Ecological dynamics of host-vector-pathogen relationships in sika deer and brown bears in Hokkaido

(エゾシカおよびエゾヒグマにおける 宿主・ベクター・病原体の生態学的関係)

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Abbreviations

16S	16 small subunit
18S	18 small subunit
AP-sd	Anaplasma sp. AP-sd
Apr.	April
Aug.	August
Bp	Base pair
BLASTn	Basic Local Alignment Search Tool for Nucleotides
DDBJ	DNA Data Bank of Japan
DNA	Deoxyribonucleic acid
Dec.	December
EDTA	Ethylenediaminetetraacetic acid
Feb.	February
GLMM	Generalized linear mixed model
Jan.	January
Jul.	July
Jun.	June
Mar.	March
MEGA	Molecular Evolutionary Genetics Analysis
No	Number
Nov.	November
Oct.	October
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SE	Standard error
Sep.	September
VIF	Variance Inflation Factor

Notes

Publications related to the dissertation

1. Shimizu K, Shimozuru M, Yamanaka M, Ito G, Nakao R, Tsubota T. Seasonal infestation patterns of ticks on Hokkaido sika deer (*Cervus nippon yesoensis*). Parasitology, 151:1317–1325, 2024.

2. Shimizu K, Shimozuru M, Yamanaka M, Ito G, Nakao R. Evaluating the vector potential of deer keds *Lipoptena fortisetosa* for selected pathogens in Hokkaido sika deer (*Cervus nippon* yesoensis). Parasitol Int, 107, 103053, 2025.

The contents of Chapter I and Chapter III have been published as publications 1 and 2, respectively.

Preface

Tick-borne diseases are a significant threat to wildlife, livestock, and human health worldwide. In recent years, climate changes, wildlife habitat alterations, and environmental modifications caused by human activity have rapidly transformed the distribution and occurrence patterns of these diseases. Hokkaido, the northernmost island in Japan, has unique climatic conditions and wildlife populations. Belonging to the subarctic climate zone, this region exhibits distinctive tick seasonal activity patterns, creating a unique host-vector-pathogen relationship. In particular, Hokkaido sika deer (*Cervus nippon yesoensis*) and Hokkaido brown bears (*Ursus arctos yesoensis*) play crucial roles in the ecosystem and serve as primary hosts for diverse tick species. Large mammals such as these are capable of extensive movement and may contribute to the spread of ticks and their associated pathogens.

Understanding the complex interplay between hosts, vectors, and pathogens is essential for elucidating the ecology of tick-borne diseases and is critical for public health and wildlife conservation. However, previous research lacks a comprehensive understanding of tick ecology and tick-borne pathogen dynamics in Hokkaido wildlife. In particular, research involving large mammals, which are key tick hosts, remains scarce due to technical challenges associated with field sampling and long-term monitoring. Clarifying these complex relationships is crucial for developing effective disease management strategies.

This study focused on the wildlife in Hokkaido, particularly sika deer and brown bears, to investigate tick-borne pathogen infection dynamics and tick infestation patterns. To address the complex ecological relationships in this system, three interrelated research chapters were designed to examine different aspects of host-vector-pathogen dynamics.

In Chapter I, the hypothesis that tick species have species-specific attachment site preferences influenced by interspecific interactions was tested. A detailed investigation of seasonal tick infestation patterns was conducted using sika deer captured in the Rusha area of the Shiretoko Peninsula. Tick infestation was specifically analyzed across different body parts of sika deer, focusing on species-specific attachment site preferences and interspecific interactions among tick species in attachment site selection. This study provides fundamental insights into tick infestation patterns at the individual host level and interspecific tick interactions.

In Chapter II, the hypothesis that pathogen infection rates and tick infestation

patterns are influenced by environmental and host factors was examined. This study investigated the prevalence and distribution patterns of tick-borne pathogens in sika deer and brown bears across four regions of Hokkaido (East, North, Central, and South). This study focused on *Anaplasma* sp. AP-sd (AP-sd) in sika deer and *Hepatozoon ursi* in brown bears as representative pathogen systems with different transmission characteristics. Generalized linear mixed models (GLMMs) were used to evaluate how environmental factors (region and season) and host factors (sex and age) influence both pathogen prevalence and tick infestation patterns. This comprehensive study provides the first large-scale assessment of tick-borne pathogen transmission dynamics in Hokkaido sika deer and brown bears. The findings establish fundamental knowledge for understanding host-vector-pathogen relationships.

In Chapter III, the hypothesis that external parasites other than ticks may be involved in pathogen transmission was tested. This study focused on deer keds (*Lipoptena fortisetosa*) parasitizing sika deer and evaluated their pathogen vector potential. This study investigated pathogen detection in sika deer, blood-fed deer keds collected from deer, and unfed deer keds collected from the environment to assess the role of deer keds in pathogen acquisition and transmission. The results of this research suggest their possible role as mechanical vectors and highlight the need for further experimental validation of their transmission capabilities.

This integrated approach reveals the complex ecological networks linking sika deer and brown bears, the representative large mammals of Hokkaido, with vectors and their associated pathogens. The findings establish a foundation for evidence-based wildlife health management and tick-borne disease prevention strategies for these key wildlife species in subarctic regions.

Chapter I

Seasonal infestation patterns of ticks on Hokkaido sika deer

Introduction

Most ectoparasites, including ticks, do not infest their hosts randomly but feed preferentially on specific body parts (Andrews et al., 1981; Baer-Lehman et al., 2012; Chilton et al., 1992; Kiffner et al., 2011; Lydecker et al., 2019; Mysterud et al., 2014; Tiffin et al., 2021). The distribution patterns depend on the characteristics of the host and tick (Kar et al., 2013). For example, it has been reported that many tick species prefer sites where they can effectively feed, such as the hairless parts of rodents (Pilosof et al., 2012). Feeding efficiency is influenced by the anatomical characteristics of the host and the morphology of the ectoparasite, that is, the compatibility of the feeding apparatus of the parasite and the feeding site on the host. Ectoparasites with short proboscises select body parts with relatively thin epidermis and capillaries close to the body surface, whereas ectoparasites with relatively long proboscises select body parts with a thicker epidermis and deeper capillaries (Pilosof et al., 2012). It is also important for ticks that the animal's mouth or claws are out of reach to prevent their removal during feeding. Host defense efforts and efficiency vary by body site (Reiczigel and Rózsa, 1998), and many ticks that feed on birds prefer their heads as feeding sites because they are difficult to remove by grooming by the host (Fracasso et al., 2019).

Another important factor affecting the spatiotemporal distribution of ticks is interspecific interactions. Such interspecific interactions include cases where one parasitic species promotes or inhibits infestation by another species (Fenton et al., 2010; Hellard et al., 2015). For example, some hard ticks (Acari: Ixodidae) that feed on the eastern rock sengis (*Elephantulus myurus*) may facilitate the invasion of other tick species onto the same host (no records of attachment sites) (Lutermann et al., 2015). In contrast, *Ixodes scapularis* and *Dermacentor albipictus* compete for preferred attachment sites on white-tailed deer (*Odocoileus virginianus*) (Fellin and Schulte-Hostedde, 2022). In addition to interspecific relationships, facilitative interactions within the same tick species have been reported, possibly due to the decreased immune response of the host as the tick infestation burden increases (Lutermann et al., 2015).

Understanding tick infestation patterns in host animals is important for efficient tick sampling, effective treatment of animals with repellents and acaricides, and, consequently, control of tick-borne diseases (Schmidtmann et al., 1998). Hokkaido sika deer (*C. nippon yesoensis*), a subspecies of the sika deer (*C. nippon*), is one of the most important hosts for several tick species of the genera *Ixodes* and *Haemaphysalis* (Elbaz et al., 2020; Iijima et al., 2022; Tsukada et al., 2014). The population of sika deer in Hokkaido has increased over the last 20–30 years (approximately 670,000 between 2014 and 2020) (Ikeda and Koizumi, 2024). Because deer are known carriers of several tick-borne pathogens such as Piroplasmida and *Anaplasma* (Elbaz et al., 2017; Lee et al., 2014), it is of critical importance to understand the spatiotemporal distribution of ticks on Hokkaido sika deer.

This study aimed to understand tick infestation patterns in Hokkaido sika deer. More specifically, the research focused on seasonal differences in infestation patterns, preferred attachment sites for each tick species, and interspecific interactions between different tick species.

Materials and methods

Study area

This study was conducted in the Rusha area of the Shiretoko Peninsula, eastern Hokkaido, Japan (44˚11'N, 145˚11'E). The area from the center of the peninsula to Shiretoko Cape, including the research site, is designated as Shiretoko National Park and is a UNESCO World Heritage Site where hunting is prohibited. This area is a narrow estuary coast that stretches from south to north for approximately 3 km, and its climate is classified as Subarctic. Climate information for the sampling period in Utoro (44°03' N, 144°59' E), the closest climate observation point to the study area, is shown in Table 1. Based on the definitions provided by the Japan Meteorological Agency, March to May is defined as spring, June to August as summer, September to November as autumn, and December to February as winter. Six native mammals, namely the Hokkaido sika deer, Hokkaido brown bear (*U. arctos yesoensis*), red squirrel (*Sciurus vulgaris*), red fox (*Vulpes vulpes*), raccoon dog (*Nyctereutes procyonoides*), and sable (*Martes zibellina*), have been identified in the northwestern part of the national park, including the study area (Kawamura et al., 2022).

Sampling

Deer were captured 48 times for tick collection between June and October 2022, totaling 37 deer, nine of which were captured more than once. Deer capture and sampling were conducted for three to four days each month during the study period. The deer were anesthetized with 0.1 mg/kg medetomidine (Dorbene Vet, Kyoritsu Seiyaku, Co., Tokyo, Japan) using a CO_2-powered gun (Dan-Inject ApS, Borkop, Denmark) for intramuscular injection and awakened with 0.1 mg/kg atipamezole (Atipame, Kyoritsu Seiyaku, Co., Tokyo, Japan) injected intramuscularly after all procedures were completed. The design of the capture process was based on previous studies (Onuma et al., 2005). Deer age was estimated based on tooth eruption and replacement (Ohtaishi, 1977). The sample population consisted of the following age and sex structure: two 1-year-old females, one 2-year-old male, eight 2-year-old females, and 26 females aged ≥ 3 years. All captured deer were tagged with earmarks for identification, and individual deer were not captured more than twice in the same month to ensure that recapture did not affect sampling. Ticks were collected from the head, pinna, body (neck: 50 × 70 mm, body side: 100 × 150 mm,

and anus tail), and legs (elbow: 50 × 70 mm, forelimb, knee: 50 × 70 mm, and hindlimb) of the sika deer; only the left half of the body was examined (Figure 1). Ticks found on the host were recorded as either feeding or questing. Owing to the extremely low number of questing ticks (only a few *Ixodes* spp. males), no distinction between the feeding and questing states was made in subsequent data analyses. The sampling sites were selected based on previous studies (Kiffner et al., 2011; Matthee et al., 1997; Mysterud et al., 2014). The collected ticks were identified by species, sex, and life stage based on their morphological characteristics (Yamaguti et al., 1971) using a stereomicroscope. Owing to the difficulty in accurately identifying larvae morphologically, they were not included in the analysis.

