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Temporal and spatial variation in *Anaplasma phagocytophilum* infection in Swedish moose (*Alces alces*)

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SUMMARY

The occurrence of *Anaplasma phagocytophilum* was investigated in spleen and serum samples from Swedish moose (*Alces alces*) in southern Sweden (island and mainland). Samples were analysed for presence of *A. phagocytophilum* DNA by real-time PCR ($n=263$), and for *Anaplasma* antibodies with ELISA serology ($n=234$). All serum samples had antibodies against *A. phagocytophilum*. The mean DNA-based prevalence was 26.3%, and significant ($P<0.01$) temporal, and spatial variation was found. Island moose had significantly ($P<0.001$) higher prevalence of *A. phagocytophilum* DNA than moose from the mainland areas. Two samples were sequenced to determine genetic variation in the 16S rRNA and *groESL* genes. Genetic sequence similarity with the human granulocytic anaplasmosis agent, equine granulocytic ehrlichiosis agent, and different wildlife-associated *A. phagocytophilum* variants were observed in the 16S rRNA and *groESL* genes. Our study shows that moose are exposed to *A. phagocytophilum* in Sweden, and represent a potential wildlife reservoir of the pathogen.

Key words: *Ehrlichia*, epidemiology, HGA, moose, PCR, serology, tick-borne fever.

INTRODUCTION

Anaplasma phagocytophilum is a widespread tick-borne pathogen reported to infect various species

of wild and domestic animals [1]. It is a Gram-negative, obligate intracellular bacterium of the order Rickettsiales that infects white blood cells (neutrophils, eosinophils, monocytes). The primary vectors are ticks including *Ixodes ricinus* (Europe [1]), *Ixodes scapularis* (eastern United States), and *Ixodes pacificus* (western United States) [2]. In cattle and sheep, *A. phagocytophilum* infection can cause tick-borne fever (TBF), a disease characterized by

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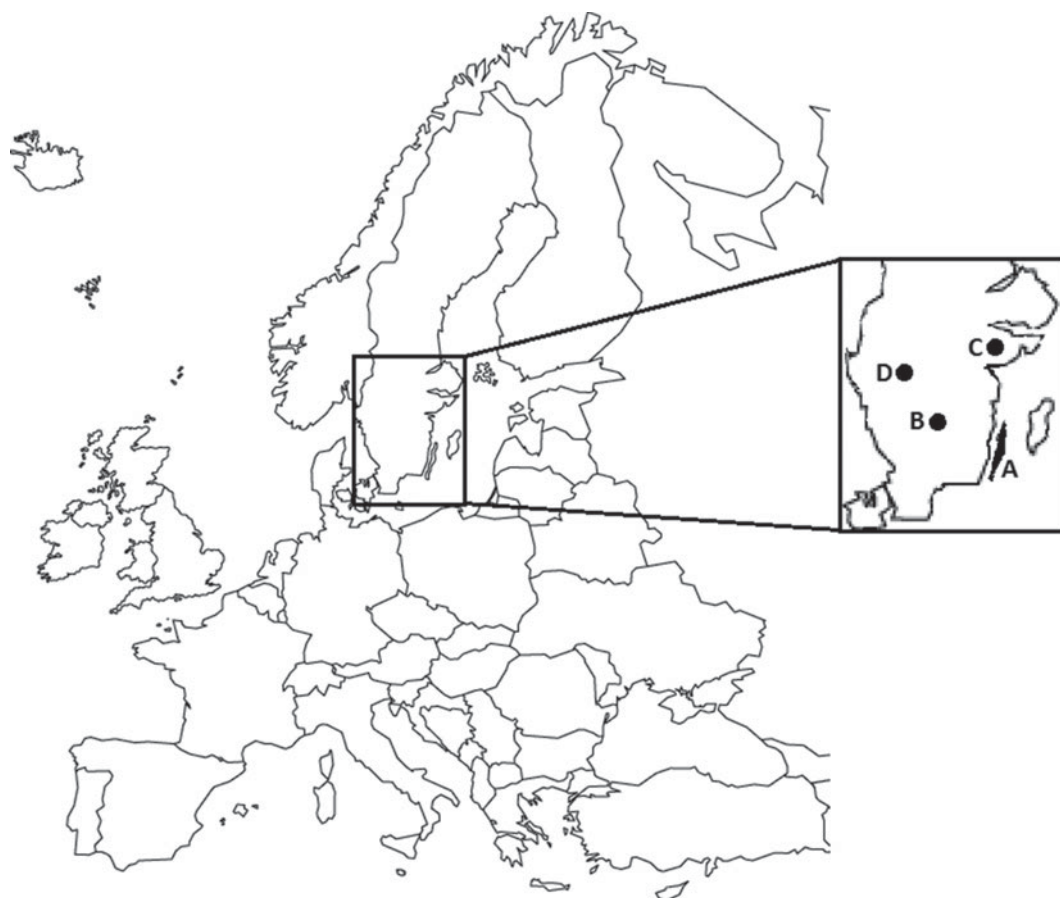


