (farm A) and on the west coast of Norway in Tingvoll municipality (62°60'N, 8°15'E) (farm B). Lambs were expected to be exposed to ticks at turnout on these farms. Between 77- 90 lambs per farm and year, giving a total of 336 lambs, were included in the study (Table 1). On each farm the following three trial groups were established with respect to age of lambs at time of pasturing and time of birth:

- 3E: Lambs  $\geq$  three weeks old when turned out to pasture and early time of birth
- 1L: Lambs  $\leq$  one week old when turned out to pasture and late time of birth
- 3L: Lambs  $\geq$  three weeks old when turned out to pasture and late time of birth

To ensure equal conditions for tick infestation the 3E and 1L trial groups were turned on to pasture at the same point of time. Consequently, these trial groups differed in time of birth and in order to correct for a possible effect of time of birth we also established trial group 3L, allowing for comparison between different times of birth.

Number of lambs per ewe was assessed by embryo screening to assist selection and allocation of ewes to the trial groups. The three trial groups were made as equal as possible with respect to age and number of lambs per ewe.

All lambs were treated against coccidian infection (Baycox Sheep vet., Bayer Animal Health GmbH) one week after pasturing. Gastrointestinal (GI) parasites in lambs were regularly monitored by faecal egg counts. Preventive treatment against GI parasites (Valbazen vet., Pfizer), was conducted on all ewes before lambing and regularly, every four weeks on lambs on pasture during the spring grazing period. Lambs were not treated with acaricides in this study. Permission from the Norwegian Animal Research Authority was obtained.

#### **Recordings and blood sampling**

Recordings of tick-bites, clinical signs of disease (including rectal temperature measurements), mortality and weight were conducted every second week, making a total of four to five observations per lamb during the spring grazing period (approx. eight weeks). In addition, the mortality and weaning weight were recorded approximately 11 weeks after the spring grazing period.

Tick-bites were registered by observing the head, flank and abdomen for attached ticks. In the statistical analyses it was treated as a binary observation depending on the presence of ticks or not. Clinical signs of disease were registered by a veterinarian. The registration of rectal temperature was used as a binary observation of fever ( $\geq 40.5$  °C) or no fever ( $< 40.5$  °C) in the statistical analysis. The observation of other clinical signs of disease apart from fever, was used as binary observation of other clinical signs or not. Mortality was registered both at the end of the spring grazing period (approx. eight weeks) and at the end of the summer grazing period (approx. 11 weeks). In the statistical analysis, the observation of mortality was used as

a binary observation of dead or alive. A routine autopsy mortem was conducted on dead lambs that were retrieved.

Whole blood samples of all lambs were collected three times; during the first week after birth, at eight weeks of age (average 56 days (SD 7)) and at 4.5 months of age (average 136 days (SD 18)). Hereafter, the antibodies are referred to as maternal, spring and autumn antibodies, respectively.

EDTA blood was sampled from all lambs during the spring at average age of 56 days for blood smear preparation. In addition, whole blood and EDTA blood was sampled from all lambs with a temperature  $\geq 41^{\circ}\text{C}$  and/or showing other clinical signs of disease. Lambs showing clinical signs of disease were treated. The mean number of grazing days (SD) on spring pasture was: 38 (6), 43 (6) and 32 (5) for 3E, 1L and 3L trial groups, respectively.

### **Serology**

Whole blood was sampled and centrifuged for 10 min at 3500 ppm within 24 hours after sampling. Serum was extracted and kept frozen until analysis. Serum samples were analysed by an indirect fluorescent antibody assay (IFA) at the Swedish Veterinary Institute, Uppsala to determine the antibody titre to a horse strain of *A. phagocytophilum* (Artursson et al., 1999; Stuen and Bergstrom, 2001a). Unfortunately, no antigen from a sheep variant of *A. phagocytophilum* was available. Briefly a two-fold dilution of sera was added to slides precoated with *A. phagocytophilum* antigen (Protatek International and Organon Teknika). Bound antibodies were visualized by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-sheep immunoglobulin (Cappel, Organon Teknika). Sera were screened for antibodies at dilution 1:40 and a titre of 1.6 ( $\log_{10}$  of the titre 1:40) was regarded as positive. If positive, the serum was further diluted to obtain an end titre.

### **Blood smears**

Blood smears were prepared from EDTA blood samples for detection of *A. phagocytophilum* infection. Blood smears were stained with May-Grünwald Giemsa. Two hundred neutrophils were examined by light microscopy to investigate the number of cells containing *Anaplasma* inclusions. The percentages of infected neutrophilic granulocytes were calculated.

### **Spring infection**

A spring infection of *A. phagocytophilum* in lambs was determined by information from serology, temperature, and blood smears. Lambs were considered to have suffered a spring infection when serology showed a spring antibody titre of 2.8 ( $\log_{10}$  of titre 1:640) or higher. In addition, lambs with rectal temperature  $\geq 41^{\circ}\text{C}$  together with a positive blood smear during the spring period were also considered to have suffered from an *A. phagocytophilum* infection.

### **Statistical analyses**

#### Incidence of fever, tick counts, disease and mortality

Initially a multivariate logistic regression was carried out using the SAS-procedure PROC LOGISTIC (SAS, 1999) in order to reveal effects on incidence of spring infection, tick-bites, fever ( $\geq 40.5^{\circ}\text{C}$ ), other clinical signs, mortality on spring pasture and mortality on summer pasture between trial groups, years and farms, including sex, rearing rank and age of mother as explanatory effects. Explanatory effects were removed by a stepwise procedure if they did not show any significant effect. The final logistic regression model for the incidences listed above included trial groups, years and farms as independent explanatory variables, and rearing rank on incidence of clinical signs of disease between years.

#### Weight gain

Gompertz growth curve model as described by Lambe et al. (2006) was fitted to the live weight data from each lamb using the NLIN procedure of SAS (SAS, 1999). The Gompertz

growth curve equation used was:  $BW_t = Ae^{-e[B(C-t)/A]}$ , where A = final body weight (BW), kg; B = maximum average daily gain, kg/day; C = age at maximum average daily gain, days; t is the age in days and e is Euler's Number (e=2.71828). The lamb performance parameters that were subjected to statistical analysis using the MIXED procedure in SAS (SAS, 1999) where: Gompertz estimates A, B and C, weaning weight and daily weight gain from birth to weaning. Initial statistical model for performance parameters included age at weighing (as regression effect) (used only on the performance parameter weaning weight), sex, rearing rank and age of mother as fixed effects and farm and year as random effects. All interactions with fixed effects were included in the initial analyses, but were removed subsequently if they did not show significant effect on weaning weight. The final model used was:

$$\text{Model 2: } T_{ijklm} = \mu + A(x_{ijklm} - \bar{x}) + F_i * Y_j + TG_k + S_l + e_{ijklm};$$

Where T is the performance variable,  $\mu$  = mean, A is the regression of age at recording on the performance parameter weaning weight (days); FY = farm\*year (random), TG = trial group, S = sex, and e = residual error.

## Results

### *A. phagocytophilum* infection

The proportion of lambs per trial group having maternal, spring and autumn antibodies to *A. phagocytophilum* is illustrated in Figure 1. Overall, 91 % of the lambs had maternal antibodies to *A. phagocytophilum*. Furthermore, 93 % of the lambs had antibodies by the end of the grazing season. For all lambs, the mean (95% confidence interval(CI)) of maternal, spring and autumn antibody titre was 2.62 (2.57, 2.67), 2.77 (2.70, 2.84) and 3.14 (3.08, 3.19), respectively. Spring antibody titres were not significantly higher (P=0.08) than the maternal antibody titres, and they were both significantly lower (P<0.01) than the autumn

antibody titres. However, for lambs considered as spring infected, the mean (95% CI) titre of maternal, spring and autumn antibodies was 2.65 (2.57, 2.73), 3.11 (3.05, 3.16) and 3.20 (3.12, 3.28), respectively. Spring antibody titres for these lambs were significantly higher ( $P < 0.01$ ) from the maternal antibody titres.

#### Incidence of spring infection of *A. phagocytophilum*

After the spring grazing period, 73 % of all lambs had antibodies to *A. phagocytophilum*. However, actual spring infection was considered to be experienced by 48 % of the lambs (Table 1), of which titre level determined 45 % of lambs as spring infected and fever and blood smears determined three percentage as spring infected. There were significant differences in incidence of spring infection between years ( $P < 0.001$ ) and between trial group 3E and 3L ( $P < 0.001$ ) and 1L and 3L ( $P = 0.001$ ), respectively.

#### Incidence of tick-bites, clinical disease and mortality

There were incidences of tick bites, clinical signs (including fever) , and mortality in all three trial groups, on both farms in 2008 and 2009 (Table 2). However, there was no mortality during the spring period for lambs in the 1L group. Other clinical signs recorded were reduced condition, respiratory symptoms and lameness.

There was no significant effect of lamb age to a natural *A. phagocytophilum* infection on incidence of other clinical signs of disease or mortality between the trial groups (Table 2).

There were, however, significantly more tick-bites in the 3E vs 3L ( $P = 0.037$ ) and in the 1L vs 3L ( $P = 0.002$ ) trial group and higher incidence of fever in the 3E vs 3L trial group ( $P = 0.007$ ) (Table 2).

Higher incidence of other clinical signs of disease ( $P<0.001$ ) and summer mortality ( $P=0.005$ ) on Farm B compared to Farm A were observed, but there were no difference in tick-bites, fever and spring mortality between farms (Table 2).

In addition, there were significantly more tick-bites ( $P<0.001$ ), incidence of fever ( $P=0.012$ ) and other clinical signs of disease ( $P<0.001$ ) in 2008 than in 2009, but there was no difference in mortality between years (Table 2).

### Weight gain

Considering all lambs during the spring grazing period, the estimated final weight (A) and maximum growth rate (B) from the Gompertz weight curve were higher for 1L lambs than for 3L lambs (Table 3A), but not different to lambs in 3E. Growth rate between birth was higher ( $P<0.05$ ) for 1L lambs than for 3E and 3L lambs (Table 3A).

All lambs were, however, not exposed to an *A. phagocytophilum* infection during the spring grazing period. When analyzing only the lambs that were considered to have actually been infected with *A. phagocytophilum* during this period, the 1L lambs obtained higher daily weight gain and higher estimated maximum weight gain (Gompertz parameter B) than those in 3E and 3L (Table 3B). The faster growth in the 1L spring infected lambs is also illustrated in Figure 2.

The high proportion (93%) of lambs with autumn antibodies indicates that *A. phagocytophilum* infection did also occur after the spring grazing period. Of the lambs that were not considered to have been undertaking a spring infection, 86 % experience an *A. phagocytophilum* infection by the end of the summer grazing period, however, there was no effect between the trial groups on weight gain and estimated maximum weight gain (Gompertz parameter B) (Table 3C).