All procedures involving the capture and sampling of live animals were conducted by the Guidelines for Animal Care and Use, Hokkaido University, and with the approval of the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Permit Number: 22–0016).

Statistical analysis

The abundance and prevalence of each tick species attached to each deer per month were determined. Tick abundance was defined as the mean number of each tick species attached per deer per month, whereas tick prevalence was defined as the proportion of infested individuals among the captured deer. The monthly mean number of ticks per body part was calculated to determine the body parts of the deer that were preferred for tick attachment. The number of ticks on different body parts was compared using the Kruskal–Wallis test (Vargha and Delaney, 1998), with three degrees of freedom. Subsequent post-hoc analysis was conducted using the Steel–Dwass method for multiple comparison tests (Neuha and Bretz, 2001). The Kruskal–Wallis test and Steel-Dwass post-hoc analysis were performed separately for each tick life stage (males, females, and nymphs) and for the total number of ticks across all stages to compare tick distributions among different body parts. Because most of the captured deer were females aged ≥ 3 years, comparisons of tick infestation patterns by deer sex or age were not made. All statistical analyses were performed using R version 4.1.0 (R Core Team, 2021), and differences were considered statistically significant at $P < 0.05$. The co-occurrence patterns of different tick species and stages across attachment sites (each body part of the deer) were investigated to evaluate the inter and intraspecific relationships between ticks.

The ‘co-occurrence’ package in R (Griffith et al., 2016) was used to conduct a pairwise co-occurrence analysis of the species for this analysis, determining whether the co-occurrences were positive, negative, or random. This package uses an algorithm that calculates the observed and expected frequencies of co-occurrence between each pair of species and stages based on the presence/absence data across a collection on each body part. These associations were interpreted as negative or positive co-occurrences when the co-occurrence frequencies were much lower or higher than expected, respectively. In this study, four body parts (head, pinna, body, and legs) were examined for each of the 48 host individuals, resulting in 192 observations (48 individuals × four body parts). Pairs with expected co-occurrence numbers of <1 were excluded from the analysis to minimize the likelihood of spurious results.

Table 1. Climate information for the sampling period in Utoro, Japan, the closest climate observation point to the study area.

	Precipitation (mm)		Temperature (°C)		
Month	Total	Highest day	Daily average	Daily high	Daily low
June	127.5	29.5	12.6	17.0	9.0
July	80.0	28.5	19.0	24.1	14.8
August	157.5	48.5	19.6	24.5	15.2
September	107.5	48.5	16.9	22.6	11.8
October	130.5	38.0	10.7	15.3	6.5

The data are provided by the Japan Meteorological Agency, Japan.

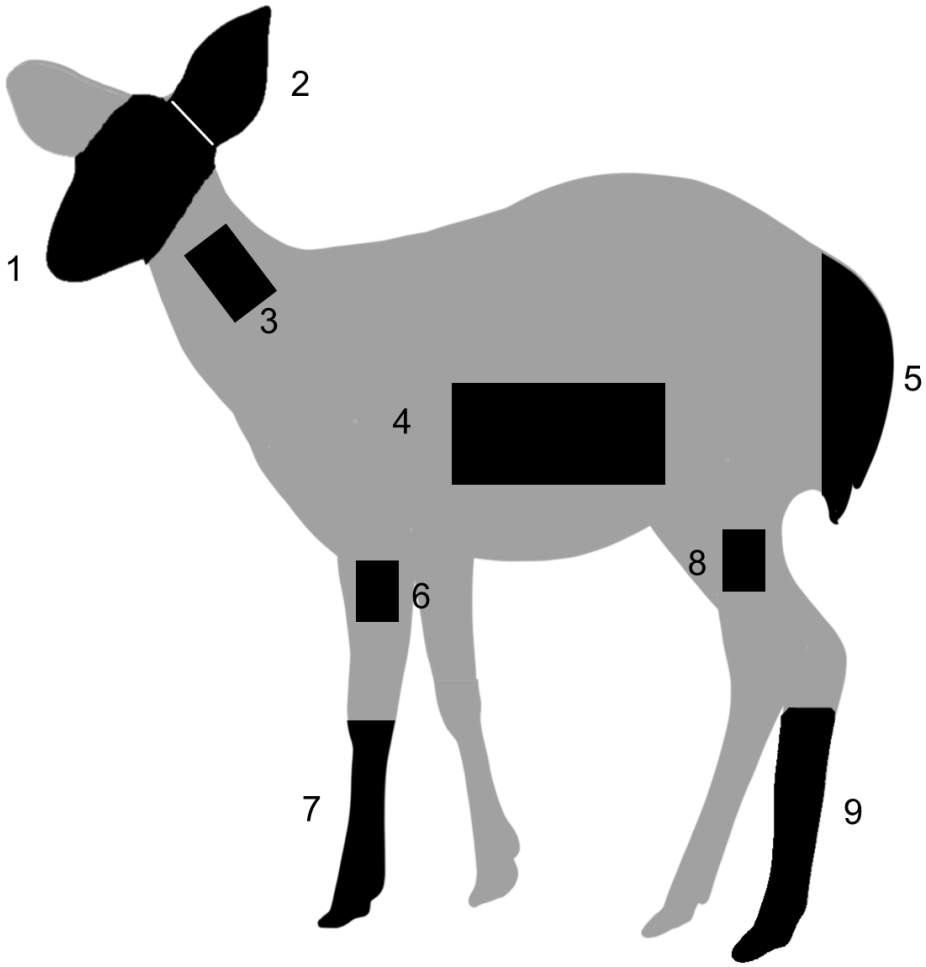

Figure 1. Diagram of the examined sites on the body of the sika deer. 1: head, 2: pinna, body (3: neck 50 × 70 mm, 4: body side 100 × 150 mm, and 5: anus and tail), and legs (6: elbow 50 × 70 mm, 7: forelimb, 8: knee 50 × 70 mm, and 9: hind limb); each part was examined only on the left side.

Results

Seasonal infestation patterns

All captured deer were infested with at least one tick, and 3026 ticks were collected during the study period. The overall composition of the tick population from 48 samplings of sika deer was 21.9% female (663/3026), 52.0% male (1574/3026), and 26.1% nymphs (789/3026). The tick species identified were *I. ovatus* (n = 441), *I. persulcatus* (n = 538), *Ha. japonica* (n = 1793), and *Ha. megaspinosa* (n = 254) (Table 2). The stage composition of attached ticks varied by species, with *I. ovatus* (males: 51, females: 379, and nymphs: 11), *Ha. japonica* (males: 1365, females: 135, and nymphs: 293), and *Ha. megaspinosa* (males: 134, females: 113, and nymphs: 7) being the predominant adults, whereas *I. persulcatus* (males: 24, females: 36, and nymphs: 478) were predominant at the nymphal stage (Table 2).

Monthly variation in tick infestation showed different patterns for each species (Table 2 and Figure 2). The highest infestation by *I. ovatus* was observed in June (n = 256), followed by that in July (n = 133), August (n = 39), September (n = 12), and October (n = 1). Infestations of *I. persulcatus* nymph were relatively consistent throughout the sampling period, with a gradual decrease from June to October (June: n = 156; July: n = 131; August: n = 151; September: n = 68; and October: n = 32). In contrast, the number of *Ha. japonica* decreased from June to August (June: n = 201; July: n = 93; and August: n = 65) but increased rapidly from September (n = 441) to October (n = 993). *Haemaphysalis megaspinosa* was not observed in June, July, or August but appeared in September (n = 126) and October (n = 126).

The peak prevalence of *I. ovatus* and *Ha. megaspinosa* was observed in the same month as their peak abundances (Table 2 and Figure 2). The prevalence of *I. persulcatus*, similar to its abundance, showed little monthly variation and was always higher than 70%. The trend in the prevalence of *Ha. japonica* was different from that of the other three species, with a high prevalence from June to August, even when the abundance was relatively low compared with September to October (June: 90%, July: 80%, and August: 100%).

Attachment site preference

This study revealed that the preference for the body parts to which ticks attach

varies significantly depending on the tick species (Figure 3). In *I. ovatus* adults, significantly higher numbers were observed on the pinna than on other body parts from June to August (June: H-statistic = 36.0; July: H-statistic = 20.8; and August: H-statistic = 18.4). Similarly, *I. persulcatus* nymphs were significantly more abundant in the pinna, and this trend was observed throughout the sampling period, whereas *I. persulcatus* adults showed a slight preference for the head (June: H-statistic = 17.5; July: H-statistic = 29.6; August: H-statistic = 27.9; September: H-statistic = 25.9; and October: H-statistic = 20.8). Conversely, *Ha. japonica* exhibited notable monthly variations in attachment sites. It was found on the body and legs in June, July, and August (June: H-statistic = 19.3; July: H-statistic = 18.0; and August: H-statistic = 28.4); however, a significant shift was observed in September and October, with a marked aggregation on pinnae across all stages (September: H-statistic = 25.5; and October: H-statistic = 17.1). This trend was observed for nymphs and adults, but only the total number of all stages and the number of males showed statistically significant differences between September and October. In contrast to the other three species, which were highly aggregated on specific body parts, *Ha. megaspinosa* was widely distributed on the head and body, with preference for the pinna (September: H-statistic = 9.2; and October: H-statistic = 12.8).

Co-occurrence patterns

In the analysis of co-occurrence patterns among ticks, 66 pairs of combinations derived from different tick species and stages were considered. Consequently, 13 pairs were removed from the analysis because their expected co-occurrence was <1, leaving 53 pairs for further analysis. Positive (28%, 15/53), negative (11%, 6/53), and random co-occurrence patterns (60%, 32/53) were observed (Figure 4). Ticks of the same species and genus tended to exhibit positive co-occurrence patterns. In particular, positive co-occurrence patterns between females and males of the same species were observed in all species and between females and nymphs in *I. persulcatus* and *Ha. japonica*. Conversely, females of *I. ovatus* showed negative co-occurrence patterns with all stages of *Ha. japonica* and both males and females of *Ha. megaspinosa*.

Table 2. Number of ticks collected from Hokkaido sika deer from June to October 2022.