Fig. 1. Map showing sampling areas (highlighted) in Sweden. A represents the island of Öland; B, C, and D represent sampling areas in three different mainland populations.

fever, depression, lethargy, reduced milk yield (dairy cattle) and abortion [1]. In horses, infection with *A. phagocytophilum* (earlier known as *Ehrlichia equi*) may cause equine granulocytic ehrlichiosis [3], and in humans it is a cause of human granulocytic anaplasmosis (HGA [4]). HGA is considered to be an emerging vector-borne infection in Europe [5] and the USA [5, 6], and is reportedly under-diagnosed due to the similarity of its clinical symptoms with infection with *Borrelia burgdorferi* [6].

The epidemiology of *A. phagocytophilum* in wildlife is poorly understood, and few reports on the pathogenic potential of *A. phagocytophilum* infection in cervids exist. These include case reports of *A. phagocytophilum*-associated mortality in roe deer (*Capreolus capreolus*) fawns and a moose calf (*Alces alces*) from Norway [7–9]. In these reports, the animals succumbed due to a fatal, presumably secondary bacterial pneumonia, and the authors suggested that the immunosuppressive effects of a primary infection with *A. phagocytophilum* may have facilitated the

lung infection. Seroprevalence in moose was reported to be 43% in a study in Norway [10], but association with disease was not investigated. Another Norwegian study reported a 79% seroprevalence in moose from a habitat known to be tick-infested [11]. Different genetic variants of *A. phagocytophilum* have been found in cervids based on 16S rRNA gene sequencing [12–14]. Red deer (*Cervus elaphus*) in the Czech Republic reportedly carry two different 16S rRNA variants of *A. phagocytophilum* [15]. However, sequences of other less conserved genes such as *msp4*, *groESL* or *anka* from different *A. phagocytophilum* isolates revealed substantial variability [16–19]. For instance, up to 11 different variants of *A. phagocytophilum* based on the *groESL* gene sequences were detected in roe deer, red deer, alpine chamois (*Rupicapra rupicapra*), Alpine ibex (*Capra ibex*), and European mouflon (*Ovis musimon*) in Austria [13].

In 2005, a dead moose calf from the island of Öland in the Baltic Sea (Fig. 1) was submitted for

post-mortem examination to the National Veterinary Institute in Uppsala, Sweden. The cause of death was a severe bacterial bronchopneumonia, with a concurrent septicaemia (caused by *Streptococcus* sp.). *A. phagocytophilum* DNA was detected by polymerase chain reaction (PCR) analysis [20]. It was suggested that the *A. phagocytophilum* infection facilitated the bacterial bronchopneumonia, resembling the findings of the report from Norway [7]. On the island of Öland local hunters have since 2006 reported low numbers of observed moose calves during the autumn hunt [21]. Several hunters on the island are also farmers (sheep, cattle), and frequently observe and treat their animals for tick-borne fever; similar information has been reported by local veterinarians [22]. Hence, it was suggested that infection with *A. phagocytophilum* had affected moose calf health and survival on the island.