The lambs that showed clinical signs of disease were treated differently on the two farms: On farm A, lambs with clinical signs of disease, except for fever, were treated with penicillin (Penovet vet, Boehringer Ingelheim). On farm B, lambs with rectal temperature  $\geq 41^{\circ}\text{C}$  and/or observations of other clinical signs were treated with tetracycline (Terramycin Prolongatum vet., Pfizer).

## Discussion

The ewes in the present study were fed differently; 1L ewes were fed indoors for about one week before they were let onto spring pastures, while the 3E and 3L ewes were fed indoors for about three weeks, potentially effecting weight gain in lambs. Indoor feeding consisted of hay, silage and concentrates. Lamb growth, particularly during the first month of life, is known to be influenced by the milk production of the ewe (Nedkvitne, 1975) and indoor feeding with concentrates has shown to be inferior to high quality pastures (Milne et al., 1981; Nedkvitne, 1990). However, if ewes get access to high quality pasture within two to three weeks after lambing, the lamb growth is shown not to be considerably influenced by the indoor feeding period (Nedkvitne, 1990; Nedkvitne, 1998). Even if spring pastures may be more nutritious than preserved forage, climate condition on pasture might also be rough and increase the ewes and lambs energy requirement for maintenance (Hocquette et al., 1992; Steinheim et al., 2004). When kept indoors, all ewes were fed according to standard recommendation and it is unlikely that milk production and consequently daily growth rate of lambs was affected substantially due to the two weeks longer indoor feeding period (Nedkvitne, 1998) in 3E and 3L compared to 1L lambs. The overall mean weight gain at the age of 17 (SD 6) days was 329 g/day and there were no statistical differences between trial groups at this age. The national average weight gain of 40.5 days old lambs is 332 g/day (Animalia, 2009), and these lambs have been kept both indoor and on pasture. Although the



data are not directly comparable, they indicate that feeding during the first three weeks in the present trial groups have not differed greatly from the national average. Even though the weight gain from birth to weaning of spring infected lambs in 3E and 3L trial groups was significant lower ( $P < 0.05$ ) than the 1L trial group, this feeding difference will always be a practical consequence of adjusting pasturing to lamb age.

#### Spring infection of *A. phagocytophilum*

The exact time of spring infection with *A. phagocytophilum* is unfortunately not known in this study. It is possible that lambs infected during spring pasturing in the 1L trial group, were not actually infected at a younger age than 3E and 3L lambs. The significant differences between years and the trial groups 3E and 1L vs 3L in incidence of spring infection and tick-bites, illustrate that annual and seasonal variations in tick-infestation pressure in tick-endemic areas is present as has been shown in other studies (Jaenson and Tälleklint, 1996; Korenberg, 2000). As these seasonal and annual variations affect the incidence of tick-bites and *A. phagocytophilum* infection, the effect of lamb age at turnout on tick-infested pastures will also vary between years.

Determining a spring infection was done on the basis of serology, high fever ( $> 41^{\circ}\text{C}$ ) and blood smears. An infection with *A. phagocytophilum* is expected to give a serological response after 2-4 weeks (Woldehiwet and Scott, 1982; Stuen et al., 1992; Stuen et al., 2003; Stuen et al., 2011). Lambs in this study were on average 37.8 (SD 6.6) days on spring pasture when spring antibodies were measured. Lambs were expected to have developed antibodies at this point if they had been exposed to an *A. phagocytophilum* infection at the time of pasturing. But the spring infected lambs were not necessarily infected during the first weeks on pasture. However, approximately 14 days after pasturing 52% of all lambs in this study had tick-bites. In a study on Norwegian White sheep conducted in Norway, only two of 16 lambs were infected after 14 days on tick-infested pasture (Ladbury et al. 2008).

The level of maternal antibodies wains over time, and Stuen et al. (1992) estimated the half-life of antibodies to *A. phagocytophilum* to 17.5 days. It is also shown that even if antibodies wain over time they may persist for longer than six months in naturally infected lambs (Stuen et al. 2001). Maternal antibodies may therefore be present in the spring blood sampled at the average age of 56 (SD 7) days, but are expected to be present at a low level. Spring antibody titre of 2.8 may therefore reflect a spring infection with *A. phagocytophilum*. The mean titre of spring antibodies in lambs considered spring infected were higher than that of maternal antibodies, supporting that spring infection has occurred in these lambs. However, there are several different variants of *A. phagocytophilum* that may show low serological response to the antigen used (Stuen et al., 2003). Lambs infected with such variants, may be concealed in this study due to a low serological response. A PCR detection of *A. phagocytophilum* in the peripheral blood after turnout would allow a more precise determination of infection time (Engvall et al., 1996). Unfortunately, this was not done in the present study. However, the risk of having erroneously included lambs with only maternal antibodies in the spring antibody group is expected to be low.

#### Incidence of tick-bites, clinical disease and mortality

Even if the 1L spring infected lambs experienced a higher growth than the 3E and 3L lambs, clinical signs of disease occurred in the 1L lambs. Turning lambs onto tick-infested pastures not later than one week after birth does not fully protect lambs from TBF, which is in accordance to previous studies (Stuen et al., 1992; Stuen, 1993). In addition, lambs may be infected with several variants of *A. phagocytophilum* during the grazing period (Ladbury et al. 2008) and it is known that the different variants provide different degrees of protection to re-infection with other variants, which might have influenced the incidence of clinical disease and mortality (Stuen et al., 2003). The variants of *A. phagocytophilum* were not identified in this study, and the issue of re-infection was not investigated.

A potential shortcoming in the present study was the farm difference in treatment of lambs with clinical signs of disease. Lambs on farm A were treated with penicillin. *A. phagocytophilum* is not affected by this drug, and such treatment is not expected to affect the development of fever and the weight reduction caused by *A. phagocytophilum* (Foggie, 1951). However, secondary infections may also cause reduced weight gain and penicillin treatment may terminate such an effect. In contrast, lambs on farm B were treated with tetracyclines. It is shown that oxytetracycline treatment given in the acute stage of the infection may effectively terminate the development of fever, and weight reduction in *A. phagocytophilum* infected lambs (Stuen and Bergström, 2001b). There was a significantly higher incidence of fever, other clinical disease and mortality on Farm B. The consequence of the different treatment regimes in the present study is, however, unknown.

#### Weight gain

For lambs experiencing an *A. phagocytophilum* infection on spring pasture the higher growth rate between birth and weaning and higher maximum daily weight gain (Gompertz parameter B) in lambs pastured at  $\leq$  one week old (1L) compared to  $\geq$  three weeks old (3E and 3L) indicates that there is a positive effect of pasturing lambs at this young age in tick endemic areas. There was no weight effect between the trial groups to lambs that were not infected in the spring period. This supports our understanding that it was the lamb age at infection that affected weight gain. However, this may again depend on the *A. phagocytophilum* variants involved during the early and late grazing period (Ladbury et al. 2008).

Animal's individual growth performance and resulting body weight can be predicted by a number of nonlinear mathematical functions of age. Various growth functions have been used to predict animal growth, and there is no consensus to what function describes the growth in sheep most accurately (Sarmiento et al., 2006). However, the Gompertz weight curve function (Laird, 1965) has shown to correspond well with lamb growth curves (Sarmiento et al., 2006;

Malhado et al., 2009) and is designed to fit the non-linearity of normal animal weight gain curves.

The Gompertz weight curve function include all weight registrations available in this study and is expected to be a more robust measure when comparing weight gain than using only weight registrations at birth, spring and autumn. The weight gain curves of some of the lambs in this study may, however, deviate from normal weight gain curves as an *A.*

*phagocytophilum* infection may have restrained growth at some point of time. Hence, a concern is that the estimated Gompertz weight curve parameters do not express the weight curve differences between the trial groups in this study. However, the difference in weight gain between the trial groups estimated by Gompertz weight curve parameter B and the observed weight gain from birth to weaning in autumn correspond well. This may indicate that the Gompertz weight curve estimates are suited to express weight differences in the present study.

## **Conclusion**

Even if pasturing of one week old lambs on tick-infested pastures did not protect lambs against TBF, the significant higher weight gain in these lambs compared to lambs that were three weeks old at turnout indicates that pasturing of young lambs can be recommended to reduce the effect of *A. phagocytophilum* infection on weight gain. Note should however be taken on annual and seasonal variations in tick activity relative to lambing, *A.*

*phagocytophilum* variants involved and turnout time, as this probably will influence the effect of pasturing young lambs.

## **Competing interests**

The authors' have no competing interests.

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Table 1. Total number (n) of lambs per trial group, farm and year, and number and percentage of lambs experiencing a spring infection, tick-bites, fever( $\geq 40.5^{\circ}\text{C}$ ), other clinical signs, mortality in spring and mortality in summer per trial group; 3E ( $\geq$  three weeks old when turned out and early time of birth), 1L ( $\leq$  one week old when turned out and late time of birth) and 3L ( $\geq$  three weeks old when turned out and late time of birth), year and farm.

Trial group	Farm A		Farm B		Total %	
	2008	2009	2008	2009		
3E	Nr of lambs	30	30	30	26	
	Spring infection	14	18	28	8	59
	Tick-bite	29	18	30	16	80
	Fever	9	3	14	6	28
	Other clinical signs	3	1	15	1	17
	Mortality spring	0	1 <sup>a</sup>	0	0	1
	Mortality summer	1 <sup>b</sup>	0	2 <sup>c</sup>	2 <sup>d</sup>	4
1L	Nr of lambs	30	30	23	28	
	Spring infection	22	15	17	4	53
	Tick-bite	30	25	22	18	86
	Fever	6	9	4	4	21
	Other clinical signs	1	0	8	3	11
	Mortality spring	0	0	0	0	0
	Mortality summer	1 <sup>e</sup>	0	1 <sup>f</sup>	4 <sup>g</sup>	5
3L	Nr of lambs	28	30	24	28	
	Spring infection	5	13	15	1	31 <sup>ii</sup>
	Tick-bite	23	16	19	16	67 <sup>ii</sup>
	Fever	4	0	6	4	13 <sup>ii</sup>
	Other clinical signs	1	0	8	2	9 <sup>ii</sup>
	Mortality spring	1 <sup>h</sup>	0	1 <sup>i</sup>	0	2
	Mortality summer	0	0	1 <sup>j</sup>	4 <sup>k</sup>	5

<sup>a</sup> TBF and septicaemia (*Mannheimia haemolytica*). Approximately 61 days old.

<sup>b</sup> Helminthiasis (*Haemonchus contortus*).

<sup>c</sup> Run over by car. Missing on summer pasture.

<sup>d</sup> Missing on summer pasture.

<sup>e</sup> Helminthiasis (*Haemonchus contortus*).

<sup>f</sup> TBF and pneumonia.

<sup>g</sup> Missing on summer pasture.

<sup>h</sup> TBF and pyaemia (*Staphylococcus aureus*). Approximately 58 days old.

<sup>i</sup> Missing on spring pasture.

<sup>ii</sup> Lamb <sup>1</sup> not included as it had no recordings during the spring period.

<sup>j-k</sup> Missing on summer pasture.