Species	Stage	Sampling month (number of deer)					
		Jun. (11)	Jul. (10)	Aug. (10)	Sep. (10)	Oct. (7)	Total (48)
Ixodes ovatus	Male	28	13	8	2	0	51
	Female	227	120	23	9	0	379
	Nymph	1	0	8	1	1	11
	Subtotal	256	133	39	12	1	441
Ixodes persulcatus	Male	12	5	2	2	3	24
	Female	23	4	2	3	4	36
	Nymph	121	122	147	63	25	478
	Subtotal	156	131	151	68	32	538
Haemaphysalis japonica	Male	76	24	0	344	921	1365
	Female	53	30	8	34	10	135
	Nymph	72	39	57	63	62	293
	Subtotal	201	93	65	441	993	1793
Haemaphysalis megaspinosa	Male	0	0	0	49	85	134
	Female	0	0	0	76	37	113
	Nymph	0	0	0	1	6	7
	Subtotal	0	0	0	126	128	254
Total		613	357	255	647	1154	3026

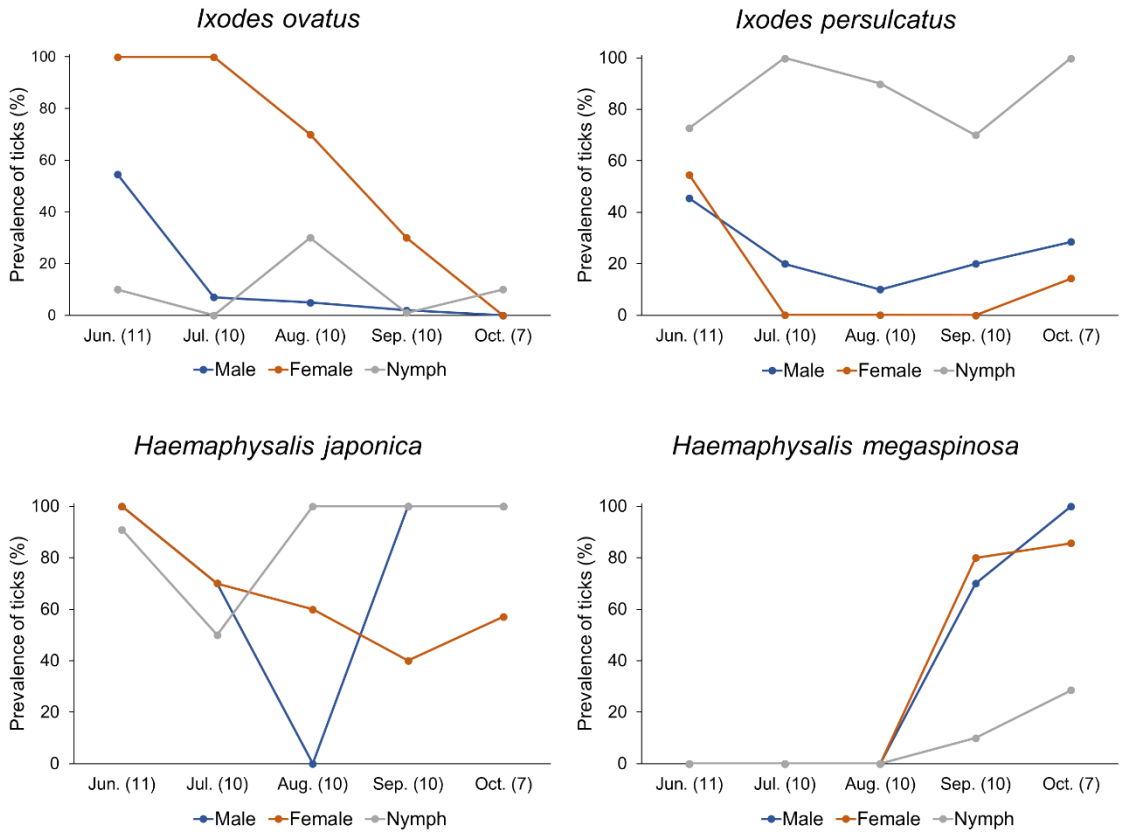

Figure 2. Seasonality of the prevalence of each tick species and their developmental stages parasitizing sika deer from June to October 2022. The horizontal axis represents the sampling month (number of deer).

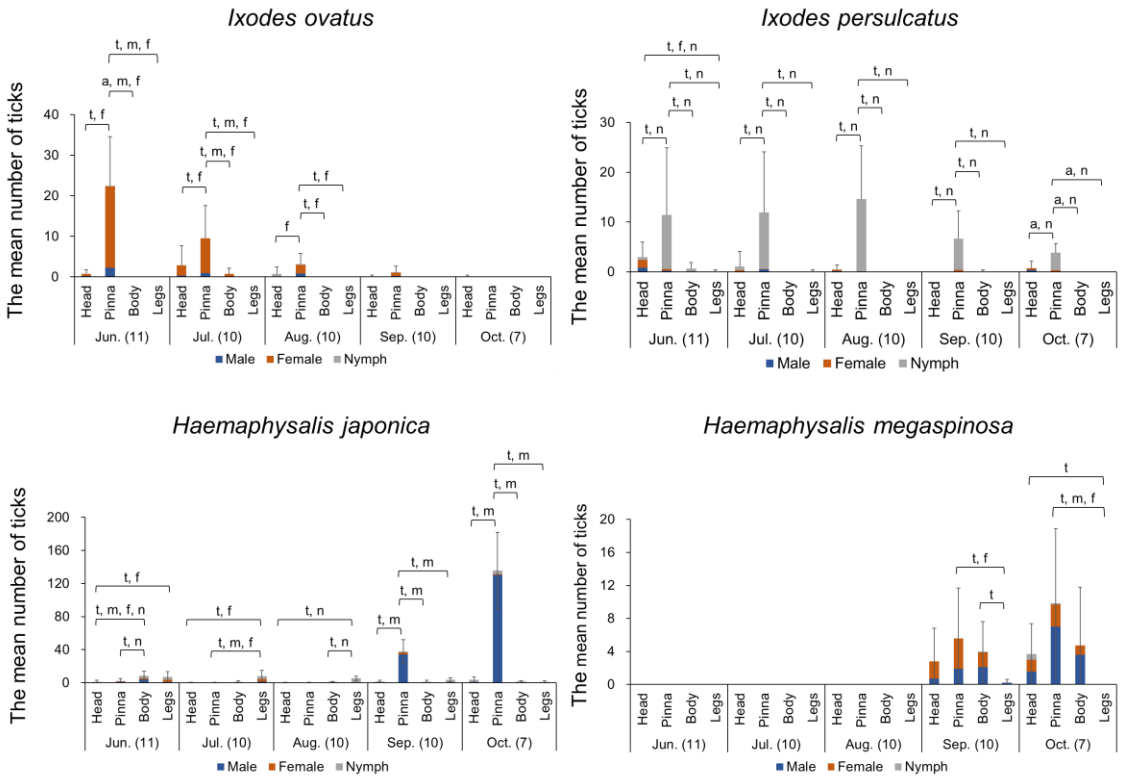

Figure 3. Seasonal occurrence of ticks on the examined attachment sites of infested sika deer from June to October 2022. Error bars represent standard errors of the total number of ticks attached to each site, and the horizontal axis represents the sampling month (number of deer).

(t, m, f, n: $P < 0.05$, based on the Steel–Dwass method for total, male, female, and nymph ticks, respectively).

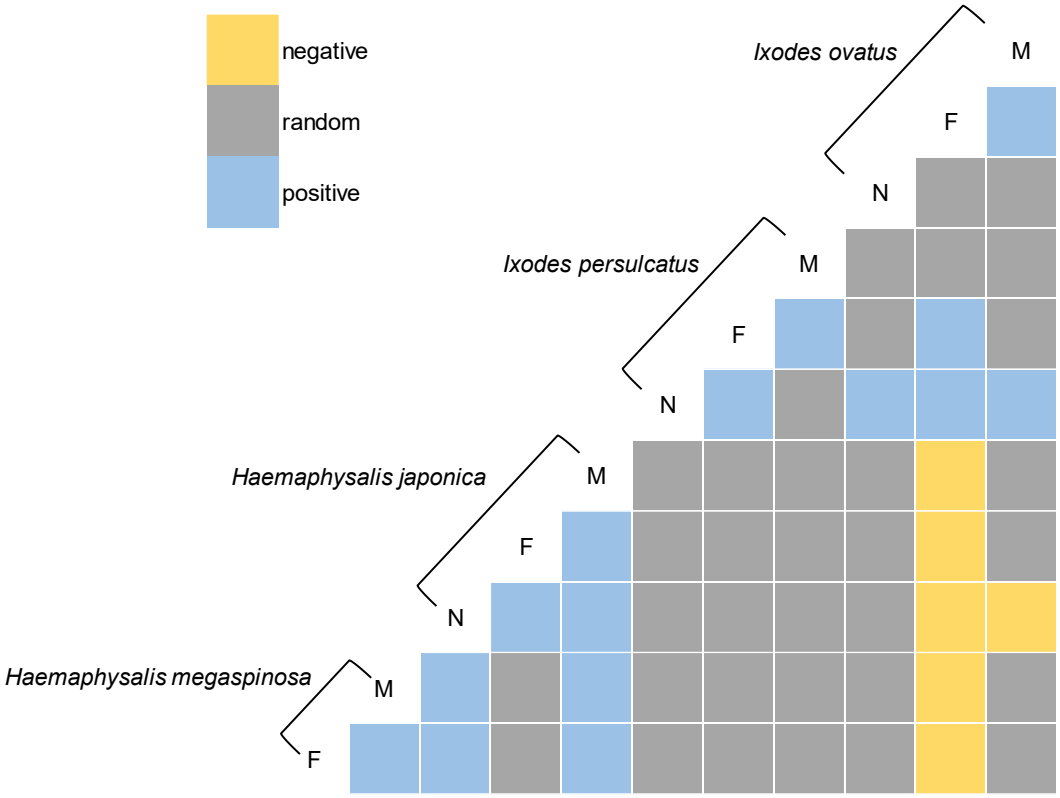

Figure 4. Heat map representing positive, negative, and random associations of tick species/stages found in each body part of individual sika deer, as determined using the stochastic co-occurrence model in the 'co-occurrence' package in R. Species names and stages are arranged to show columns and rows representing pairwise relationships with other species/stages.

(M; male, F; female, N; nymph)

Discussion

This is the first detailed study of tick infestation patterns in the Hokkaido sika deer. Seasonal activity, attachment site preferences, and co-occurrence patterns of tick species were investigated. In this study, four tick species, *I. ovatus*, *I. persulcatus*, *Ha. japonica*, and *Ha. megaspinosa*, were collected from Hokkaido sika deer, and each species showed unique seasonal fluctuations. Specific attachment site preferences were observed for each tick species, with *Ha. japonica* showing seasonal variation in attachment site preferences. Ticks showed various co-occurrence patterns on the body surfaces of deer, suggesting the presence of inter and intraspecific interactions between ticks and their attachment sites. These results provided robust evidence that complex host-tick and tick-tick interactions exist during the selection of tick attachment sites, as previously suggested (Andrews et al., 1981; Baer-Lehman et al., 2012; Chilton et al., 1992; Kiffner et al., 2011; Lydecker et al., 2019; Mysterud et al., 2014; Tiffin et al., 2021).