The epidemiology of *A. phagocytophilum* infection in moose populations based on both serological and PCR-based data has not been reported previously. The aim of the present study was to investigate the serological and DNA-based prevalence of *A. phagocytophilum* in hunter-harvested moose from the island of Öland, and three areas on the mainland of Sweden.

MATERIALS AND METHODS

Samples from harvested moose were collected from the island of Öland (Fig. 1, area A; 2007–2010), and from three mainland populations (Fig. 1, areas B, C, D; 2008–2010) during the moose hunt in October and November. Area A, the island of Öland, is a habitat with a mix of agricultural land and deciduous forest in the southern part, and boreal forest in the northern parts. Areas B, C, and D consist of mostly boreal forest [23]. Trained field assistants collected fresh samples (spleen, kidney, liver, faeces, lower jaws, ectoparasites, rumen contents, blood) from harvested moose, according to a standardized protocol. Blood (primarily from vena cava caudalis or the heart) was collected in non-additive blood tubes (BD Vacutainer®, BD Diagnostics, USA). Serum was separated from the blood samples and stored at -20°C . A 4×4 cm piece of the spleen, mandibles, and remaining samples were stored at -20°C awaiting further analysis. The body condition (below normal or normal) based on the presence of coronary fat deposits and signs of muscular atrophy, was recorded, and a specific identity was assigned to each sampled moose. The hunters recorded the weight of the carcass

after removal of skin, head, internal organs, and metapodials, according to Wallin [24].

For age determination in adult moose, the first molar of the lower jaw was sectioned and cementum layers counted as described by Wolfe [25]. The animals were divided into three age groups: calf (<1 year), subadult (1–2 years), and adult (>2 years).

The screening for presence of *A. phagocytophilum* DNA was performed with a diagnostic commercial real-time PCR test for detection of 16S rDNA using GER3 (TAGATCCTTCTTAACGGAAGGGCG) and GER4 (AAGTGCCCGGCTTAACCCGCTG-GC) primers as described by Goodman *et al.* [26], but converted to a real-time PCR format by adding a TaqMan probe [27]. Total DNA was extracted from spleen samples, excising a cube with sides of 0.5 cm, mashing the tissue with sterile scalpels, subsequently followed by lysis and DNA extraction according to the manufacturer's instructions, using the QIAamp DNA Extraction kit and an EZ1 BioRobot (Qiagen GmbH, Germany). PCR was performed according to Jäderlund *et al.* [27] in an ABI 7500 FAST PCR machine (Life Technologies, USA), and was run for 40 cycles. Each PCR included a control with the DNA template substituted with sterile water, a positive and a negative reference *A. phagocytophilum* template, extracted from blood from *A. phagocytophilum*-infected and non-infected horses, respectively. To prevent contamination from environmental DNA, the preparation of PCR mixtures, DNA extraction, PCR and detection of products were all performed in separated laboratory facilities, and aerosol-resistant filter pipette tips were used throughout the experiment.

Of the 10 spleen samples submitted for genetic sequencing, direct sequencing using GER3 and GER4 primers for 16S rDNA, yielded good quality sequences without the need for cloning for two samples (915 bp), one from a mainland moose and one from an island moose. From these two samples also partial *groESL* sequences were obtained (1275 bp). The PCR and sequencing protocol used was identical to that used by Franzén *et al.* [28].