Table 2. Incidence (%) and association of incidence of spring infection, tick-bites, fever, other clinical disease, mortality on spring pasture and mortality on summer pasture between the trial groups: 3E ( $\geq$  three weeks old when turned out and early time of birth), 1L ( $\leq$  one week old when turned out and late time of birth) and 3L ( $\geq$  three weeks old when turned out and late time of birth), year and farm.

Factor	Level	Incidence (%)	Odds Ratio (95%CI)	P-value
<b>Spring infection</b>				
trial group	3E	0.586a	3.1 (1.8, 5.4)	<0.001
	1L	0.527a	2.5 (1.4, 4.3)	0.001
	3L	0.312b	1	-
year	2008	0.620	3.1 (2.0, 4.9)	<0.001
	2009	0.343	1	-
farm	Farm A	0.490	1.1 (0.7, 1.7)	0.664
	Farm B	0.465	1	-
<b>Tick-bites</b>				
trial group	3E	80.2a	1.9 (1.0, 3.5)	0.037
	1L	86.4a	3.0 (1.5, 5.9)	0.002
	3L	67.3b	1.0	-
year	2008	92.7	8.8 (4.3, 18.0)	<0.001
	2009	63.4	1.0	-
farm	Farm A	79.2	1.1 (0.7,1.9)	0.636
	Farm B	76.1	1.0	-
<b>Fever</b>				
trial group	3E	27.6a	2.6 (1.3, 5.2)	0.007
	1L	20.9ab	1.8 (0.9, 3.7)	0.114
	3L	12.7b	1.0	-
year	2008	26.1	2.0 (1.2, 3.5)	0.012
	2009	15.1	1.0	-
farm	Farm A	17.4	0.7 (0.4, 1.1)	0.127
	Farm B	23.9	1.0	-
<b>Other clinical signs of disease</b>				
trial group	3E	17.2a	1.9 (0.8, 4.1)	0.124
	1L	10.9a	1.1 (0.5, 2.6)	0.844
	3L	9.1a	1.0	-
year	2008	21.8	8.6 (3.4, 21.7)*	<0.001
	2009	4.1	1.0	-
farm	Farm A	3.4	0.1 (0.0, 0.3)	<0.001
	Farm B	23.3	1.0	-
<b>Spring mortality</b>				
trial group	3E	0.9a	0.5 (0.0, 5.3)	0.540
	1L	0a	0	0.962
	3L	1.8a	1.0	-
year	2008	1.2	2.1 (0.2, 23.5)	0.543
	2009	0.6	1.0	-
farm	Farm A	1.1	1.8 (0.2, 19.9)	0.638
	Farm B	0.6	1.0	-

Summer mortality				
trial group	3E	4.3a	0.9 (0.3, 3.3)	0.919
	1L	5.5a	1.2 (0.4, 4.0)	0.781
	3L	4.5a	1.0	-
year	2008	3.6	0.6 (0.2, 1.7)	0.365
	2009	5.8	1.0	-
farm	Farm A	1.1	0.1 (0.0, 0.5)	0.005
	Farm B	8.8	1.0	-

<sup>a,b</sup> Prevalence values for trial groups followed by different letters were statistically different ( $P < 0.05$ )

\* Rearing rank was included as an explanatory effect

Table 3: Estimated LSMMeans (s.e.) for the parameter estimates (A, B, and C) obtained by Gompertz function, weight gain from birth to autumn and weaning weight for the three trial groups: 3E ( $\geq$  three weeks old when turned out and early time of birth), 1L ( $\leq$  one week old when turned out and late time of birth) and 3L ( $\geq$  three weeks old when turned out and late time of birth)

A) All lambs					
Trial group	Gompertz parameter estimates			Observed	
	A. kg	B. g/day	C. day	Growth birth– autumn. g/day	Weaning weight. kg
3E	44.4(3.27) ab	336(8.9) ab	38.1 (3.18) a	240 (13.9) b	37.1 (1.83) a
1L	47.4 (3.29) a	347 (9.0) a	38.5 (3.22) a	256 (13.9) a	37.0 (1.81) a
3L	41.8 (3.29) b	323 (9.0) b	34.7 (3.23) a	238 (13.9) b	35.3 (1.78) b
B) Spring infected lambs					
Trial group	Gompertz parameter estimates			Observed	
	A. kg	B. g/day	C. day	Growth birth– autumn. g/day	Weaning weight. kg
3E	44.3 (3.15)ab	334 (9.8) b	37.5 (2.92) a	240 (14.1) b	37.9(1.85)b
1L	46.9 (3.19) a	358(10.2) a	36.4 (3.02) a	267 (14.3) a	38.5(1.80) a
3L	42.5 (3.37) b	310(11.9) c	34.5 (3.04) a	239 (14.9) b	36.1 (1.80) b
C) Not spring infected lambs					
Trial group	Gompertz parameter estimates			Observed	
	A. kg	B. g/day	C. day	Growth birth– autumn. g/day	Weaning weight. kg
3E	43.4 (4.31) a	340(10.3) a	37.5 (5.63) a	238 (13.6) a	37.0 (1.68) a
1L	47.7 (4.17) a	336(10.0) a	40.3 (5.16) a	246 (13.4) a	35.1 (1.60) b
3L	41.0 (3.74) a	329 (9.0) a	34.2 (5.03) a	237 (13.0) a	34.6 (1.50) b

<sup>a,b,c</sup> Estimated LSMMeans values followed by the same letters were not statistically different ( $P > 0.05$ )

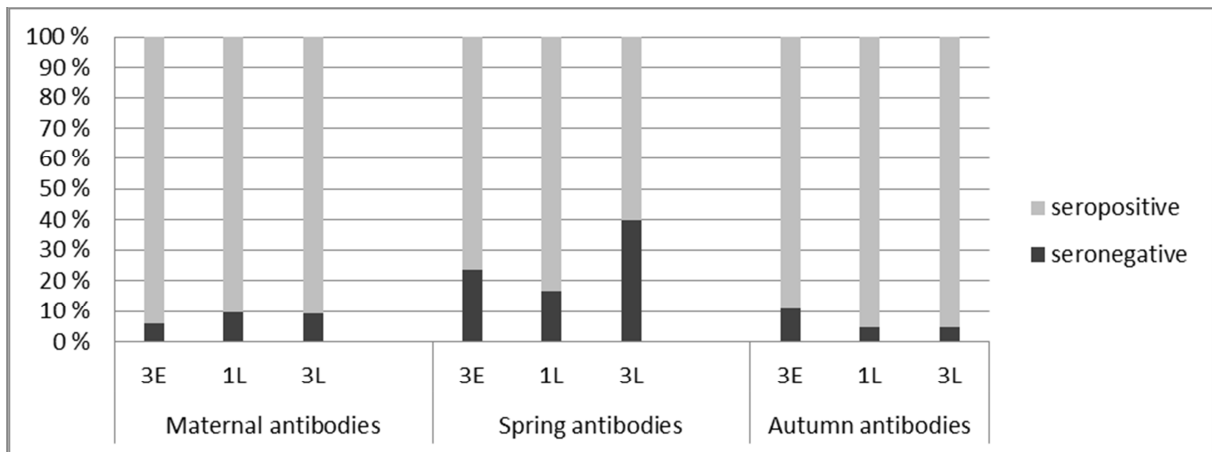


Figure 1. Proportion of seropositive and seronegative lambs per trial group: 3E ( $\geq$  three weeks old when turned out and early time of birth), 1L ( $\leq$  one week old when turned out and late time of birth) and 3L ( $\geq$  three weeks old when turned out and late time of birth) for maternal-, spring- and autumn antibodies.

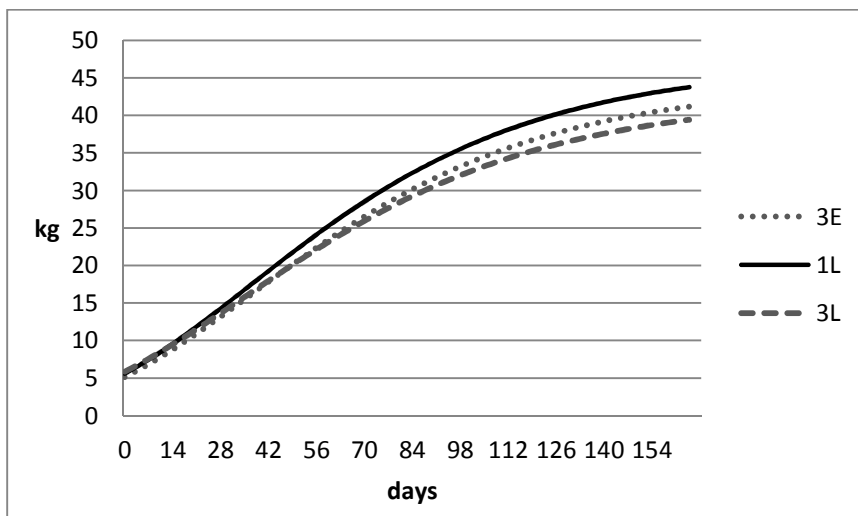


Figure 2. Predicted Gomperts weight curve for spring infected lambs in the three trial group: 3E ( $\geq$  three weeks old when turned out and early time of birth), 1L ( $\leq$  one week old when turned out and late time of birth) and 3L ( $\geq$  three weeks old when turned out and late time of birth)

# Paper III



RESEARCH

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# A comparative study of clinical manifestations, haematological and serological responses after experimental infection with *Anaplasma phagocytophilum* in two Norwegian sheep breeds

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## Abstract

**Background:** It has been questioned if the old native Norwegian sheep breed, Old Norse Sheep (also called Norwegian Feral Sheep), normally distributed on coastal areas where ticks are abundant, is more protected against tick-borne infections than other Norwegian breeds due to a continuously high selection pressure on pasture. The aim of the present study was to test this hypothesis in an experimental infection study.

**Methods:** Five-months-old lambs of two Norwegian sheep breeds, Norwegian White (NW) sheep and Old Norse (ON) sheep, were experimentally infected with a 16S rRNA genetic variant of *Anaplasma phagocytophilum* (similar to GenBank accession number M73220). The experiment was repeated for two subsequent years, 2008 and 2009, with the use of 16 lambs of each breed annually. Ten lambs of each breed were inoculated intravenously each year with 0.4 ml *A. phagocytophilum*-infected blood containing approximately  $0.5 \times 10^6$  infected neutrophils/ml. Six lambs of each breed were used as uninfected controls. Half of the primary inoculated lambs in each breed were re-challenged with the same infectious dose at nine (2008) and twelve (2009) weeks after the first challenge. The clinical, haematological and serological responses to *A. phagocytophilum* infection were compared in the two sheep breeds.

**Results:** The present study indicates a difference in fever response and infection rate between breeds of Norwegian sheep after experimental infection with *A. phagocytophilum*.

**Conclusion:** Although clinical response seems to be less in ON-lambs compared to NW-lambs, further studies including more animals are needed to evaluate if the ON-breed is more protected against tick-borne infections than other Norwegian breeds.