Adults of *I. ovatus* were collected in the highest numbers in June and then declined toward October. Previous studies on the seasonal activity of *I. ovatus* in the natural environment of Honshu, Japan, have reported that *I. ovatus* was highly active from April to mid-July but declined markedly in late August (Fujimoto, 1997, 1996). Although sampling before June was not possible in this study, the activity pattern of *I. ovatus*, with a gradual decline beginning in June, was consistent with the activity pattern of this species reported in previous studies. In contrast, nymphs peaked in abundance and prevalence in August, but the number collected was very small throughout the study period. In general, larvae and nymphs of two- and three-host ticks prefer smaller animals, whereas adults prefer larger animals (McCoy et al., 2013). Given that several small- and medium-sized animal species are present in the study area (Kawamura et al., 2022), it is likely that other animal species serve as primary hosts for *I. ovatus* nymphs. This preference for smaller hosts likely contributed to the low number of nymphs collected from the deer in this study.

In contrast to the other three species that displayed clear seasonal variation, *I. persulcatus* abundance remained stable throughout the study period. A study in Finland has reported that *I. persulcatus* nymphs survived for long periods without exhibiting clear seasonal fluctuations (Pakanen et al., 2020), which is consistent with the results of the present study. Nymphs were predominant throughout the sampling period, and adult ticks were present in very small numbers. As mentioned above, nymphal ticks prefer small to medium-sized mammals as hosts, while adult ticks tend to parasitize larger mammalian

species (McCoy et al., 2013). However, the scarcity of *I. persulcatus* adults in this study suggested that there are other suitable hosts for these adults in Hokkaido. The most likely host of *I. persulcatus* adults is the Hokkaido brown bear, another large mammal present in Hokkaido. A previous study examining ticks infesting Hokkaido brown bears has reported that most *I. persulcatus* infesting bears were adult ticks (Ozawa and Kadosaki, 1996). However, the previous and present studies have been conducted in different areas of Hokkaido (Ozawa and Kadosaki, 1996). Further investigations involving multiple wild animals at the same location are necessary to gain a better understanding of the host preferences of *I. persulcatus*.

The total number of *Ha. japonica* was low in the summer but increased significantly in the autumn. Furthermore, the increase in number from September onward was primarily due to the presence of males, whereas the numbers of females and nymphs did not show such significant fluctuations. A similar seasonal variation was observed for *Ha. megaspinosa*, which was collected only from September onward, with males predominating in October. A previous study on Hokkaido brown bears has reported that the abundance of *Ha. japonica* and *Ha. megaspinosa* increased in November, which is somewhat different from the results of the present study (Ozawa and Kadosaki, 1996). Although geographic and temporal gaps between this and previous study should be noted, these differences in results suggested that ticks establish different relationships with different animal species. The male-biased attachment in *Haemaphysalis* spp. observed in the present study has also been previously reported and may be related to reproductive strategies (Ozawa and Kadosaki, 1996; Tsunoda, 2012). Metastriate ticks, including *Haemaphysalis* spp., secrete pheromones that control aggregation, mating, and ejaculation after attachment to the host, and they mate only on the body surface of the host, whereas *Ixodes* spp. frequently mate before attachment to the host (Kiszewski et al., 2001). Therefore, it is advantageous for males of *Haemaphysalis* spp. to attach to their hosts earlier than females, wait for mates, and remain on the host longer after mating (Tsunoda, 2012). Another possibility is that unmated males attempt to overwinter on the host (Doi et al., 2018; Ozawa and Kadosaki, 1996); however, research on the overwintering strategies of ticks is limited (Kim et al., 2020), and no conclusive statements can be made.

Co-occurrence analysis revealed positive co-occurrence patterns between male and female ticks of the same species, and these patterns were observed in all tick species. These results suggest that the reproductive strategy of ticks promotes male-female

aggregation, as previously reported (Sonenshine, 2004, 1985). Additionally, positive co-occurrence patterns between females and nymphs were observed for *I. persulcatus* and *Ha. japonica*. Differences in the attachment sites at different life stages have been reported in several species (Tiffin et al., 2021). *Ixodes persulcatus* nymphs were clustered on the pinna, whereas adults were attached to the pinna and head, with a slightly higher frequency of attachment to the head. However, these two stages shared the same attachment sites on the head and pinna, possibly resulting in a positive co-occurrence pattern. These results are interesting when considering disease dynamics in nature via co-feeding transmission. Co-feeding transmission occurs when pathogens are transmitted between infected and naïve vectors that feed in close temporal and spatial proximity to hosts that have not yet developed systemic infections (Belli et al., 2017). Co-feeding transmission has been confirmed in several animals and tick species for some pathogens, such as tick-borne encephalitis virus (Labuda et al., 1996, 1993) and *Borrelia burgdorferi* (Gern et al., 1998; Gern and Rais, 1996; Ogden et al., 1997; Randolph et al., 2003). Both pathogens are vectored by *I. persulcatus* in Hokkaido (Hayasaka et al., 1999; Muto et al., 2015; Okado et al., 2021). The results of this study suggested that similarities in attachment site preference between ticks at different stages could potentially facilitate co-feeding transmission. Hence, this study has important implications for understanding the dynamics of tick-borne diseases in wildlife and highlights the need for further research to understand how ticks of different species and stages interact with each other.

The results of the co-occurrence analysis of different species showed negative co-occurrence patterns between *I. ovatus* and *Haemaphysalis* spp. Three hypotheses may explain the positive and negative co-occurrence patterns of ticks among different species.

The first hypothesis was that similarities and differences in phenology between the same or different genera and species affect co-occurrence patterns. Previous studies have reported phenological differences between *Ixodes* spp. and *Haemaphysalis* spp. and similarities within the same genus (Kanamori et al., 2009; Ozawa and Kadosaki, 1996). In the present study, *I. ovatus* and *Ha. megaspinosa* peaked in abundance and infestation rates in summer and autumn, respectively. A previous study has also reported clear phenological differences between the two species (Ozawa and Kadosaki, 1996). Based on these results, phenological differences may be the most plausible explanation for the negative co-occurrence patterns observed between the two species.

The second hypothesis was that morphological similarities within the same taxon promote positive co-occurrence among tick species of the same genus. A previous study

on similar attachment site preferences within the same subgroups of ectoparasites supports this hypothesis (Pilosof et al., 2012). The most prominent morphological difference between *Ixodes* spp. and *Haemaphysalis* spp. is the length of their mouthparts, which consists of a pair of chelicerae and a single hypostome. *Ixodes* spp. have longer and sharper mouthparts than *Haemaphysalis* spp. and secrete fewer cementing substances to hold the body in place during engorgement (Balashov, 1972). Such morphological differences could influence the availability of host blood vessels and resistance to grooming (Pilosof et al., 2012) and may explain the similarities in attachment site preferences among the same genus groups. The fact that *I. ovatus* adults and *I. persulcatus* nymphs were primarily attached to the pinna during the study period suggests a high degree of compatibility with the pinna. The pinna has been reported to be the preferred feeding site for certain ticks that feed on ungulates because it is difficult for the host to groom this area with its mouth and legs (Handeland et al., 2013; Matthee et al., 1997; Mysterud et al., 2014). In contrast, the distribution of *Ha. megaspinosa* was wider than that of the other tick species found in the present study and was distributed on the head, pinna, and body. Similar attachment patterns have been observed in other tick species (Sundstrom et al., 2021; Tiffin et al., 2021). This suggested that some species may have adapted to a wide range of host body parts. However, the negative co-occurrence patterns of *I. ovatus* and *Ha. japonica* could not be explained by phenological and morphological differences alone. This is because, although both species had a high prevalence in June and July, the attachment sites were separate, and *Ha. japonica* showed dynamics such that it moved to the pinna from September onward when *I. ovatus* was no longer present. This suggested that other factors play a role in the co-occurrence patterns.

The third hypothesis was that negative co-occurrence patterns were formed to avoid interference due to similarities in semiochemical communication, which may explain the patterns of *I. ovatus* and *Ha. japonica*. Semiochemicals are chemical compounds that arthropods secrete outside their bodies to communicate with other individuals, guiding behaviors such as mate location, host finding, and predator avoidance (Sonenshine, 2004). Semiochemical-mediated changes in tick behavior and intraspecific and interspecific communication are well known (Sonenshine, 2004), and in some cases, some species use the same chemicals. For example, it has been reported that *I. ricinus* and *I. scapularis* exhibit similar arrest behaviors by guanine and xanthine (Sonenshine, 2004). In metastriate ticks, differences in the concentrations of 2,6-dichlorophenol induce different reactions depending on the species (Sonenshine, 1985). Furthermore, when

reptile ticks, such as *Bothriocroton hydrosauri*, *Amblyomma albolirnbatum*, and *Am. limbatum* infest the same host, the movement of males searching for sexually receptive females is constrained, and males display courtship behavior toward females of different species (Andrews et al., 1982a). It has been suggested that such signal interference may promote spatial separation among tick species (Andrews et al., 1982b; Chilton et al., 1992). Although there have been no previous reports on the interference of semiochemical communication between *I. ovatus* and *Ha. japonica*, this is the first study to suggest an interspecific interaction between these two species at the field level. Laboratory-based behavioral studies are necessary to investigate the semiochemical-mediated relationship between these two species.

Random co-occurrences were the most frequently observed among all the co-occurrence patterns. It is well known that ticks of different species feed on the same host (Baer-Lehman et al., 2012; Cayol et al., 2018; Gómez-Rodríguez et al., 2015). Therefore, it is not surprising that the neutral interspecific relationships suggested by the random co-occurrence patterns were most frequently observed. Neutral interspecific relationships are beneficial when sufficient resources (hosts) are available for tick survival (Butler et al., 2021; Canard et al., 2012). The tick species collected in this study are generalists that feed on various hosts (Elbaz et al., 2020; Ozawa and Kadosaki, 1996; Sashika et al., 2011; Tsunoda, 2012). Furthermore, sika deer are bigger than other land mammals (the average was 76.2 kg in this study), providing ticks with a large feeding area and abundant blood meals (Iijima et al., 2022), and deer density in the study area has been reported to be very high (Uno et al., 2022). Therefore, it is likely that ticks have sufficient feeding resources in the study area and that a neutral relationship prevailed.

This study suggests that each tick species has unique attachment site preferences on the Hokkaido sika deer body surface and positive, negative, or random co-occurrence patterns with ticks of both conspecific and heterospecific groups. However, the observed interactions between tick species might result from various confounding factors. The risk of inferring species interactions from observational data has been previously discussed. Confounding effects may occur at the host individual (host age and sex), population (host sex ratio, age structure, and density), parasite (presence of risk factors shared by two or more parasites), or landscape level (geological and climatic factors) (Hellard et al., 2015). In particular, it was not possible to examine how deer host characteristics, such as sex and age affect tick infestation. Nonetheless, this is the first study to suggest the possibility of tick species interactions on wild sika deer, highlighting the need for further experimental

studies to elucidate the underlying mechanisms.