For detection of *Anaplasma* antibodies, an immunofluorescence assay (IFA) was used [27]. Briefly, two-fold dilutions of sera were added to slides precoated with *A. phagocytophilum* antigen (Protatek, USA). To visualize the bound antibodies, a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-deer IgG (KP Laboratories, USA) was added and slides were examined by fluorescence microscopy. For negative control, a seronegative moose sample was used, and

Table 1. Results of a logistical regression model with factors associated with PCR-based detection of *Anaplasma phagocytophilum* in Swedish moose (*Alces alces*) spleens

Variable		Crude OR* (95% CI)	Adjusted OR† (95% CI)	P (Wald's test)‡	P (LR test)§
County	Ref.: A (Öland)	1	1		0.001
	B (Småland)	0.82 (0.19–3.62)	1.45 (0.26–8.2)	0.675	
	C (Sörmland)	0.36 (0.08–1.70)	0.37 (0.06–2.23)	0.276	
	D (V-Götland)	0.11 (0.01–0.84)	0.19 (0.02–1.75)	0.141	
Sex	Male vs. female	1.26 (0.69–2.28)	0.94 (0.44–1.99)	0.869	0.869
Year	Ref.: 2007	1	1		<0.001
	2008	9.84 (2.21–43.86)	19.57 (3.91–97.93)	<0.001	
	2009	1.99 (0.41–9.77)	4.73 (0.88–25.49)	0.07	
	2010	9.43 (1.95–45.66)	28.93 (5.17–162.05)	<0.001	
Age group	Ref.: calf	1	1		0.485
	Yearling	0.22 (0.09–0.53)	0.26 (0.10–0.63)	0.637	
	Adult	0.35 (0.18–0.7)	0.1 (0.05–0.18)	0.263	
Carcass weight		0.99 (0.99–1.00)	0.99 (0.95–1.02)	0.464	1
Age group: carcass weight	Ref.: calf	1	1		0.716
	Yearling	—	1.00 (0.95–1.06)	0.858	
	Adult	—	1.01 (0.98–1.05)	0.495	

OR, Odds ratio; CI, confidence interval; LR, likelihood ratio.

* The OR of one specific factor without consideration of confounding effects of other factors.

† The OR of one factor, with consideration of confounding effects of other factors.

‡ The *P* value based on the sample estimate.

§ Likelihood ratio test.

for a positive control the opposite. All samples were screened at a dilution of 1:40. For further titration, 77 high-quality (minimal hemolysis) serum samples from 2009 and 2010 were selected (51 from area A, 26 from areas B, C, D). These samples were further titrated in twofold steps to a dilution of 1:1280. An IFA titre \geq 1:40 was considered positive, in accordance with Stuen *et al.* [10]. A further titration of samples with titres 1:1280 was not performed.

Statistical analyses of risk factors affecting PCR presence of *A. phagocytophilum* were performed using logistic regression in R (R Foundation for Statistical Computing, Austria). An exploratory analysis using multiple possible risk factors was conducted (Table 1). Crude and adjusted odds ratios (OR) with 95% confidence intervals were calculated to identify which factors (sampling area, sex, sampling year, age group, carcass weight) were associated with higher or lower PCR presence of *A. phagocytophilum*. The crude OR is an estimation of one specific factor without consideration and possible confounding effects of other factors. The adjusted OR is estimated with consideration of other factors (stated previously) included in the model. In addition, statistical

significance calculations (*P* values) between sampling area, sex, sampling year, age group, and carcass weight (in relation to age group) were performed using Wald's test within the same logistic regression, as seen in Table 1.

RESULTS

Spleen samples from 263 harvested moose (141 females, 122 males) were collected (Table 2). Blood was obtained from a total of 234 moose. From the remaining 29 moose, blood could not be collected, mostly due to contamination by rumen contents, or an insufficient collectable volume that occurred when the moose had been eviscerated prior to sampling by field assistants. Mean carcass weight for calves ($n=73$) was 60.5 kg (range 25–87 kg), for sub-adults ($n=57$) 127.3 kg (range 75–180 kg), and for adults ($n=109$) 193.1 kg (range 118–302 kg). For 24 moose, no carcass weight was reported by the hunters. Twelve moose were found to be in subnormal body condition, and of these, clinical signs of disease (emaciation of unknown cause) were observed in