## Background

Tick-borne fever (TBF) caused by the bacteria *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) is an endemic disease of sheep in tick (*Ixodes ricinus*) infested areas of Norway. Natural infection with *A. phagocytophilum* has been reported in humans and a variety of domestic and wild animal species [1]. TBF is seldom fatal, unless complicated by secondary infections. However, TBF causes immunosuppression, leaving sheep

vulnerable to secondary infections, such as tick pyaemia caused by *Staphylococcus* spp. [2], and *Pasteurella* (*Mannheimia*) septicaemia [3,4]. Complications also include abortion in pregnant ewes [5], reduced milk yield in cattle [6], impaired spermatogenesis in rams [7], and reduced weight gain in lambs [8]. The infection in sheep may cause considerably animal welfare problems and has for decades been one of the main scourges for the Norwegian sheep industry [8].

A serological survey in sheep indicated that *A. phagocytophilum* infection is widespread along the coast of southern Norway. However, clinical TBF was only diagnosed in half of these seropositive sheep flocks [9].

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The reason for this diagnostic deficit may be attributed to the existence of genetic variants of the agent causing different clinical symptoms and immunological reactions [2,10]. Based on a 16S rRNA gene sequence study, it has recently been shown that genotypes of *A. phagocytophilum* may co-exist in the same sheep flock and even in the same animal [11]. The geographical distribution of these variants is however unknown.

The management of sheep flocks may vary considerably in Norway. While the dominant Norwegian sheep breed, Norwegian White (NW) sheep, are normally housed indoors during the winter season and treated regularly against ticks and gastro-intestinal parasites, the Old Norse (ON) sheep breed can be on pasture the whole year around with limited parasitic treatment [12]. A British study indicated earlier, that there may be a difference in breed susceptibility to *A. phagocytophilum* infection [13]. Based on a continuous high selection pressure and possible also breed differences, it has been hypothesized that the ON-breed is more protected against tick-borne infections than other Norwegian breeds. The aim of the present study was therefore to test this hypothesis by experimental infection, to compare the clinical, haematological and serological responses to *A. phagocytophilum* infection in two Norwegian sheep breeds.

## Materials and methods

### Source of *Anaplasma phagocytophilum* and DNA sequencing

Blood samples were collected from a flock of Norwegian sheep known to be infected with *A. phagocytophilum*. Based on partial sequencing of the 16S rRNA gene, a variant of *A. phagocytophilum* was found in one lamb, similar to GenBank accession number M73220 [11]. Both EDTA and heparinised blood samples were taken from the infected lamb. The EDTA blood samples were used to measure haematological values and to prepare blood smears. The absolute number of infected cells per unit volume was determined by multiplying the total number of neutrophils per unit volume by the percentage of infected neutrophils counted on a May-Grünwald Giemsa stained blood smear. The heparinised blood was stored at  $-70^{\circ}\text{C}$  in 5 ml aliquots with 10% dimethyl sulphoxide (DMSO) as cryoprotectant without any propagation in cell culture or sequence passage through other sheep.

### Animals, experimental design, and haematology

A total number of 64 lambs, 5 months old, were used in this trial, 32 lambs of the NW-breed and 32 of the ON-breed. The experiment was approved by the National Animal Research Authority (Norway). The study was conducted for two following years, 2008 and 2009, with the yearly use of 16 lambs of each breed. The ON-

lambs came from two different sheep herds in Rogaland county, lambs from one herd were used each year, while all NW-lambs belonged to the experimental sheep flock at the Department of Production Animal Clinical Sciences. All ON-lambs were adapted and housed at the department three to four months before start of the experimental period. None of the lambs had previously been exposed to pasture with *I. ricinus* and were kept indoors during the whole experimental period of four to five months. The lambs of each breed were grouped in infected and controls, according to equal distribution of sex and mean live weight. Ten lambs of each breed were inoculated intravenously (day 0) with 0.4 ml of the above mentioned DMSO-stabilate of *A. phagocytophilum* using one aliquot for each breed. The infectious blood contained approximately  $0.5 \times 10^6$  infected neutrophilic granulocytes/ml. Six lambs in each group were left as uninfected controls. Nine weeks (2008) and twelve weeks (2009) after the primary inoculation, five of the earlier inoculated lambs of each breed were selected by random sampling and re-challenged with the same infectious dose of the homologous variant.

The lambs were observed at least twice a day. Rectal temperature was measured daily in all lambs throughout the experiment. The incubation period was defined as the period between inoculation and the first day of fever ( $\geq 40.0^{\circ}\text{C}$ ), and the duration of fever was recorded as the number of days with elevated body temperature ( $\geq 40.0^{\circ}\text{C}$ ). The magnitude of fever of each lamb was estimated from the area of plots of daily temperature on 5 mm grids and calculated according to the Trapezium Rule [14]. For this purpose  $40^{\circ}\text{C}$  represented the baseline.

Daily EDTA blood samples were collected during the fever period, and on a weekly basis after the fever had subsided. In addition, EDTA blood samples were later collected from individual lambs when intermittent rectal temperatures above  $40.0^{\circ}\text{C}$  were recorded. Total and differential leucocyte counts were determined electronically (ADVIA<sup>®</sup>, Bayer) and blood smears were prepared and stained with May-Grünwald Giemsa. Four hundred neutrophils were examined on each smear by light microscopy, and the number of cells containing *Anaplasma* inclusions was recorded. The infection rate (percentage of infected neutrophils) was calculated. The body weight of all lambs was measured weekly, during the whole experimental period. The average live weights ( $\pm$  SD) of the lambs at the start of the experiment were  $52.8 \pm 6.86$  (NW-2008),  $44.9 \pm 6.10$  (NW-2009),  $28.8 \pm 4.10$  (ON-2008), and  $21.3 \pm 3.44$  (ON-2009), respectively.

### Extraction of DNA and real time PCR for the identification of *A. phagocytophilum* positive samples, targeting *msp2* (p44)

In order to investigate *A. phagocytophilum* infection of non-reactive lambs, the ON-lambs (2009) were analysed



for *Anaplasma* -DNA by PCR [15]. Briefly, an automated isolation procedure based on magnetic bead technology was performed by the application of the MagNA Pure LC instrument (Roche) and the MagNA Pure LC DNA Isolation Kit I Blood Cells High Performance (Roche). EDTA blood samples from the inoculated animals were thawed at room temperature and 200  $\mu$ l blood was transferred to the DNA isolation procedure according to the instruction manual (Roche). The isolated DNA was eluted with 100  $\mu$ l low salt buffer and stored at -20°C awaiting PCR analysis. The concentration of DNA in each sample was determined by OD<sub>260</sub> spectrophotometry (GeneQuant II, Pharmacia Biotech, Cambridge, UK). The samples were diluted 1:100 before PCR analysis.

The primers were Ap MSP2 252 5' ACAGTC-CAGCGTTTAGCAAGA and Ap MSP2 459 5' CAC-CACCAATACCATAACCA, amplifying a product of 208 bp at the N-terminal hyper variable region of the *msp2(p44)* expression site. The primers were manufactured by TIB Molbiol (Germany). A Light Cycler 480 instrument (Roche) was used for the real-time PCR analysis. A total of 96 well white plates were loaded with a reaction mix consisting of 0.5  $\mu$ l (5  $\mu$ M) ApMSP2 252 primer, 0.5  $\mu$ l (5  $\mu$ M) ApMSP2 459 primer, 1.5  $\mu$ l RNase free H<sub>2</sub>O, 5  $\mu$ l LightCycler 480 DNA SYBR Green I Master and 2.5  $\mu$ l sample. Plates were sealed by sealing foil and centrifuged at 1200 rpm for two minutes. Samples and non-template controls were run in duplicates on each plate. The C<sub>q</sub> values (quantification cycle) were determined by the 2<sup>nd</sup> derivative maximum method and verified by melting point analysis (T<sub>m</sub>) [15].

### Serology

Sera were collected at days 0, 7, 14, 21, 28, 42, and 63 (only first challenge) after each inoculation and analysed using an indirect immunofluorescence antibody assay (IFA) to determine the antibody titre to *Ehrlichia equi* [9,16]. Briefly, two-fold dilutions of sera were added to slides precoated with *A. phagocytophilum* (formerly *E. equi*) antigen (Protatec, St. Paul, Minn.). Bound antibodies were visualized by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-sheep immunoglobulin (Cappel, Organon Teknika, West Chester, PA). Sera were screened for antibodies at dilution 1:40. If positive, the serum was further diluted and retested. A titre of 1.6 (log<sub>10</sub> reciprocal of 1:40) or more was regarded positive.

### Statistics

Statistical calculations were done using Statistix, version 4.0 (Analytical Software), and a two-sample *t* test was used to analyse clinical, haematological and serological variables. A *P* value of < 0.05 was considered significant.

## Results

### Clinical parameters, haematology, PCR-detection and serology

In general, a one to two days period with reduced appetite was observed in the NW-lambs infected with *A. phagocytophilum*, while infected ON-lambs showed no signs of clinical illness.

In 2008, all inoculated lambs from both groups developed fever. Significant differences in duration of fever and magnitude of fever were recorded between the two sheep breeds (Table 1). In addition, infection rate was significantly different on days 3, 6, 7 and 8 (Table 2). However, no significant difference was observed in the serological response for the first 63 days (Figure 1). Recurrent fever periods of one to two days duration, were observed in five (NW) and four (ON) lambs, respectively.

After re-challenge on day 63 (2008), clinical signs were not observed, and only one lamb of each breed reacted with a detectable infection rate (Table 3). No significant difference in the antibody titre was observed in the challenged and unchallenged lambs in either of the two breeds (data not shown).

In contrast, only seven of the infected ON-lambs in 2009 reacted with fever after the primary inoculation (Table 1). The most remarkable finding was that the incubation time in these seven ON-lambs varied from six to thirteen days (mean  $\pm$  SD: 9.6  $\pm$  2.44) and the fever period lasted for one to ten days (mean  $\pm$  SD: 5.4  $\pm$  3.11). Significant differences in incubation period, maximum temperature, magnitude of fever and weight gain were recorded between the two sheep breeds (Table 1). Two infected ON-lambs had fever for one day, with a max. temperature of 40.0°C and 40.4°C, respectively. The infection rate was displaced by several days and therefore difficult to compare from day to day (Table 3). However, the max. infection rate was not significantly different (Table 1). In addition, no significant difference between the two breeds was observed in the serological reaction for the first 84 days, except for day 14 (*p* < 0.05) (Figure 2).

The three ON-lambs that did not show any clinical reaction after *A. phagocytophilum* inoculation were also found negative by blood smear examination and serology. In addition, real time PCR for identification of *A. phagocytophilum* positive samples was negative in these three lambs on days 3-15 (Table 4). In the infected ON-lambs, *Anaplasma*-DNA was detected as early as five days before inclusions were observed by blood smear microscopy. Recurrent fever periods of one to two days duration, were observed in two lambs of each breed, respectively.