Summary

Ticks prefer specific feeding sites on the host that are influenced by host-tick and tick-tick interactions. This study focused on the spatiotemporal distribution of ticks in Hokkaido sika deer, an important tick host in Hokkaido, Japan. Tick sampling was performed on the sika deer in the Shiretoko National Park between June and October 2022. Ticks were collected from nine different body parts of the deer to compare their attachment site preferences. Interspecific and intraspecific relationships among ticks were examined using co-occurrence analysis. The collected ticks were nymphal and adult stages of four species: *I. ovatus*, *I. persulcatus*, *Ha. japonica*, and *Ha. megaspinosa*. Seasonal variations in tick burden were observed, with *I. ovatus* and *I. persulcatus* in June and declining toward October; *Ha. japonica* showing low numbers in July and August and increasing from September; and *Ha. megaspinosa* appearing from September onward with little variation. Attachment site preferences varied among species, with a significant preference for the pinna in *I. ovatus* and *I. persulcatus*. *Haemaphysalis japonica* was mainly found on the body and legs between June and August and shifted to the pinna in September. *Haemaphysalis megaspinosa* showed a general preference for areas other than the legs. Co-occurrence analysis revealed positive, negative, and random co-occurrence patterns among the tick species. Ticks of the same genus and species exhibited positive co-occurrence patterns; *I. ovatus* showed negative co-occurrence patterns with *Haemaphysalis* spp. This study revealed the unique attachment site preferences and distinct seasonal distributions of tick species in the Hokkaido sika deer.

Chapter II

Patterns of tick infestation and tick-borne pathogen prevalence in sika deer and brown bears of Hokkaido

Introduction

Tick-borne diseases pose significant risks to wildlife and human populations, and the ecology of these diseases is shaped by complex interactions among hosts, vectors, and pathogens. These transmission processes are shaped by environmental conditions, host characteristics, and vector behavior, creating distinctive epidemiological patterns across different ecosystems. Ticks are hematophagous ectoparasites that, in many species, require multiple hosts to complete their life cycle. Most ixodid ticks in Japan are three-host tick species that require different hosts at each developmental stage: larva, nymph, and adult (Takano, 2015). These developmental stages require suitable temperature and humidity for survival and development, and their distribution and seasonal activity are largely constrained by climatic conditions (Doi et al., 2021; Nuttall, 2022; Yang et al., 2021).

Hokkaido, the northernmost island of Japan, has a humid continental climate with distinct regional variations, with winter temperature commonly falling below freezing temperatures throughout most regions (Aurich, 2008; Beck et al., 2018). Winter conditions vary markedly across the island; snow depth decreases from West to East due to reduced precipitation, while the East regions experience more severe cold, resulting in soil freezing depths of 35–50 cm (Christopher et al., 2008; Okawa, 1992). This substantial climatic difference may have affected the seasonal fluctuations in arthropods and their hosts in the region (Burtis et al., 2016).

Stage-specific host preferences have been reported for ticks, with larvae and nymphs primarily parasitizing small and middle mammals and adults targeting larger hosts (McCoy et al., 2003). These preferences can be influenced by environmental conditions, host availability, and interspecific competition among tick species (Doi et al., 2021; Gandy et al., 2021). Host-related factors also influence tick infestation patterns. Sex differences in the parasite burden are commonly observed, with male mammals often exhibiting higher infestation levels owing to testosterone-induced immunosuppression and sexual dimorphism (Dhakal et al., 2023; Hillegass et al., 2008; Takatsuki et al., 2024).

Age-related effects are more complex, as aging leads to acquired immunity that can reduce parasite loads while simultaneously resulting in increased body size and movement range that enhances exposure (Anderson et al., 2013; Butler et al., 2020; Vor et al., 2010). Additionally, defensive behaviors such as grooming and tick avoidance strategies also play a role in shaping infestation patterns by influencing tick attachment success (Blaise et al., 2023; Chamberlin, 2021; Mamillapalli et al., 2016).

The abundance and diversity of available wildlife hosts further shape local tick assemblages (Keesing et al., 2006). Several mammalian species in Hokkaido serve as major tick hosts, ranging from small mammals (small Japanese field mouse, *Apodemus argenteus*; large Japanese field mouse, *Ap. speciosus*; grey red-backed vole, *Myodes rufocanus*; and northern red-backed vole, *M. rutilus*) to medium-sized mammals (raccoon, *P. lotor*; and raccoon dog, *N. procyonoide*s) and large mammals (Hokkaido sika deer, *C. nippon yesoensis*; and Hokkaido brown bear, *U. arctos yesoensis*) (Elbaz et al., 2020; Ito et al., 2024; Ozawa and Kadosaki, 1996; Sashika et al., 2011; Shimizu et al., 2024; Taylor et al., 2013). Among these, Hokkaido sika deer and Hokkaido brown bears are widely distributed across Hokkaido, forming a predator-prey relationship (Sato et al., 2005; Shirane et al., 2021). In Hokkaido, sika deer and brown bear populations exhibit distinct regional genetic structures and dynamics. Brown bear populations in Hokkaido show mitochondrial DNA differentiation into three geographic clusters (Central, Eastern, and Southwestern regions), indicating limited female-mediated gene flow (Matsuhashi et al., 1999), whereas sika deer populations have a relatively homogeneous genetic structure across the island (Nabata et al., 2007; Terada et al., 2013). These differences in population connectivity might influence the distribution patterns of host-specific pathogens and their vectors. Their long-distance movement capacity plays a critical role in tick dispersal, and their large body size provides a large amount of blood meals for many ticks (Al-Warid et al., 2017; Doi et al., 2020; Iijima et al., 2022; Merrill et al., 2018). However, the tick fauna associated with these species has only been studied in limited geographic areas and seasons (Ozawa and Kadosaki, 1996; Shimizu et al., 2024).

In Hokkaido sika deer, AP-sd has been frequently reported and is characterized as a deer-specific strain of *An. phagocytophilum* based on studies conducted in Japan and China (Kawahara et al., 2006; Liu et al., 2012; Moustafa et al., 2015; Wu et al., 2015; Yang et al., 2013). This organism is transmitted by both *Ixodes* and *Haemaphysalis* spp. (Ybañez et al., 2012) and has been detected in multiple host species, with the sika deer serving as the primary reservoir host (Kawahara et al., 2006; Moustafa et al., 2015). In

Hokkaido brown bears, a preliminary survey (unpublished data) revealed high infection rates of *He. ursi*, representing the first documentation of this organism in bear populations in Hokkaido. *Hepatozoon ursi*, which infects ursids through the ingestion of *Haemaphysalis* spp. containing sporulated oocysts, exhibits high infection rates but low pathogenicity in bear populations (Kubo et al., 2010; Moustafa et al., 2020; Pawar et al., 2011; Tsai et al., 2024). Previous surveys in Hokkaido reported extremely low prevalence of *Hepatozoon* spp. infections in brown bears, with no molecular identification at the species level (Kubo et al., 2010), and even recent studies in the Shiretoko Peninsula failed to detect *He. ursi* in brown bears (Moustafa et al., 2020). This temporal pattern suggests that *He. ursi* may represent an emerging pathogen in Hokkaido brown bear populations.

Understanding the ecology of ticks and their interactions with wildlife hosts is fundamental for elucidating the dynamics of tick-borne organisms (Benavides et al., 2012; Bournez et al., 2020; Newman et al., 2015). Although tick distribution and host infection patterns have been investigated in various regions of Japan, studies on tick fauna and pathogenic infections in large mammals in Hokkaido remain limited, especially in sika deer and brown bears. Based on extensive surveys across Hokkaido from April 2021 to February 2024, this study investigated how environmental (region and season) and host-related (sex and age) factors influence tick infestation and pathogen infection patterns in these two species. Widespread prevalence of AP-sd and *He. ursi* in their respective hosts has enabled robust statistical analyses to understand the ecological dynamics among hosts, ticks, and pathogens. By examining AP-sd in sika deer and *He. ursi* in brown bears—two pathogen systems with different host and vector associations—this study aimed to comprehensively understand the disease ecology of tick-borne pathogens in these two major large mammals in Hokkaido.

Materials and methods

Study area

For this study, Hokkaido was divided into four regions with reference to the regional classification of the Management scheme of Yezo sika deer in Hokkaido (sixth period; in Japanese) (Hokkaido, 2022a) and Brown bear management plan in Hokkaido (second period; in Japanese) (Hokkaido, 2022b). These regional classifications, while partially reflecting subpopulations based on geographic barriers, genetic clusters, and population distribution status for each species (Hokkaido, 2022a, 2022b), were artificially established for this study to ensure comparable sample sizes and facilitate statistical analyses and comparison between the two animal species. The four regions were the East (Okhotsk, Tokachi, Kushiro, and Nemuro subprefectures), North (Sorachi, Kamikawa, Rumoi, and Soya subprefectures), Central (Ishikari, Iburi, and Hidaka subprefectures), and South (Shiribeshi, Oshima, and Hiyama subprefectures) regions (Figure 5). The seasons were defined according to the Japan Meteorological Agency standard classification: spring (March–May), summer (June–August), autumn (September–November), and winter (December–February).

Sample collection

Samples for tick infestation and pathogen infection surveys were collected from sika deer and brown bears in Hokkaido, between April 2021 and February 2024. For sika deer, samples for tick collection and pathogen screening were obtained from three regions: East, North, and South Hokkaido. For brown bears, tick samples were collected from three regions (East, Central, and South Hokkaido), while samples for pathogen screening were obtained from all four regions, including the North region. Sample collection employed two methods: collected from harvested individuals of both sika deer and brown bears, and live captured sika deer. Pinna (either left or right) and liver samples were collected from the harvested individuals. Some of the brown bear samples were provided by the Hokkaido Research Organization, which has collected samples including livers and teeth from brown bears killed by nuisance control and hunting for monitoring purposes, with some of these samples also containing pinnae. In East Hokkaido, sika deer were repeatedly captured from June to October 2022 following the protocols described in previous studies (Shimizu et al., 2024, 2025). During this period, 37 individuals were

captured during 48 capture events, ticks on the left pinna, and blood samples were collected from each deer. The total number of samples collected from each region is presented in Table 3. The dataset lacks tick samples from brown bears in North Hokkaido and all samples (both tick and pathogen screening) from sika deer in Central Hokkaido.