Table 2. Geographical and biological distribution, and numbers of *Anaplasma phagocytophilum* DNA-positive samples in moose spleen samples from Sweden collected from 2007 to 2010

Variable	No. of samples	<i>A. phagocytophilum</i> DNA positive (%)
Sampling area*		48 (36.6)
A (Öland)	131	15 (17.6)
B (Småland)	85	3 (7.9)
C (Sörmland)	38	3 (33.3)
D (Västergötland)	9	
Sex		
Male	141	34 (24.1)
Female	122	35 (28.7)
Year		
2007	35	4 (11.4)
2008	90	33 (36.7)
2009	90	14 (15.6)
2010	48	18 (37.5)
Age group†		
Calf	81	37 (45.7)
Yearling	66	10 (15.2)
Adult	113	22 (19.5)
Not recorded	3	0
Overall	263	69 (26.2)

* Four different counties in southern Sweden according to Figure 1.

† Sampled moose divided in to three age groups: 0–1 year, 1–2 years, and >2 years.

two moose. The mean age of sampled moose was 2.8 years (range 0.5–15.5).

The overall prevalence of *A. phagocytophilum* DNA was 26.3%. On a spatial scale, moose from area A (island) had a significantly ($P < 0.006$) higher prevalence of *A. phagocytophilum* DNA (36.6%) than the combined prevalence in moose from areas B, C, and D (15.9%). The area variable was highly significant (likelihood ratio test $P < 0.01$), indicating significant spatial heterogeneity of *A. phagocytophilum* prevalence. Area A (island) had the highest recorded prevalence, 36.6%, and area C had the lowest recorded prevalence of 7.9%. No significant difference in prevalence ($P = 0.141$ – 0.657 , Table 1) between individual counties was found. Regardless of sampling areas, samples from 2008 and 2010 had significantly ($P < 0.01$) higher *A. phagocytophilum* DNA prevalence than samples collected in 2007 (Table 1) and 2009 (results from separate model using 2009 as reference, not shown). For all moose samples from 2007 to 2010, sex, age group, and age did not have significant effects on the presence of *A. phagocytophilum* DNA.

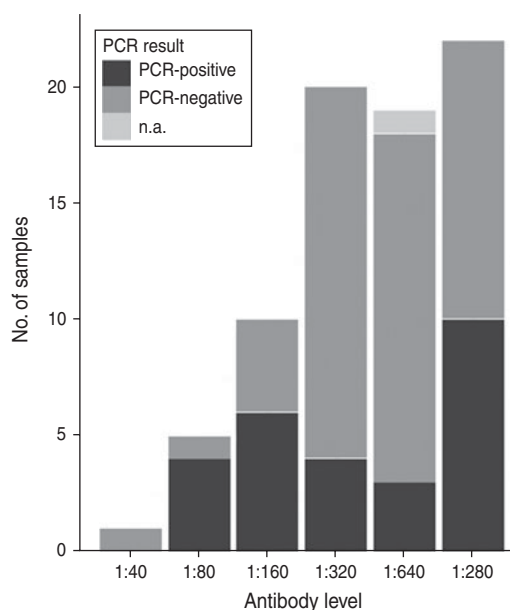


Fig. 2. An overview of numbers of samples with antibody titres and DNA presence of *Anaplasma phagocytophilum* in Swedish moose (*Alces alces*) sampled in 2009 and 2010. n.a., Not available.

All tested samples were serologically positive (titre $\geq 1:40$). Titration was performed on 77 samples from 2009 and 2010, with titres ranging from 1:40 to 1:1280. Figure 2 shows an overview of serological titres related to presence of *A. phagocytophilum* DNA. An additional model fitted to a subset of 77 animals that included *A. phagocytophilum* titre levels (results not shown) indicated no significant predictive effect of titre levels, but a significant effect ($P < 0.001$) of body weight when controlled for age.