After re-challenge on day 84, all five ON-lambs reacted with a clinical response. One of the lambs, which did not react after the first inoculation, now responded with a typical TBF- infection, i.e. incubation

**Table 1 Mean and standard deviation ( $\pm$  SD) of different clinical variables in 5-month-old lambs of two Norwegian Sheep breeds, Norwegian White (NW) and Old Norse (ON), respectively, infected with one variant of *A. phagocytophilum***

	2008			2009		
	NW	ON	P-value	NW	ON <sup>#</sup>	P-value
Incubation period (days)	3.0 $\pm$ 0.0	2.9 $\pm$ 0.3	ns	3.0 $\pm$ 0.0	9.6 $\pm$ 2.4	p < 0.0001
Max. temp. (°C)	41.87 $\pm$ 0.18	41.64 $\pm$ 0.11	ns	41.86 $\pm$ 0.13	40.96 $\pm$ 0.54	p < 0.001
Duration of fever (days)	8.9 $\pm$ 2.77	5.6 $\pm$ 1.02	p < 0.01	8.0 $\pm$ 3.07	5.4 $\pm$ 3.11	Ns
Magnitude of fever (mm <sup>2</sup> )	912 $\pm$ 158	503 $\pm$ 89	p < 0.001	737 $\pm$ 231	275 $\pm$ 174	p < 0.001
Nadir of neutropenia (<0.7 $\times$ 10 <sup>9</sup> litre <sup>-1</sup> )	0.22 $\pm$ 0.06	0.22 $\pm$ 0.08	ns	0.26 $\pm$ 0.10	0.28 $\pm$ 0.15	Ns
Duration of neutropenia (days)	9.1 $\pm$ 2.38	10.1 $\pm$ 2.21	ns	6.7 $\pm$ 2.24	5.7 $\pm$ 2.49	Ns
Max. infection rate (%)	49.0 $\pm$ 3.46	44.6 $\pm$ 5.66	ns	52.9 $\pm$ 1.79	46.6 $\pm$ 9.76	Ns
Weight loss (%) <sup>a</sup>	- 10.7 $\pm$ 3.21	-9.5 $\pm$ 4.63	ns	-9.6 $\pm$ 2.81	1.4 $\pm$ 4.20	p < 0.001

<sup>a</sup> weight loss was measured one week after inoculation; ns - not significant.

<sup>#</sup> only seven lambs were infected.

The study was performed in two subsequent years, 2008 and 2009, respectively. Ten lambs in each group were inoculated. A two-sample *t* test was used for statistical analysis.

period: 3 days, max. temperature: 40.9°C; magnitude of fever: 273 mm<sup>2</sup> and duration of fever: 7 days. In addition, blood smears displayed cytoplasmic inclusions (max. infection rate: 60%), duration of neutropenia (7 days) and a serological response (titre value on day 98: 1/1280). All the other four ON-lambs also reacted with fever of 1-2 days duration, cytoplasmic inclusions (max. infection rate: 6-56%), and neutropenia (duration: 3-5 days) after re-challenge. A significant increase in antibody titre (p < 0.05) was observed in the re-challenged lambs compared with the unchallenged lambs on days 98, 105 and 112, respectively (data not shown). In contrast, the five NW-lambs that were re-challenged on day 84, did not react with a clinical response, cytoplasmic inclusions or a titre increase (Table 3, Figure 2). Clinical symptoms, haematological reaction or seroconversion were not detected in the control lambs during the experimental period.

Comparison of results from 2008 and 2009, showed no difference in clinical signs or haematological reaction between the NW-lambs (two-sample *t* test). The infection rate was significantly different only on days 3 and 4 (p < 0.001). However, significant differences were

observed in the antibody titres on days 14, 21, 28, 42 and 63 (p < 0.001). Significant differences in the antibody responses were also observed between the ON-lambs in 2008 and 2009, i.e. on days 14, 21, 28, 42 and 63 (p < 0.01), respectively. In addition, variation in clinical and haematological responses and infection rates were observed within the ON-breed, such as in the maximum temperature (p < 0.05), magnitude of fever (p < 0.01), duration of neutropenia (p < 0.01) and weight gain (p < 0.001) (two-sample *t* test).

#### Weight gain

In 2008, the mean daily weight gains ( $\pm$  SD) for the first nine weeks in the infected and control lambs were 180  $\pm$  21.2 g/d (NW- infected), 204  $\pm$  35.9 g/d (NW-control), 155  $\pm$  47.8 g/d (ON-infected), and 164  $\pm$  44.5 g/d (ON-control), respectively. No significant differences between the infected and control lambs within each breed were observed.

Similarly, the mean daily weight gains ( $\pm$  SD) for the first twelve weeks in the infected and control lambs in 2009 were 137  $\pm$  30.0 g/d (NW-infected), 165  $\pm$  16.1 (NW-control), 78.1  $\pm$  29.0 g/d (ON-infected), and 105  $\pm$  18.9 (ON-control),

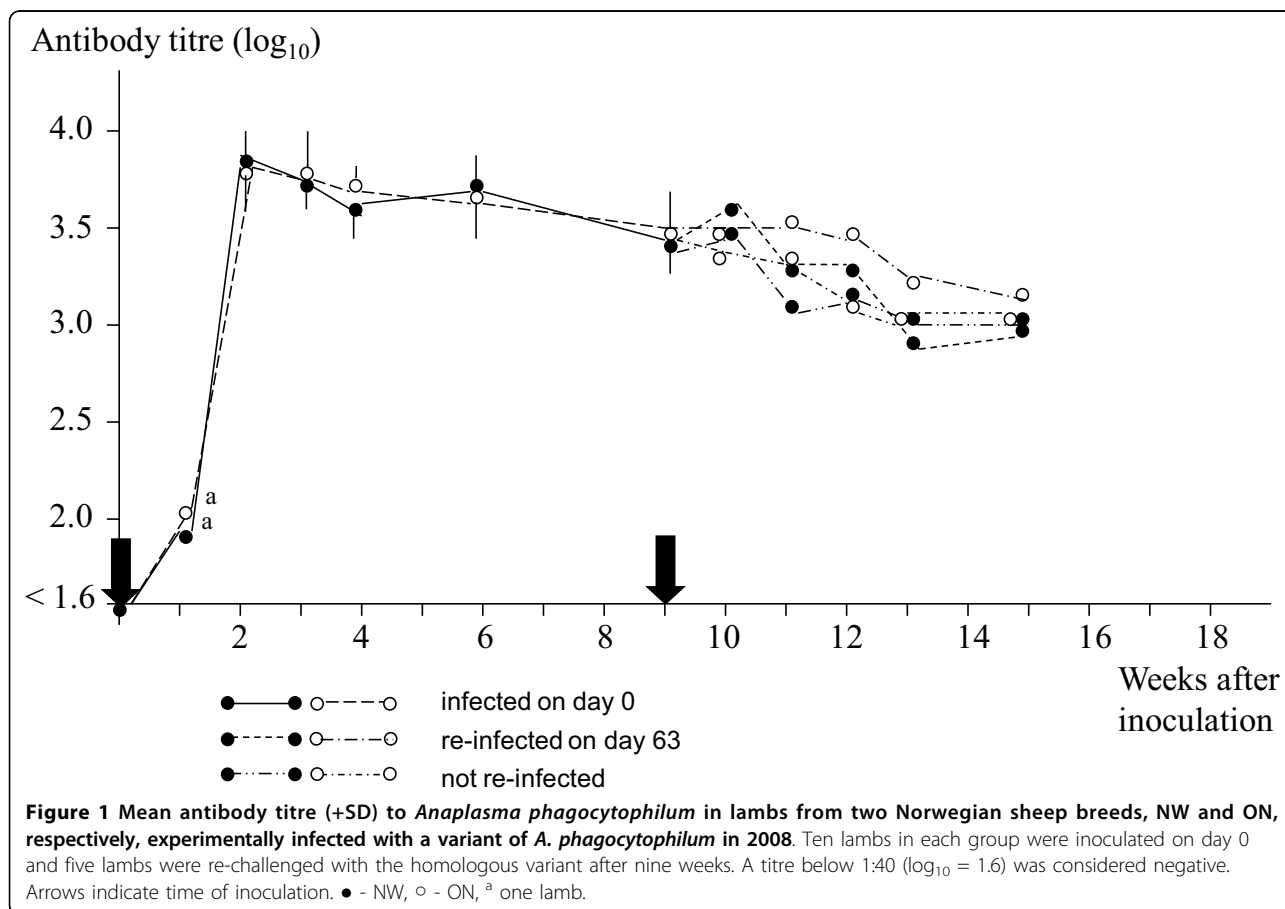
**Table 2 Mean percentage of infected neutrophils observed in groups of ten lambs<sup>5</sup> of two different breeds of Norwegian sheep, Norwegian White (NW) and Old Norse (ON), respectively, inoculated with one variant of *A. phagocytophilum***

Sheep breeds	Days after inoculation/challenge										
	3	4	5	6	7	8	10	11	12	13	17
NW - 2008	43.2 <sup>#</sup>	45.4	36.4	41.2 <sup>#</sup>	37.8 <sup>##</sup>	32.0 <sup>#</sup>	22.6	3.3	<1	-	-
ON - 2008	35.1	44.4	35.8	32.8	25.0	11.7	2.1	1.9	<1		
NW - 2009	7.9	57.4	45.2	39.8	35.2	27.6	3.3	<1	-		
ON - 2009	-	-	-	-	13.0 <sup>a</sup>	26.0 <sup>b</sup>	47.5 <sup>b</sup>	24.5 <sup>c</sup>	32.1 <sup>d</sup>	22.9 <sup>d</sup>	3.8 <sup>b</sup>

<sup>5</sup> only seven ON-2009 lambs were infected.

Two-sample *t* test (only 2008): p < 0.05<sup>#</sup>, p < 0.001<sup>##</sup>, <sup>a</sup>3 lambs, <sup>b</sup>4 lambs, <sup>c</sup>6 lambs, <sup>d</sup>7 lambs.

The study was performed in two subsequent years, 2008 and 2009, respectively.



respectively. No significant differences between the infected and control lambs within each breed were observed.

### Discussion

In the present study, only a limited number of animals of each breed were included. Earlier experimental studies in NW-lambs indicate a variation in clinical, serological and haematological reactions to an *A. phagocytophilum* infection [8]. In order to evaluate the susceptibility in outbred sheep breeds, more animals should have been included. In addition, the study should have been conducted within

one year to exclude annual variation in experimental conditions. However, due to limited number of lambs available, housing facilities and other practical reasons these criteria were difficult to fulfil.