For each sampled individual, comprehensive metadata were recorded including sex, age, and harvest or capture date and location. This classification was established based on the dispersal age of each species (Jiang et al., 2022; Shimozuru et al., 2017; Shirane et al., 2019; Takii et al., 2012). For deer, age estimates were based on comprehensive assessment of physical morphological characteristics, reproductive status, and dispersal status (Hayden et al., 1994; Jiang et al., 2022; Suzuki et al., 2001; Takii et al., 2012), supplemented by tooth eruption and replacement patterns when feasible for more precise age estimation (Ohtaishi, 1977). For bears, age estimates were determined using cementum annuli analysis of teeth (Yoneda, 1976). All data of killed bears provided by the Hokkaido Research Organization were obtained from the HRO Bear Database ver. 20250121 maintained by the Hokkaido Research Organization.

Based on the results from Chapter I, the pinna was confirmed to be a particularly preferred attachment site for ticks. Ticks were collected from thawed pinna samples by using a systematic method. The specimens were meticulously examined under optimal lighting conditions, including comprehensive inspection of the sample containers. Ticks were collected by palpation from the base of the pinna to the tip. Identification was performed using morphological characteristics under a stereomicroscope using an identification key (Yamaguti et al., 1971), with classification based on the species, sex, and developmental stage. Owing to morphological identification challenges, the larvae were excluded from subsequent statistical analyses.

All procedures involving the capture and sampling of live animals were conducted by the Guidelines for Animal Care and Use, Hokkaido University, and with the approval of the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Permit Number: 22–0016). The study samples of killed bears collected by Hokkaido Research Organization were obtained in accordance with the Hokkaido administrative plan and do not require any specific ethical approval.

Molecular analysis

DNA was extracted from blood and liver samples using a DNeasy Blood and

Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and stored at −30°C until analysis. Polymerase chain reaction (PCR) was performed to detect AP-sd in sika deer, while brown bear samples were screened for *He. ursi*. Both blood and liver samples were used to detect AP-sd in sika deer and it was confirmed that the results were consistent between both sample types (data not shown). PCR was performed in 25-μL reaction mixtures containing 12.5 μM of 2× Gflex PCR Buffer (Mg^{2+} dNTP plus) (Takara Bio Inc., Kusatsu, Japan), 0.5 μL of Tks Gflex DNA polymerase (1.25 units/μL) (TaKaRa Bio Inc.), 10 μM of each primer, and 1.0 μL of template DNA. The primers used to detect AP-sd and *He. ursi* are listed in Table 4. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator. Positive bands were excised and purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

Sequencing was performed using the forward primers used for PCR amplification and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), purified using the BigDye X Terminator Purification Kit (Applied Biosystems) and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The obtained sequences were identified by comparison with reference sequences in GenBank using BLASTn searches.

The selected samples were sequenced to confirm pathogen identity. For each pathogen, representative positive samples (up to five) from each region were selected for bidirectional sequencing using the same primers as those used for PCR. The sequences obtained in this study were submitted to DDBJ with accession numbers LC871429-LC871434 for AP-sd and LC871435-LC871444 for *He. ursi*.

Statistical analysis

GLMMs were used to evaluate pathogen infection status and tick infestation patterns using only individuals with complete data for all variables of interest. For pathogen analysis, the infection status (positive/negative) of each host (AP-sd: sika deer, *He. ursi*: brown bears) was used as a response variable, assuming a binomial distribution. For tick analyses, the number of ticks of each species and developmental stage were used as response variables, assuming a negative binomial distribution with a log-link function.

Environmental (sampling region and season) and host factors (sex and estimated age) were used as explanatory variables. Table 5 presents the details of the explanatory

variables. To account for potential inter-annual variations in tick infestation and pathogen prevalence across the three-year study period and to control for sampling effort, sampling year (April–March of the following year) was included as a random effect in all models. This approach allowed statistically control for year-to-year fluctuations, while focusing on the effects of our primary variables of interest (region, season, host sex, and age).

To avoid overfitting and estimation instability, spring samples from sika deer, which were collected from only one individual, were excluded from the tick infestation model construction, and categories in which no ticks were collected for a particular species or developmental stage were excluded from the explanatory variables. Potential multicollinearity among explanatory variables was assessed using variance inflation factors (VIF), with variables showing VIF values greater than 10 considered multicollinear and were excluded before model selection.

GLMMs were constructed and evaluated using the glmmTMB package (Brooks et al., 2017) in R version 4.1.0 (R Core Team, 2021), with model convergence assessed using the default optimization algorithm nlminb. The convergence was assessed based on standard criteria, including a maximum gradient magnitude of 0.001, a relative convergence tolerance of 1e-10, and a maximum of 300 iterations. Models that failed to meet these criteria were deemed nonconvergent and excluded from further analysis to ensure reliability.

All possible combinations of explanatory variables were evaluated and the best models were selected based on the Akaike Information Criterion. Statistical significance was set at $P < 0.05$. To evaluate the best models, both marginal R^2 (the proportion of variance explained by fixed effects alone) and conditional R^2 (the proportion of variance explained by both fixed and random effects) were computed for each GLMM. The contribution of the random effect of the sampling year was evaluated by comparing the marginal R^2 and the conditional R^2 for each model. The car package (Fox J and Weisberg S, 2019) and the performance package (Lüdecke et al., 2021) in R version 4.1.0 (R Core Team, 2021) were used to calculate the VIF values and R^2 values, respectively.

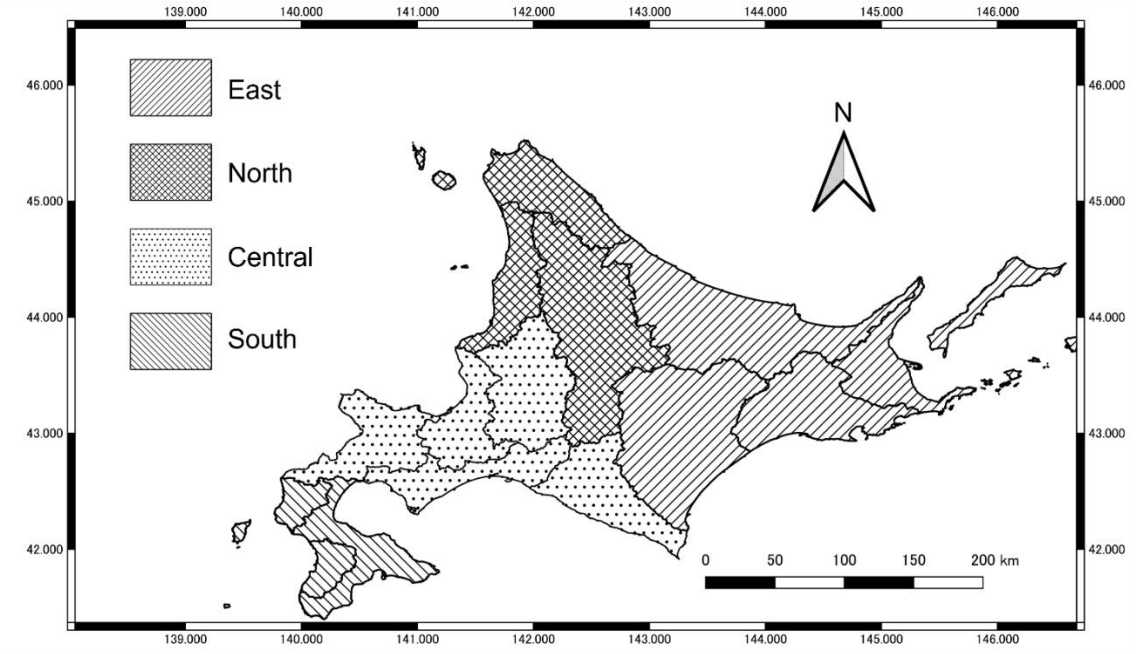

Figure 5. Study areas in Hokkaido, Japan, divided into four regions based on subprefectures. East region: Okhotsk, Tokachi, Kushiro, and Nemuro subprefectures; North region: Sorachi, Kamikawa, Rumoi, and Soya subprefectures; Central region: Ishikari, Iburi, and Hidaka subprefectures; South region: Shiribeshi, Oshima, and Hiyama subprefectures.

Table 3. Number of samples collected from sika deer and brown bears in Hokkaido.

	Region	Year	No. of pinna samples	No. of liver samples
Sika deer	East Hokkaido	2021	0	22
		2022 [a]	48	48
		2023	10	0
	North Hokkaido	2021	0	23
		2022	49	49
	South Hokkaido	2021	0	24
		2022	21	21
		2023	29	36
Brown bear	East Hokkaido	2021	14	37
		2022	17	25
		2023	128	133
	North Hokkaido	2021	0	21
		2022	0	23
		2023	0	13
	Central Hokkaido	2021	9	42
		2022	20	38
		2023	39	39
	South Hokkaido	2021	4	28
		2022	10	24
		2023	14	14

[a], During this study period, 37 deer were captured in 48 capture events, and ticks were collected from the left pinna along with blood samples from each animal.

Table 4. List of primers used for pathogen detection.

Target organism	Target gene	Primer name	Primer sequence (5'−3')	Expected size (bp)	Reference
Anaplasma sp. AP-sd	16S rRNA	EC9	TACCTTGTTACGACTT	~1,462	(Sashika et al., 2011)
		EC12A	TGATCCTGGCTCAGAACGAACG		
	16S rRNA	AP-f1	CATGCAAGTCGAACGGGTTA	~770	
		AP-r1	CATCAACACGGAGATAAATTATC		
Hepatozoon ursi	18S rRNA	Piro0F	GCCAGTAGTCATATGCTTGTGTTA	~450	(Kawabuchi et al., 2005)
		Piro6R	CTCCTTCCTYTAAGTGATAAGGTTCAC		
	18S rRNA	HepF	ATACATGAGCAAAATCTCAAC	~667	(Inokuma et al., 2002b)
		HepR	CTTATTATTCCATGCTGCAG		

Table 5. Description of explanatory variables used to analyze tick infestation patterns and pathogen infection status.

Factor	Variable	Definition
Environmental	Region	Sampling regions: Sika deer (East [a], North, South Hokkaido); Brown bear (East [a], North [*Hepatozoon ursi* only], Central, South Hokkaido)
	Season	Season in which the host animal was sampled (Spring: March–May, Summer: June–August, Autumn [a]: September–November, Winter: December–February of the following year)
Host related	Sex	Sex of the animal (male, female [a])
	Estimated age	Estimated age categories: cub/subadults [a] (bears: <3 years, deer: <2 years) and adults (bears: ≥3 years, deer: ≥2 years)

[a], Reference category for each variable.