The genetic sequencing revealed a 99% sequence similarity between the two samples for the 16S rRNA part. Furthermore, the island sample had 16S rRNA sequence similarities with an equine variant previously detected in Sweden (GenBank accession no. AY527214; 912/915 bp, 99.7%) [27], a roe deer sequence from Austria (496/497 compared base pairs, 99.8%, GenBank accession nos. FJ812391–FJ81239 (type S) [13]). The *groESL* sequence from the island moose had a 99% sequence similarity (1267/1275 compared base pairs) with an HGA agent detected in humans in California (GenBank accession no. AF172163 [29]), unlike the mainland sample which shared 1252/1276 bp with the HGA agent (98.6%). Furthermore, the island *groESL* sequence was similar to a red deer and a roe deer sequence from Austria (530 bp compared, 99.6% and

99.4% sequence similarity, respectively, GenBank accession nos. GQ988767 and GQ988765, respectively) [13].

The 16S rRNA sequence from the mainland moose sample had a 99.9% sequence similarity with the equine variant reported previously from Sweden (GenBank accession no. AY527214; 914/915 bp [27], and was identical (497/497 compared base pairs) to an Austrian roe deer sequence (GenBank accession nos. FJ812406–FJ812408, type Y) [13]. A shorter section (*groEL*) of the *groESL* gene sequence in the mainland moose sample showed a 100% sequence similarity (455/455 bp) with a roe deer *A. phagocytophilum* sequence detected in Austria (GenBank accession no. GQ988754), and a 99.8% sequence similarity (529/530 bp) with sequences found in both red deer and roe deer in the same paper [13] (GenBank accession no. GQ988763).

GenBank accession numbers for the novel moose sequences are KC800983 (island) and KC800984 (mainland) for the 16S rRNA sequences and KC800985 (island) and KC800986 (mainland) for the *groESL* sequences.

DISCUSSION

This is the first study on *A. phagocytophilum* infection in moose in Sweden, based on the presence of *A. phagocytophilum* DNA, and of antibodies. No similar large-scale study in cervids has previously been reported, apart from a Norwegian study of archived moose serum samples (collected 1994–2000), where a 43% seroprevalence in moose was found [10]. In 2012 seroprevalence of *A. phagocytophilum* antibodies in 33 captured moose from a reported tick-infested area in southern Norway was 78% [11]. Although the performance of the serological test in moose has not been validated, we consider the results to be reliable since the test used in three Norwegian studies [10, 11, 30] is the same as the one used in this study. In addition, there are no reports of possible cross-reacting *Anaplasma* variants (e.g. *A. marginale*) in Sweden.

In the present study the overall prevalence of *A. phagocytophilum* DNA was 26.3%, with significant spatial difference between the island population (A) and the mainland populations (B, C, D). Spatial variation in prevalence of vector-borne pathogens has been described previously. Chaput *et al.* [31] reported spatial variation in incidence of the HE agent in humans in Connecticut, USA. In the present study,

one reason might be the annual arrival of high numbers of tick-carrying migrating birds, which reportedly carry *Ehrlichia* sp. bacteria (HE agent [32]). DNA in the present study also showed a temporal difference, with higher prevalence in 2008 and 2010, respectively. Temporal variation in *A. phagocytophilum* antibody prevalence has previously been reported by Rejmanek *et al.* [33] in small mammals in California where the rate of exposure (antibody presence) varied over a 2.5-year period.

The range in *A. phagocytophilum* antibody titres (1:40–1:1280) observed, may be a result of several factors including the time of infection/exposure, re-infection, host immune response, variant of the bacterium, cyclic bacteraemia in the infected animal, and the sensitivity of the detection method [16, 34]. Antibodies against different *Anaplasma* variants have been shown to persist for at least 5 months in experimentally infected dogs (*Ehrlichia* sp. [35]), lambs (*A. phagocytophilum* [36]) and horses (*Ehrlichia phagocytophila* [37]), and for at least 2.5 months in red deer [30].

The observed effect of *A. phagocytophilum* DNA presence on moose carcass weight described earlier in this report will not be further commented upon due to the small sample size ($n=77$).