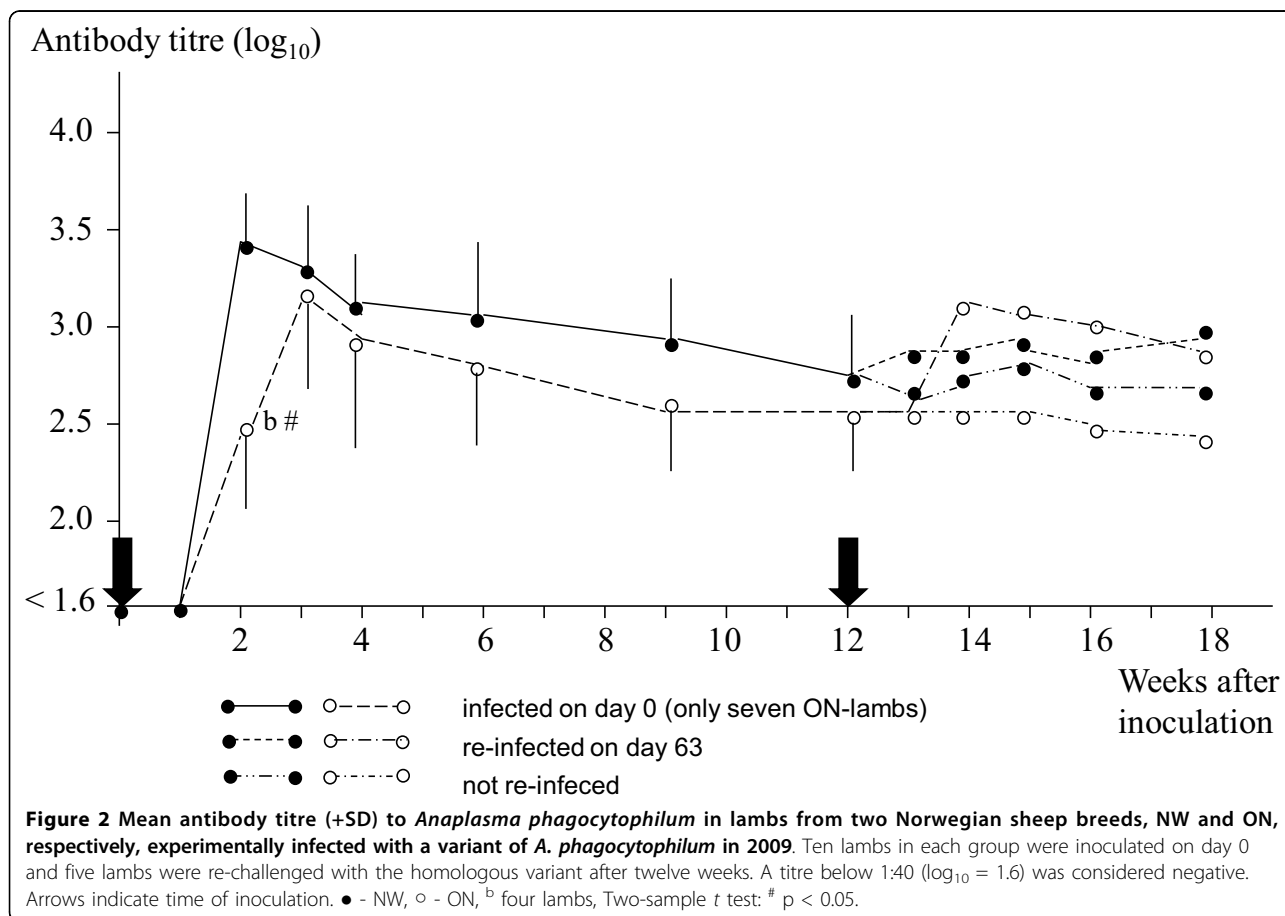
Significant differences in the clinical reaction and infection rate to a primary *A. phagocytophilum* infection were detected in the two sheep breeds. In 2008, no differences in the haematological and the serological reaction were observed. In contrast, marked differences were observed in 2009, where seven ON-lambs reacted with a prolonged incubation period and no evidence of clinical response in three lambs. Breed differences in response to *A. phagocytophilum* have, as mentioned earlier, been shown among British sheep breeds. Blackface sheep reacted less severely to tick-borne fever than other breeds and their crosses, however this seems not entirely attributable to past ancestral exposure to the pathogen [13]. The reason and implication of these breed differences need further elucidation.

A significant difference in the antibody titre response was recorded between the two years. This may be due to different batches of antigen used, since the sensitivity of the IFA-test may vary between batches (Sandstedt K, personal information). The sensitivity of the present

**Table 3** Mean percentage of infected neutrophils observed in groups of five lambs inoculated with one variant of *A. phagocytophilum* nine weeks (NW/ON-2008) and twelve weeks (NW/ON-2009) after the first inoculation, respectively

Sheep breeds	Days after inoculation/challenge							
	3	4	5	6	7	8	10	11
NW - 2008	-	<1	-	-	-	-	-	-
NW - 2009	-	-	-	-	-	-	-	-
ON - 2008	-	-	10 <sup>a</sup>	24 <sup>a</sup>	16 <sup>a</sup>	1 <sup>a</sup>	-	-
ON - 2009	13.5 <sup>b</sup>	30.6	25.4	20.8	11.5 <sup>c</sup>	5.5 <sup>b</sup>	<1 <sup>a</sup>	-

<sup>a</sup> 1 lamb, <sup>b</sup> 2 lambs, <sup>c</sup> 4 lambs.



antibody test may have been increased by use of a more proper antigen, i.e. an ovine variant of the bacterium. Strong serological cross-reactions between all members of the *A. phagocytophilum* group have been reported, but the titre response to a heterologous variant is

normally less than against a homologous variant [17]. Unfortunately, *E. equi* was the only antigen available for use in the present study.

**Table 4** Detection of *A. phagocytophilum* infection by real-time PCR, targeting *msp-2(p44)* in ON-lambs

ON-lambs 2009	Day 0	Day 3	Day 5	Day7	Day10	Day15
1	-	-	+	+	+ <sup>#</sup>	+ <sup>#</sup>
2	-	-	-	nd	+	+ <sup>#</sup>
3	-	-	+	+ <sup>#</sup>	+ <sup>#</sup>	+ <sup>#</sup>
4	-	-	-	-	-	-
5	-	-	-	nd	+ <sup>#</sup>	+ <sup>#</sup>
6	-	-	-	-	-	-
7	-	-	+	+	+ <sup>#</sup>	+ <sup>#</sup>
8	-	-	-	-	-	+ <sup>#</sup>
9 <sup>##</sup>	-	-	-	-	-	-
10	-	-	-	+ <sup>#</sup>	+ <sup>#</sup>	+ <sup>#</sup>

- negative, + positive, nd - not done.

<sup>#</sup> positive by light microscopy.

<sup>##</sup> this lamb became infected with *A. phagocytophilum* only after re-challenge on day 84.

All lambs were inoculated experimentally with *A. phagocytophilum* infected blood on day 0.

In contrast to NW-lambs, marked differences in clinical and haematological reactions were observed between years in the ON-lambs. The reason for these differences among ON-lambs is unknown. The animals were from two different geographical areas with no direct relationship, and the lambs had been adapted to the same experimental conditions for several months. In addition, earlier studies indicate that the individual diversity is not significantly different within the two sheep breeds involved [18].

In 2009, seven ON-lambs reacted with a long incubation period, a low maximum temperature and a short duration of fever [8]. These reactions may indicate an innate resistance to *A. phagocytophilum*. However, an innate immune response in infected lambs may be inefficient since *A. phagocytophilum* have lost all genes for synthesis of LPS and most genes for biosynthesis of peptidoglycans [19,20].

Earlier studies indicate that the amplitude of clinical and haematological reaction is independent of the dose of the *A. phagocytophilum* inoculum, however, the incubation period may be longer with a low infection dose.

As little as one *A. phagocytophilum* infected cell is enough to transmit the infection [21]. In the present study, the inoculation dose was approximately  $0.2 \times 10^6$  infected neutrophils, which should be more than sufficient to create a clinical reaction. However, reduction of the infectious dose in the aliquot used during freezing and thawing cannot be excluded.

Most of the inoculated blood used in 2009 must have been infectious. However, if a small infection dose in the thawed aliquot was divided into ten infectious doses, some of these batches may have lacked infectivity. This statement is supported by the fact that *Anaplasma*-DNA was not detected in the three non-responsive lambs during the first fourteen days. The detection threshold of real-time PCR used was 10 copies of DNA [15]. These three lambs were also confirmed seronegative. In addition, when one of these three lambs was challenged on day 84, it was fully susceptible to the infection. Further studies are needed to elucidate the reason for the delayed onset of clinical reactions in ON-lambs, experimentally infected with *A. phagocytophilum*.

In the present study, the daily weight gain in lambs varied between the two years. This variation may be due to the quality of the roughage involved. Unfortunately, the quality of the silage used was not measured. The daily weight gain between the infected and non-infected animals of each breed was not significantly different. One study indicates that an early *A. phagocytophilum* infection will have a negative effect on the autumn live weight of lambs [22]. However, in that study the infected lambs were grazing on pasture, while in the present trial the experimental lambs were housed indoors under favourable environmental conditions.

Resistance to experimental re-infections rises with increasing frequency of challenge [23]. Earlier experimental studies have shown that the immunity after primary *A. phagocytophilum* infection varies and that sheep may resist homologous challenge for a period from a few months to more than one year [2,24-26]. In the present study, only the ON-lambs (2009) reacted with clinical symptoms, detectable infection rate and an increase in antibody titre after re-infection, indicating that they were not fully protected after the primary infection. The implications of relapses of clinical symptoms and bacteraemia during the persistent period are unknown. The effects of re-infections are usually less severe than the primary reaction. However, it depends on the variants of *A. phagocytophilum* involved [10].

Earlier investigations have shown that lambs are susceptible to secondary infections, especially in the bacteraemic and neutropenic periods of TBF [14,27-29]. In the present study, only the rate of bacteraemia is different between the two breeds. To evaluate if *A. phagocytophilum* infected ON-lambs are more resistant to other

infectious agents compared to NW-lambs needs further investigation, since factors such as management and other external stress factors may be important to the outcome of infection [30].

## Conclusion

The present results indicate a difference in fever response and infection rate between breeds of Norwegian sheep after experimental infection with *A. phagocytophilum*. Whether these differences are due to genetic resistance that can be implemented in a breeding programme to increase the resistance against TBF, needs further exploration. However, studies including more animals are needed to evaluate if the ON-breed is more protected against tick-borne infections than the NW-breed. In addition, analyses of diseased and fatal cases of TBF must be performed in order to elucidate differences in pathogenicity and protective immunity between Norwegian sheep breeds.