Results

Tick-borne pathogens screening in sika deer and brown bears

Between April 2021 and February 2024, 223 sika deer (70, 72, and 81 from East, North, and South Hokkaido, respectively) and 437 brown bears (195, 57, 119, 66 from East, North, Central, and South Hokkaido, respectively) samples from different regions of Hokkaido were analyzed for tick-borne pathogen infection. GLMM analyses included 223 sika deer and 381 brown bears with complete datasets for all variables of interest. The models showed that the conditional R^2 was nearly identical to the marginal R^2, indicating that the variance attributed to the sampling year was close to zero, suggesting consistent patterns across the study period. All pathogen gene sequences obtained from Hokkaido sika deer and brown bears in this study showed high identity with previously reported reference sequences. AP-sd sequences from sika deer showed 99.3–100% identity with those previously reported from Hokkaido sika deer (GenBank accession numbers MH489010, MH489005, and MH489004), while *He. ursi* sequences from brown bears displayed 99.9–100% identity with those previously identified in Japanese black bears (*U. thibetanus japonicus*) (GenBank accession numbers EU041717 and EU041718).

AP-sd prevalence in sika deer showed marked regional differences, with higher rates in South (91%) and East Hokkaido (76%), compared to the notably lower prevalence in North Hokkaido (13%) (Table 6 and Figure 6). This regional pattern remained consistent across the different sampling periods, suggesting stable transmission dynamics within each region. The GLMM analysis incorporated both region and season as variables in the best-fit model, with region emerging as the only statistically significant factor (Table 7).

Hepatozoon ursi infection in brown bears exhibited pronounced regional variation, creating a clear geographical pattern across Hokkaido (Table 8 and Figure 7). Infection rates were substantially higher in South (77%) and Central Hokkaido (80%) compared to the markedly lower prevalence in East (10%) and North Hokkaido (4%) (Table 8). The GLMM analysis identified both region and host age as significant explanatory variables (Table 9). Additionally, host age emerged as a significant predictor, with adult bears showing lower infection rates than cubs/subadults.

Tick infestation patterns in sika deer and brown bears

Tick infestation patterns were examined across 157 sika deer (8, 49, and 50 from East, North, and South Hokkaido, respectively) and 255 brown bears (159, 68, and 28 from East, Central, and South Hokkaido, respectively). In total, 21,096 ticks were collected from the sika deer and brown bears. Of these, 6,566 ticks (2,394 males, 2,467 females, and 1,705 nymphs) were collected from sika deer, while 14,530 ticks (5,873 males, 5,615 females, and 3,042 nymphs) were obtained from brown bears. These ticks belong to six species from two genera: *Ixodes* (*I. ovatus* and *I. persulcatus*) and *Haemaphysalis* (*Ha. japonica*, *Ha. megaspinosa*, *Ha. flava*, and *Ha. longicornis*) (Table 10). The total number of ticks (collected from sika deer and brown bears, respectively) for each species was as follows: *I. ovatus* (2,143 and 4,970); *I. persulcatus* (729 and 116); *Ha. japonica* (1,748 and 3,385); *Ha. megaspinosa* (1,819 and 5,899); *Ha. flava* (30 and 135); and *Ha. longicornis* (97 and 25).

Robust GLMMs were obtained for the following host-tick combinations after evaluating model convergence and reliability criteria using up to 157 sika deer and 224 brown bears with complete datasets. In sika deer, *I. ovatus* (females), *I. persulcatus* (nymphs), *Ha. japonica* (males, females, and nymphs), and *Ha. megaspinosa* (males and nymphs). For brown bears, reliable models were obtained for the same combinations with the addition of females of *Ha. megaspinosa* and *Ha. flava* (males and females). Similar to the pathogen models, the random effect of sampling year had a minimal influence on tick infestation patterns, with conditional R^2 values nearly identical to marginal R^2 values in most cases. In some tick models, the variance component for the year was so small that the conditional R^2 could not be reliably computed, further suggesting a negligible influence of inter-annual variation on the observed patterns. The GLMM results for these validated models are summarized in Tables 11 and 12.

Ixodes ovatus was widely distributed across all study regions in both host species. In sika deer, infestation levels were highest in North Hokkaido (Figure 8A), whereas in brown bears, infestation peaked in East Hokkaido, with relatively lower levels in South and Central Hokkaido (Figure 9A). Seasonal patterns were similar between the host species, with peak infestations occurring in May–June, followed by a decline in autumn. GLMM analysis of adult female ticks identified region, season, and host sex as significant factors in sika deer, with male deer exhibiting higher levels of infestation (Table 11). In brown bears, the region and season significantly affected the infestation levels of adult female ticks, but no significant host-related factors were detected (Table 12).

Ixodes persulcatus exhibits contrasting distribution patterns among host species. In sika deer, infestation was high in East and North Hokkaido, with nymphs being the most predominant and showing no significant seasonal variation, while only six nymphs were identified in South Hokkaido (Figure 8B). In brown bears, infestation was low, peaking in East Hokkaido, where adult female ticks were the most abundant from April to August (Figure 9B). After autumn, adult ticks were rarely detected and the number of nymphs remained consistently low. In other regions, tick presence was minimal, with only three ticks recorded in spring in Central Hokkaido and none found in South Hokkaido. GLMM analysis of *I. persulcatus* nymphs identified region as a significant factor in sika deer (Table 11), whereas both region and host sex were significant factors in brown bears, with lower infestation levels in male bears (Table 12).

Haemaphysalis spp. demonstrated a more pronounced regional variation than *Ixodes* spp., with a notable absence in North Hokkaido (Table 10). *Haemaphysalis japonica* exhibited marked regional and seasonal variation. Sika deer in East Hokkaido showed few tick infestations before September, but infestation levels increased sharply from September onward, with adult males comprising the majority of collected ticks (Figure 8C). In South Hokkaido, nymphs predominated from June to July, whereas the number of adult females increased after August. In brown bears, infestation was the highest in East Hokkaido, with a notable increase in adult male ticks from October to December and March (Figure 9C). In South and Central Hokkaido, infestation levels were lower, with nymphs peaking in June–August and adult ticks increasing after autumn. GLMM analysis revealed significant effects of region and season on tick number across all stages in both host species (Tables 11 and 12). The host age significantly influenced the number of adult male ticks in sika deer and adult female ticks in brown bears, with adult hosts showing higher infestation levels.

Haemaphysalis megaspinosa infestation exhibited clear seasonality. In sika deer, this species was most abundant in South Hokkaido, first appearing in September, increasing sharply after October, and persisting until February (Figure 8D). In East Hokkaido, the infestation levels were lower, with adult ticks being predominant after September. In brown bears, infestation was the highest in East Hokkaido, followed by South and Central Hokkaido (Figure 9D). Adult ticks were present in small numbers from April to August but increased after September, mirroring patterns in sika deer. GLMM analysis indicated significant regional effects on all analyzed stages in both host species (Tables 11 and 12). Seasonal effects were significant in all models, except for nymph

number in sika deer. No significant host-related effects were detected in any of the analyzed models.

Haemaphysalis flava was restricted to South Hokkaido by the sika deer, where adult ticks were collected from autumn to winter (Figure 8E). In brown bears, the infestation levels were low across all regions, with the highest levels in South Hokkaido, followed by Central and East Hokkaido (Figure 9E). Both host species showed peak infestations during October–December, with predominant adult ticks. GLMM analysis revealed significant regional and seasonal effects on adult tick infestation levels in brown bears (Table 12). Additionally, adult male ticks are influenced by host age.

Haemaphysalis longicornis had the most restricted infestation for sika deer, found only in South Hokkaido in June (Figure 8F). In brown bears, only a few adult female ticks were collected from the South and Central Hokkaido (Figure 9F).

Table 6. Prevalence of *Anaplasma* sp. AP-sd in sika deer in Hokkaido.

Region	Month	No. of samples	No. of PCR-positive samples (%)
East Hokkaido	May	22	19 (86)
	June	11	10 (91)
	July	10	4 (40)
	August	10	9 (90)
	September	10	5 (56)
	October	7	6 (86)
	Subtotal	70	53 (76)
North Hokkaido	May	11	1 (9)
	June	20	2 (10)
	July	21	2 (10)
	August	9	2 (22)
	September	11	2 (18)
	Subtotal	72	9 (13)
South Hokkaido	May	5	5 (100)
	June	17	17 (100)
	July	11	9 (82)
	August	6	4 (67)
	September	11	11 (100)
	October	10	10 (100)
	November	4	3 (75)
	December	5	4 (80)
	January	3	3 (100)
	February	9	8 (89)
	Subtotal	81	74 (91)
Total		223	136 (61)

Table 7. Outputs of the best generalized linear mixed model for *Anaplasma* sp. AP-sd infection status in sika deer.

Response variable	Explanatory variable	Estimate	Standard error	*P*	Conditional R^2	Marginal R^2
Anaplasma sp. AP-sd in sika deer	Intercept	1.01	0.48	<0.05	na	0.53
	Region = North Hokkaido	−2.90	0.48	<0.001		
	Region = South Hokkaido	1.59	0.57	<0.01		
	Season = Spring	0.50	0.63			
	Season = Summer	−0.25	0.52			
	Season = Winter	−0.59	0.96			

na, Conditional R^2 could not be computed because of the negligible variance of the random effect, suggesting a minimal influence of the random factor (sampling year) on model variability.

Table 8. Prevalence of *Hepatozoon ursi* in brown bears in Hokkaido.

Region	Month	No. of samples	No. of PCR-positive samples (%)
East Hokkaido	March	1	0 (0)
	April	3	0 (0)
	May	14	3 (21)
	June	30	4 (13)
	July	26	4 (15)
	August	23	0 (0)
	September	23	4 (17)
	October	53	3 (6)
	November	18	2 (11)
	December	4	0 (0)
	Subtotal	195	20 (10)
North Hokkaido	March	3	0 (0)
	April	9	1 (11)
	May	6	0 (0)
	June	6	0 (0)
	July	5	0 (0)
	August	7	1 (14)
	September	6	0 (0)
	October	7	0 (0)
	November	6	0 (0)
	December	2	0 (0)
	Subtotal	57	2 (4)
Central Hokkaido	April	18	17 (94)
	May	16	12 (75)
	June	11	9 (81)
	July	10	10 (100)
	August	14	11 (78)
	September	18	14 (78)
	October	20	14 (70)
	November	10	5 (50)
	December	2	2 (100)
	Subtotal	119	95 (80)

South Hokkaido	April	8	7 (88)
	May	1	1 (100)
	June	2	2 (100)
	July	9	7 (78)
	August	15	13 (87)
	September	10	6 (60)
	October	16	12 (75)
	November	5	3 (60)
	Subtotal	66	51 (77)
Total		437	168 (38)

Table 9. Outputs of best generalized linear mixed model for *Hepatozoon ursi* infection status in brown bear.

Response variable	Explanatory variable	Estimate	Standard error	*P*	Conditional R^2	Marginal R^2
Hepatozoon ursi in brown bears	Intercept	−1.80	0.34	<0.001	0.55	0.53
	Region = North Hokkaido	−1.40	0.78			
	Region = Central Hokkaido	3.59	0.37	<0.001		
	Region = South Hokkaido	3.67	0.42	<0.001		
	Estimated Age = Adult	−0.79	0.30	<0.01		

Table 10. Number of ticks collected from sika deer and brown bears in Hokkaido.