A 42.6% *A. phagocytophilum* DNA prevalence has been reported previously in 237 sampled roe deer in Denmark [38]. Other European studies have shown varying *A. phagocytophilum* DNA prevalence ranging from 18% to 86% (roe deer [12–15, 39, 40]), 29–86% (red deer [12, 13, 40, 41]), 50–72% (fallow deer, *Dama dama* [15, 42]), and 40% (sika deer, *Cervus nippon* [15]). These reports suggest that infection with *A. phagocytophilum* is common in cervids in Europe. However, more knowledge is needed about the pathogenesis, immune response, and clinical manifestation of *A. phagocytophilum* infection in order to assess the clinical and pathological significance of infection in free-ranging cervids such as moose. Experimentally infected Norwegian captive red deer (*Cervus elaphus atlanticus*) and reindeer (*Rangifer tarandus*) showed individual differences in clinical signs, time of sero-conversion, and duration of antibody titre [30, 43]. In reindeer, fever and neutropenia were noted, but not in red deer, other than a calf which showed a moderate increase in rectal temperature.

In the present study, antibodies against *A. phagocytophilum* were found in both *A. phagocytophilum* DNA-positive, and DNA-negative moose. The varying titres (1:40–1:1280) is an indication of when in

time the animal is infected, or re-infected. Adding to this, no end-titration was performed which could have rendered even higher titres in some samples. Presumably, and as observed in sampled moose, the vectors (ticks) are probably active not only in the summer months but also during the autumn. Antibodies are reportedly known to persist longer than circulating bacteria, which could explain the observed discrepancy between seroprevalence and *A. phagocytophilum* DNA-based prevalence. This has been reported in a various *A. phagocytophilum*-infected ruminants including sheep [36, 44] and horses [45]. Similar discrepancies have previously been reported in roe deer, where Zeman & Pecha [15] showed a 100% seroprevalence, and a 30% *A. phagocytophilum* DNA-based prevalence. In the same study, the discrepancy in mouflon was 100% and 3.5%, respectively. In wild boar from Slovenia, the discrepancy was 72% (serology) and 6.2% (PCR), respectively [40].

No association between antibody titre and *A. phagocytophilum* DNA presence was identified in this study (Fig. 2). Infection and host humoral immune response may be dependent on several factors, as stated previously. For instance, Stuen *et al.* [46] reported that experimentally infected lambs had a detectable bacteraemia on several different occasions over a 13-week period. In the present study, both mainland and island moose had been exposed to *A. phagocytophilum*, but the *A. phagocytophilum* DNA prevalence varied between areas, and between sampling years (Table 1). A cyclic variation may also be present in moose infected with *A. phagocytophilum*, and if sampled on another occasion, such as during the summer months, the *A. phagocytophilum* DNA presence, and antibody prevalence, may have been different. The significance of body weight on the presence of *A. phagocytophilum* DNA was noted, and could be caused by a reduced weight gain, as reported by Stuen *et al.* [47] in lambs infected on pasture. However, since this effect was not present in all sampled moose (sampled from 2007 to 2010), no conclusions have been drawn.

The results of the genetic sequencing *A. phagocytophilum* DNA-positive samples provide no evidence that moose are a host or a reservoir for different genetic variants. One of the sequenced samples from an island moose had genetic similarities with the HGA agent, which has previously been reported in Sweden [5, 48]. More spleen samples from moose need to be sequenced before any further conclusions can be drawn.

In conclusion, this study demonstrated that moose from southern Sweden were commonly exposed to and infected with *A. phagocytophilum*. The *A. phagocytophilum* DNA prevalence varied between years (temporal variation) and between regions (spatial variation). Future studies should focus on the characterization of different genetic variants of *A. phagocytophilum* in free-ranging Swedish moose, and their pathogenic potential for moose and other mammalian species, such as domestic animals and humans.

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DECLARATION OF INTEREST

None.

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