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## Authors' contributions

SS, LS, IO and HS have designed the experimental study. SS performed the experimental study, carried out the statistical analysis and drafted the manuscript. EGG carried out the molecular genetic analysis. KS performed the serology. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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# Paper IV





# Heritability of lamb survival on tick-exposed pastures

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## Abstract

Grøva, L.<sup>1</sup>, Olesen, I.<sup>2,3</sup> and Ødegård, J.<sup>3</sup> (<sup>1</sup> Bioforsk Organic Food and Farming Division, Gunnars vei 6, 6630 Tingvoll, Norway, <sup>2</sup> Norwegian University of Life Sciences, Post-box 5003, 1432 Ås, Norway, and <sup>3</sup> Nofima, Post-box 5010, 1432 Ås, Norway). Heritability of lamb survival on tick-exposed pastures. *Acta Agric. Scand., Sect. A, Animal Sci.*

Sheep farming in Norway is based on grazing on unfenced rangeland and mountain pastures in summer. A major welfare and economic issue in Norwegian sheep farming is the increase in lamb loss on such pastures. Tick-borne fever (TBF), caused by the bacteria *Anaplasma phagocytophilum* and transmitted by the tick *Ixodes ricinus*, is pointed out as one important challenge facing lambs during summer grazing. Breeding for disease resistance has shown to be a possible preventive measure against numerous diseases in many farmed species. The current knowledge on genetic resistance to ticks and tick-borne diseases is to a large extent based on work done on cattle and the cattle tick *Rhipicephalus microplus* (formerly *Boophilus microplus*) in tropical environments. Reported estimates of heritability for tick infestation vary from very low to moderate in cattle, and it has been suggested that there may be breed differences in susceptibility to TBF in sheep and cattle. The objective of this study was to identify possible within breed genetic variation in lamb survival on tick-exposed pasture. Data on lambs within the normal distribution area of ticks, from flocks participating in ram circles with recordings in the Norwegian Sheep Recording System and registered with cases of TBF or using prophylactic treatment against ectoparasites at any time in 2000 to 2008 were included, making a total of 126,732 lambs with an average mortality of 3.8%. The data were analysed by a linear model, and the estimated heritability (on the observed scale) for the direct genetic effect on lamb survival was  $0.220 \pm 0.005$ . The estimated maternal variance in proportion to phenotypic variance of lamb survival was  $0.000 \pm 0.0004$  indicating that maternal environment had very little effect on lamb survival. This indicates potential for genetic

selection to improve survival in the studied population. The heritability cannot, however, be accurately attributed to resistance to *A. phagocytophilum* infection and TBF as the infection status of the lambs is unknown. Improved registrations of TBF and tick-exposure by farmers in ram circles in the Norwegian Sheep Recording System is therefore recommended to enable the use of such data for studying genetic variation in robustness in environments with and without ticks and a possible implementation in selection programs.

## **Background**

Sheep farming in Norway is based on grazing on unfenced rangeland and mountain pastures during summer. A major welfare and economic issue in Norwegian sheep farming is the increase in lamb loss on such pastures, from about 4.8% in 1990 to 8.1% in 2010 (Norwegian Forest and Landscape Institute, 2011). The main welfare challenges that lambs are faced with during the summer grazing period in Norway are predators, blow flies (*Myiasis externa*), alveld (a photosensitivity disease) and tick-borne fever (TBF) (Dahl and Lystad, 1998; Norges forskningsråd, 2005). The causes of death and reduced welfare on summer pasture do vary between areas and farms, but the exact causes of deaths of lambs on pasture are seldom documented as only a few lost lambs are found (Warren and Mysterud, 1995; Warren et al., 2001; Hansen, 2006; Grøva, 2010). Survival is further a complex trait, affected by a number of different behavioural and physiological factors (Dwyer and Lawrence, 2005).

Ticks and TBF have received increased attention during the recent years. TBF is caused by the bacteria *Anaplasma phagocytophilum*, transmitted by the tick *Ixodes ricinus*, and may cause direct (lamb deaths) and indirect losses (reduced growth) in sheep farming. A direct loss of approximately 30% lamb mortality in a flock due to *A. phagocytophilum* infection has been observed (Stuen and Kjølleberg, 2000). *A. phagocytophilum* infected lambs are commonly found on the south west coast of Norway where ticks are widespread (Tambs-Lyche, 1943;

Mehl, 1983; Stuen, 2001; Jore et al., 2011). Climate change (i.e., warmer winter climate), changes in land use (i.e. bush encroachment) and an increase in populations of wild ungulates are factors expected to increase the populations of ticks (Sonenshine, 1992), giving rise to concerns that challenges with TBF will increase in Norway in the coming years. TBF is proposed to be an explanatory factor of the increased lamb loss observed (Norwegian Forest and Landscape Institute, 2011) on summer pastures in some areas in Norway (Grøva, 2010; Grønn Forskning, 2010).

Disease control measures in general often include both prevention and treatment; i.e., vaccination, culling, prophylactic treatment, environmental adjustments and selection of resistant animals. Genetic variation in disease resistance allows for selection of genetically resistant animals and thereby improves the disease control. Genetic variation in robustness is shown in many farmed species, where numerous diseases are involved (Bishop et al., 2010) i.e., gastrointestinal nematode infections (Stear, 1994), mastitis (Rupp, 2009), footrot (Raadsma, 1994) (Raadsma, 2011), ectoparasites (flies and lice) (Raadsma, 1991) (Pfeffer, 2007) and scrapie (Dawson, 1998) in sheep. Genetic variation in resistance may reflect the ability to tolerate infection and to resist infection and/or further disease. The current knowledge on genetic resistance to ticks and tick-borne diseases is to a large extent based on work done on cattle and the cattle tick *Rhipicephalus microplus* (previously *Boophilus microplus*) in tropical regions (Regatiano and Prayaga, 2010). Various levels of host resistance to tick-infestation are found to occur in different breeds of cattle and have been implemented in breeding schemes (Utech et al., 1978) (Lemos et al., 1985) (Prayaga, 2003). Variation in host resistance to ticks is also reported within cattle breeds and heritability estimates of tick infestation varied from 0.13 to 0.44 (Regatiano and Prayaga, 2010).

Quantifying deaths caused by *A. phagocytophilum* infection is difficult as most lost lambs are lost on summer pasture where few dead lambs are retrieved (Dahl and Lystad, 1998; Warren

et al., 2001). It is shown that a number of factors affect survival of sheep on summer pasture in Norway; i.e., birth weight, sex, age of mother and type of birth and rearing affect lamb survival on summer pasture in Norway (Steinheim et al., 2008). Heritability for survival of lambs on pasture have been estimated in various studies and range from very low to moderate (0.002 to 0.33 (Brien et al., 2010; Burfening, 1993; Hatcher et al., 2010; Matos, 2000; Sawalha et al., 2007). Heritability of survival of lambs exposed to ticks, has, to our knowledge, not been estimated. Hence, the objective of this study is to identify possible within-breed genetic variation in lamb survival on tick-exposed pastures.

## **Methods**

### **Data**

The data used for this analysis was extracted from the Norwegian Sheep Recording system (Animalia, 2011). Data from lambs of the Norwegian White (NW) sheep breed in flocks participating in the National sheep breeding program through ram circles (group of farms collaborating to progeny test and select rams for further elite matings) in counties in South-western Norway within the normal distribution area of ticks (Mehl, 1983) were extracted. Further selection criteria used for extracting flocks were registrations of tick-borne fever and/or registrations of preventive treatment against ectoparasites in 2000 – 2008. Preventive treatment against ectoparasites may include preventive treatment of other parasites than ticks, particularly against blow flies. All farms included were within the counties of: Aust-Agder, Vest-Agder and Rogaland in Norway. Restrictions made in this original dataset were as follows: Lambs that died during the indoor period before being turned out on pasture and lambs that were registered as lost on summer pasture to predators were excluded from the dataset. Only litters born between 1<sup>st</sup> March and 30<sup>th</sup> June and litters born by ewes that were 1 – 7 years old were included. Lambs that were hand-reared, added for fostering by another ewe or from litters with more than five lambs were excluded. These restrictions removed

approximately 14 % of the originally extracted dataset. Lambs killed by predators constituted 0.2 % of the extracted dataset. The final dataset included 126,732 individuals from 44 farms in the years 2000-2008 making a total of 383 farm-year combinations (Table 1). The survival rate was 96.2 % in the final dataset, and varied between 100 – 78 % (Table 1). Lambs were assigned dead or alive based on registrations from the National sheep recording system on time and cause of death, as well as information on weaning weight. Number of lambs for each year, sex, rearing rank (where the first digit is birth rank (single, twin, triplet, etc.) and the second digit is rank when turned out on pasture) and age of mother is presented in Table 2.

### **Study animals**

Sheep farming in Norway is commonly characterized by indoor feeding during winter with mating in early winter and lambing indoor in early spring. The sheep are usually released to spring pasture soon after lambing. After a short period on spring pasture about 80 % (1,8 million) of the lambs and sheep in Norway (Garmo and Skurdal, 1998) are released on unfenced rangeland pasture without daily herding, but with weekly attention by the farmer (LMD, 2005). During the summer period, lambs are at foot of their mother until autumn (September-October), when they are collected and weaned at an average age of 139 days (Animalia, 2011). The sheep breed involved in this study was the Norwegian White Sheep (NW) which is a composite meat-type breed originating from crosses of British breeds and older Norwegian sheep types (Dahl and Lystad, 1998).

### **Statistical analysis**

Survival on pasture was initially analysed with both linear and threshold animal models using Gibbs sampling with the DMU software (Madsen and Jensen, 2010). In these analyses, additive genetic variance was estimated with an algorithm based on parental breeding values only. The latter method is appropriate for avoiding problems typical seen in animal threshold models and is expected to improve mixing properties of quantitative genetic analyses based

on Gibbs sampling in general (Ødegård, et al., 2010). However, due to the low mortality observed in the current dataset (only 3.8%) there is still a high risk of bias due to extreme category problems for threshold models in general (high probability of having only surviving lambs within one or more fixed effect classes). Hence, in the final analysis, only the linear model was considered. The model was as follows:

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{Z}_a\mathbf{a} + \mathbf{Z}_c\mathbf{c} + \mathbf{e},$$

where  $\mathbf{y}$  is a vector of binary observations of survival (i.e., dead = 0 and alive = 1) observed at the end of summer grazing period,  $\mathbf{b}$  is a vector of fixed effect solutions,  $\mathbf{a} \sim N(\mathbf{0}, \mathbf{A}\sigma_A^2)$  is a vector of additive genetic effects,  $\mathbf{c} \sim N(\mathbf{0}, \mathbf{I}\sigma_C^2)$  is a vector of maternal effects (i.e., the effect of the maternal environment),  $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I}\sigma_E^2)$  is a vector of random residual effects, the matrices  $\mathbf{X}$ ,  $\mathbf{Z}_a$ ,  $\mathbf{Z}_c$  are the appropriate incidence matrices,  $\mathbf{A}$  is the numerator relationship matrix,  $\mathbf{I}$  is an identity matrix of appropriate size,  $\sigma_A^2$  is the additive genetic variance,  $\sigma_C^2$  is the maternal variance and  $\sigma_E^2$  is the residual variance. Fixed effects included in the initial model were flock×year of birth (383 combinations), sex (male, female), rearing rank (11, 21, 22, 31, 32, 33, 41, 42, 43, 44); age of mother in classes (1, 2, 3, 4 and 5+), and a regression of day of birth within year. Fixed effects and dispersion parameters were all assigned flat priors. Sex, rearing rank and age of mother were not significant and therefore not included in the final model.

Based on the convergence diagnostics of the Gibbs chain (Raftery and Lewis, 1992), a chain consisting of 55,000 rounds were chosen, for which the initial 5000 rounds were discarded as burn-in. Parameter estimates are in the following presented as posterior means and posterior standard deviations.

## Results and discussion

### Fixed effects

Day at birth within year had a significant effect on lamb survival (estimate- 0.0004, SD 0.00007). The fixed effects of sex, rearing rank and age of mother did not have a significant effect on survival in this study. In other studies of lambs on pastures in Norway, significant effects of birth weight, sex, litter size and age of mother was found on mortality (Warren and Mysterud, 1995; Warren et al., 2001; Vatn et al., 2007; Steinheim et al., 2008). This may indicate that the current trait, survival on tick-exposed pastures, behaves differently from survival on summer pastures in general.

### Genetic parameters

The heritability, estimated by a linear model, for the direct effect on lamb survival was  $0.220 \pm 0.005$  (Table 3). The estimated maternal variance as proportion of phenotypic variance of lamb survival was close to zero ( $0.0007 \pm 0.0004$ ). Hence, there is likely substantial genetic variation in survival of lambs on these pastures, but differences in maternal environment are seemingly a rather non-significant source of variation.

For a linear model applied to a binary trait these estimates are expected to be lower than the corresponding estimates on the underlying scale, especially at extreme frequencies. Hence, a threshold model is statistically and biologically more correct than a linear model for binary traits. However, it is also more subject to bias as a result of extreme-category problems, which typically occurs at extreme frequencies and in animal threshold models with one observation per animal. Anyhow, the chosen algorithm for sampling of additive genetic variance is adapted to solve the latter problem by sampling additive genetic variance based on parental breeding values only (Ødegård et al., 2010). However, as the frequency of the binary trait is rather extreme (0.96) there is still a risk that a considerable number of dams will be evaluated based on surviving offspring only. For this reason, the proposed method for sampling of



genetic variance may still be prone to bias under an animal threshold model applied to such extreme frequencies. Furthermore, these extreme frequencies dramatically increase the risk of extreme category problems due to the fixed effect structure (i.e., for some of the flock×year combinations all lambs likely survive). Consequently, a linear model was chosen for analysis of the current data set.

#### Data quality

The estimated heritability for lamb survival suggests that there is a potential for improving lamb survival in the studied population. This heritability cannot, however, be directly attributed to resistance to *A. phagocytophilum* infection as the status of infected or not is unknown and exposure of lambs to ticks is unconfirmed.

When studying disease resistance, using field records allow for large data sets with phenotypes of interest, but the data are often noisy, particularly when it comes to bacterial or viral diseases where the actual exposure or prevalence of disease is not known. It is however suggested that such imperfect data are not necessarily fatal flaws for demonstrating host genetic differences in resistance (Bishop and Woolliams, 2010). Bishop and Woolliams (2010) showed that imperfect exposure to infection likely reduces the estimated heritability and that the impact of this incomplete exposure is relatively linearly related to the prevalence of disease or infection. They further show that including knowledge on prevalence of disease can improve heritability estimates. However, variation in field survival may also include other factors not attributed to the disease in question, and may cause field survival to be less relevant with respect to the disease in question.

Knowing that tick activity and *A. phagocytophilum* prevalence in lambs show annual and seasonal variations (Jaenson and Tälleklint, 1996; Korenberg, 2000), a flock of sheep having a single indication of presence of ticks and/or TBF within a single year class (as may be the case in this material) does not necessarily imply that they are equally exposed to ticks over all

years as assumed here. It is however shown that *A. phagocytophilum* infected sheep are commonly found in areas with ticks (Stuen and Bergstrom, 2001; Grøva, 2009; Grøva et al., 2011a), and it is suggested that the prevalence of *A. phagocytophilum* infection in flocks that are exposed to ticks is high (Stuen and Bergstrom, 2001a; Grøva et al., 2011a) and even up to 100% (Ogden et al., 1998). This increases the potential for field survival on tick-exposed pasture as an indicator trait for resistance to *A. phagocytophilum* infection.

Previous studies of lamb survival show that the heritability of lamb survival on summer pasture range from low to moderate, and some studies concluded that survival cannot be improved by selection on this trait (Burfening, 1993; Hatcher et al., 2010), while other studies suggest that survival can be improved by selection (Sawalha et al., 2007; Brien et al., 2010).

As mentioned, survival is a complex trait, and in relation to TBF a number of questions arise. *A. phagocytophilum* infection does not necessarily cause clinical symptoms and TBF. Stress factors such as reduced general condition, poor management and weather conditions as well as other infections may affect the outcome of an infection with *A. phagocytophilum*. Also, a number of different variants of *A. phagocytophilum* exist and cause different clinical signs with varying haematological and serological response (Stuen et al., 2003; Ladbury et al., 2008). Some variants of *A. phagocytophilum* give a low response and are not associated with lamb losses. TBF alone is not likely to be fatal, but the immunosuppression that follows TBF frequently leads to secondary infections, and these secondary infections are often decisive to the final outcome of *A. phagocytophilum* infections and TBF (Stuen, 2003). A lamb loss as high as 30% has been observed in one flock due to *A. phagocytophilum* infection (Stuen and Kjølleberg, 2000). Lamb survival on tick-exposed pasture may therefore be considered a trait for genetic study of resistance to *A. phagocytophilum* infection. However, in the current study only 3.8 % of the lambs were lost during summer pasture (range from 0 to 22% for different

flock-year of birth classes). Lamb mortality may also contribute to a natural selection on this trait, but with the low mortality rates in this dataset selection will be weak.

Preventive treatment against ectoparasites was imposed as a criterion for the data extract in this study. However, it does not necessarily imply treatment against ticks only, as it may also reflect treatment against blow flies (*Myiasis externa*). Still, having selected flocks within the distribution area of ticks it was considered likely that lambs in these flocks were in fact exposed to ticks. Anyhow, the use of preventive treatment with acaricides may have affected the prevalence of *A. phagocytophilum* infection and TBF and hence resulting survival of the lambs in this study. It is shown that the use of acaricides can reduce production loss (Mitchell, 1986; Hardeng, 1992) and a resulting reduce in lamb loss would also affect the heritability estimated here (as animals with poor resistance may survive as a result of prophylactic treatment). However, Grøva et al. (2011a) found no correlation between prophylactic treatment against ticks and lamb loss or prevalence of *A. phagocytophilum* infection in flocks, and it is shown that lambs seroconvert from not infected to infected with *A. phagocytophilum* even if acaricides are used (Hardeng, 1992).

The variance due to maternal effects on lamb survival on tick-infested pasture was close to zero (0.0007) in this study. The importance of maternal effects on lamb survival have however been shown in other studies (Matos, 2000; Sawalha et al., 2007), but again survival in this study behaved differently. Older lambs released on tick infested pasture (> three weeks old) seem to be more susceptible to *A. phagocytophilum* infection than lambs that are younger (<one-two weeks old) (Stuen et al., 1992; Stuen, 1993; Grøva et al., 2011b). This age effect is common to lambs with the same dam, and is a potential factor of maternal variance (if not accounted for). A breed difference in grazing behaviour of sheep is shown (Steinheim et al., 2005), and there might be differences in grazing behaviour of ewes potentially affecting the risk of tick-exposure in lambs. Further, behaviour of the ewe and lamb and variation in the

ewes ability to care for the lamb has shown to have an effect on lamb survival (Larsgard and Olesen, 1998) (Dwyer and Lawrence, 2005). Our study, however, showed negligible effect of mother on survival.

Health registrations in the Norwegian Sheep Recording System are recorded by 45% of the member flocks that participate in ram circles (Animalia, 2011). This lack of recording restricts the possibility to use these data for studying the genetics of disease resistance in general, and for its use in selective breeding. The data extracted for this study also clearly indicates that we only managed to extract a small proportion of farms in tick-exposed areas as numerous farms in other counties than Aust-Agder, Vest-Agder and Rogaland are also within the distribution area of ticks (Mehl, 1983) and hence with frequent *A. phagocytophilum* infections (Stuen and Bergstrom, 2001; Stuen, 2003; Grøva, 2009; Grøva et al., 2011a). Improved disease registrations, with registrations of cases of TBF and information on preventive treatment against ticks, by farmers in ram circles in the Norwegian Sheep Recording System is recommended to enable the use of such data for more accurate studies of genetic variation in robustness on tick-exposed pastures and possibly resistance to TBF. Further, comparing performance and ranking of rams with progeny in environments with and without ticks might allow for estimation of genotype by environment interactions for resistance to TBF. Also, it is suggested that possible breed differences in response to *A. phagocytophilum* infection should be studied more in details (Stuen et al., 2011). Covariation between resistance to TBF and growth should also be investigated as weaning weight is heavily emphasized in the current selection program for sheep in Norway.

## **Conclusions**

This study shows that there is genetic variation in lamb survival on tick-exposed pasture. The direct heritability estimate of lamb survival was 0.22 and implies a potential for improving survival by selection. The heritability of lamb survival cannot, however, be directly attributed

to resistance to *A. phagocytophilum* infection as the actual infection status of the lambs in this study is unknown. However, breeding for improved survival on pasture in general is of interest. Knowing that the genetic interpretation of disease data using a linear model is likely to give an underestimated heritability compared with the underlying scale and that registrations in the National Sheep Recording System are uncompleted one may expect that there is potential to improve the quality of both analysis and recording system. Improved registrations of TBF and tick-exposure by farmers might enable the use of such data for studying genetic variation in robustness on tick-exposed pastures and a possible implementation in selection programs.

### **Competing interests**

The authors' have no competing interests.

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**Table 1 Descriptive statistics of the data I**

Individuals (no)	126 732
Farms (no)	44
Lambs/farm (average, min and max)	2880 (362 - 5907)
Sires total (no)	5551
Lambs/sire (average, min and max)	23 (1 - 381)
Average survival (%) (min and max farm-year)	96.2 % (100 – 78)
Day of birth within year (mean (SD))	107 (10)

**Table 2 Descriptive statistics of the data II: Total number of lambs born by year, sex, type of 'birth rank - rearing rank' at pasturing and age of dam.**

Year	Sex	Birth rank - rearing rank	Age of mother
2000	male	11	1
2001	female	12	2
2002		13	3
2003		21	4
2004		22	5
2005		23	6
2006		31	7
2007		32	
2008		33	
		41	
		42	
		43	
		51	
		52	
		53	

**Table 3**

**Estimates of variance components, heritability ( $h^2$ ) and maternal variance as proportion of phenotypic variance ( $c^2$ ) for survival on tick infested summer pasture with standard errors**

Parameter	Estimate	s.e.
Direct additive variance	0.0075	0.00019
Maternal variance	0.00003	0.00001
Phenotypic variance	0.0342	0.00015
Residual variance	0.0266	0.00017
Heritability ( $h^2$ )	0.2200	0.00501
Maternal variance as proportion of phenotypic variance ( $c^2$ )	0.0007	0.00037