Animal species		Sika deer			Brown bear		
Sampling region		East Hokkaido	North Hokkaido	South Hokkaido	East Hokkaido	Central Hokkaido	South Hokkaido
No. of samples		58	49	50	159	68	28
Tick species	Stage						
Ixodes ovatus	Male	75	118	48	607	191	98
	Female	638	848	402	3036	690	347
	Nymph	5	8	1	0	1	0
	Subtotal	718	974	451	3643	882	445
Ixodes persulcatus	Male	15	0	0	14	0	0
	Female	13	10	0	64	1	0
	Nymph	480	205	6	35	2	0
	Subtotal	508	215	6	113	3	0
Haemaphysalis japonica	Male	1278	0	18	1627	35	8
	Female	42	0	159	439	54	46
	Nymph	47	0	204	1009	101	66
	Subtotal	1367	0	381	3075	190	120
Haemaphysalis megaspinosa	Male	72	0	760	2819	211	183
	Female	58	0	248	757	58	68
	Nymph	1	0	680	1672	99	32
	Subtotal	131	0	1688	5248	368	283
Haemaphysalis flava	Male	0	0	10	4	44	32
	Female	0	0	20	4	7	19

	Nymph	0	0	0	0	18	7
	Subtotal	0	0	30	8	69	58
Haemaphysalis longicornis	Male	0	0	0	0	0	0
	Female	0	0	29	0	1	24
	Nymph	0	0	68	0	0	0
	Subtotal	0	0	97	0	1	24
Total		2724	1189	2653	12087	1513	930

Table 11. Outputs of the best generalized linear mixed models for each tick species and developmental stage parasitizing sika deer in Hokkaido.

Response variable	Explanatory variable	Estimate	Standard error	*P*	Conditional R^2	Marginal R^2
Ixodes ovatus female	Intercept	0.46	0.32		na	0.67
	Region = North Hokkaido	0.50	0.33			
	Region = South Hokkaido	−0.20	0.34			
	Season = Summer	1.98	0.29	<0.001		
	Season = Winter	−1.98	0.77	<0.05		
	Sex = Male	0.80	0.29	<0.01		
Ixodes persulcatus nymph	Intercept	1.62	0.54	<0.01	0.724	0.60
	Region = North Hokkaido	−0.81	0.26	<0.01		
	Region = South Hokkaido	−3.73	0.49	<0.001		
Haemaphysalis japonica male	Intercept	1.06	1.26		0.805	0.73
	Region = North Hokkaido	NA				
	Region = South Hokkaido	−1.96	0.76	<0.01		
	Season = Summer	−4.07	0.47	<0.001		
	Season = Winter	−0.92	1.16			
	Estimated Age = Adult	1.96	0.93	<0.05		
Haemaphysalis japonica female	Intercept	0.23	0.41		na	0.49
	Region = North Hokkaido	NA				
	Region = South Hokkaido	2.01	0.52	<0.001		
	Season = Summer	−1.44	0.52	<0.01		
	Season = Winter	−3.50	0.92	<0.001		
Haemaphysalis japonica nymph	Intercept	0.30	0.30		na	0.65

	Region = North Hokkaido	NA				
	Region = South Hokkaido	2.53	0.45	<0.001		
	Season = Summer	−1.70	0.45	<0.001		
	Season = Winter	−3.86	0.76	<0.001		
Haemaphysalis megaspinosa male	Intercept	1.30	0.38	<0.001	na	0.88
	Region = North Hokkaido	NA				
	Region = South Hokkaido	2.20	0.54	<0.001		
	Season = Summer	−5.57	0.73	<0.001		
	Season = Winter	−0.80	0.60			
Haemaphysalis megaspinosa nymph	Intercept	−2.89	1.07	<0.01	na	0.87
	Region = North Hokkaido	NA				
	Region = South Hokkaido	5.95	1.10	<0.001		

NA, Variables not included in the statistical model; na, the Conditional R^2 could not be computed because of the negligible variance of the random effect, suggesting a minimal influence of the random factor (sampling year) on the model variability.

Table 12. Outputs of best generalized linear mixed models for each tick species and developmental stage parasitizing brown bear in Hokkaido.

Response variable	Explanatory variable	Estimate	Standard error	*P*	Conditional R^2	Marginal R^2
Ixodes ovatus female	Intercept	1.38	0.28	<0.001	0.58	0.51
	Region = Central Hokkaido	−0.60	0.19	<0.01		
	Region = South Hokkaido	−0.36	0.25			
	Season = Spring	1.87	0.21	<0.001		
	Season = Summer	2.07	0.17	<0.001		
	Season = Winter	NA				
	Estimated Age = Adult	0.29	0.16			
Ixodes persulcatus nymph	Intercept	−0.97	0.40	<0.05	0.41	0.37
	Region = Central Hokkaido	−1.93	0.79	<0.05		
	Region = South Hokkaido	NA				
	Sex = Male	−1.26	0.47	<0.01		
Haemaphysalis japonica male	Intercept	2.60	0.16	<0.001	na	0.82
	Region = Central Hokkaido	−2.80	0.33	<0.001		
	Region = South Hokkaido	−3.00	0.58	<0.001		
	Season = Spring	−0.47	0.33			
	Season = Summer	−4.45	0.42	<0.001		
	Season = Winter	1.67	0.91			
Haemaphysalis japonica female	Intercept	1.13	0.22	<0.001	na	0.50
	Region = Central Hokkaido	−0.87	0.36	<0.05		
	Region = South Hokkaido	0.20	0.50			
	Season = Spring	−1.19	0.41	<0.01		

	Season = Summer	−2.69	0.37	<0.001		
	Season = Winter	NA				
	Estimated Age = Adult	0.49	0.28	<0.05		
Haemaphysalis japonica nymph	Intercept	2.61	0.37	<0.001	0.50	0.45
	Region = Central Hokkaido	−0.98	0.32	<0.01		
	Region = South Hokkaido	−0.29	0.43			
	Season = Spring	−0.88	0.41	<0.05		
	Season = Summer	−2.37	0.34	<0.001		
	Season = Winter	−1.89	1.16			
	Estimated Age = Adult	−0.38	0.26			
Haemaphysalis megaspinosa male	Intercept	3.36	0.20	<0.001	na	0.67
	Region = Central Hokkaido	−1.19	0.40	<0.01		
	Region = South Hokkaido	−1.22	0.45	<0.01		
	Season = Spring	−1.55	0.46	<0.001		
	Season = Summer	−3.95	0.33	<0.001		
	Season = Winter	−1.37	1.21			
Haemaphysalis megaspinosa female	Intercept	2.06	0.24	<0.001	na	0.56
	Region = Central Hokkaido	−1.67	0.57	<0.01		
	Region = South Hokkaido	−1.01	0.53			
	Season = Spring	−0.56	0.64			
	Season = Summer	−3.25	0.41	<0.001		
	Season = Winter	NA				
Haemaphysalis megaspinosa nymph	Intercept	2.88	0.19	<0.001	na	0.70

	Region = Central Hokkaido	−1.28	0.44	<0.01		
	Region = South Hokkaido	−1.02	0.49	<0.05		
	Season = Spring	−3.92	0.55	<0.001		
	Season = Summer	NA				
	Season = Winter	NA				
Haemaphysalis flava male	Intercept	−3.78	0.61	<0.001	na	0.62
	Region = Central Hokkaido	3.01	0.62	<0.001		
	Region = South Hokkaido	3.57	0.62	<0.001		
	Season = Spring	−0.58	0.56			
	Season = Summer	−1.54	0.62	<0.05		
	Season = Winter	NA				
	Estimated Age = Adult	0.94	0.45	<0.05		
Haemaphysalis flava female	Intercept	−3.28	0.55	<0.001	na	0.42
	Region = Central Hokkaido	1.64	0.73	<0.05		
	Region = South Hokkaido	3.42	0.75	<0.001		
	Season = Spring	NA				
	Season = Summer	−1.15	0.69			
	Season = Winter	NA				

NA, Variables not included in the statistical model; na, the Conditional R^2 could not be computed because of the negligible variance of the random effect, suggesting a minimal influence of the random factor (sampling year) on the model variability.

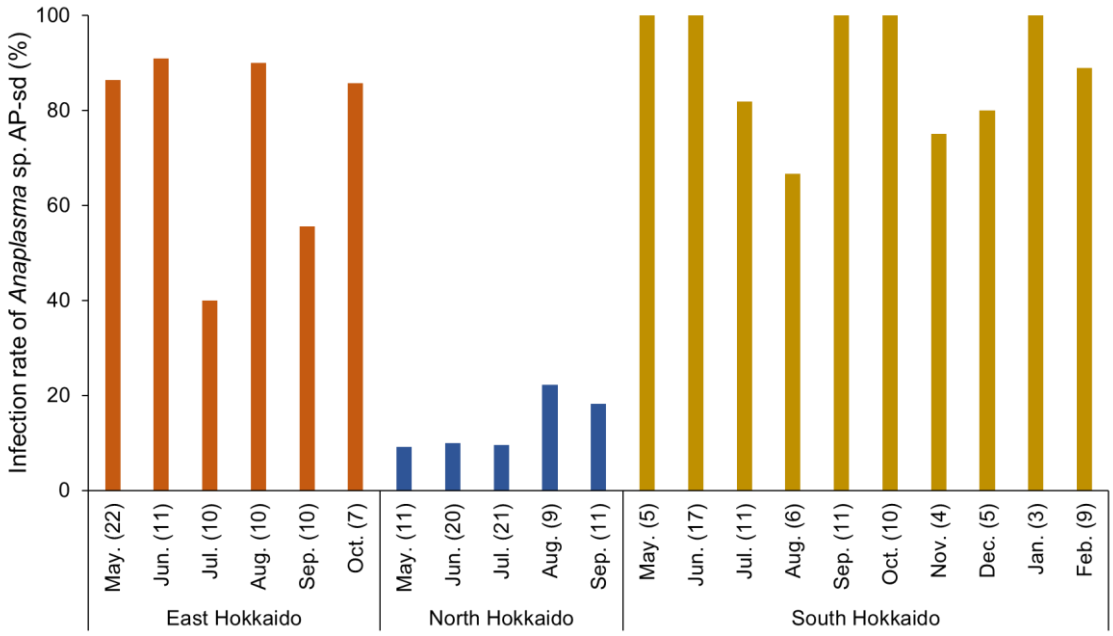

Figure 6. Seasonal changes in the prevalence of *Anaplasma* sp. AP-sd in sika deer in Hokkaido. The x-axis indicates the sampling month (number of deer). Different colors represent different regions of Hokkaido (East, North, and South Hokkaido).

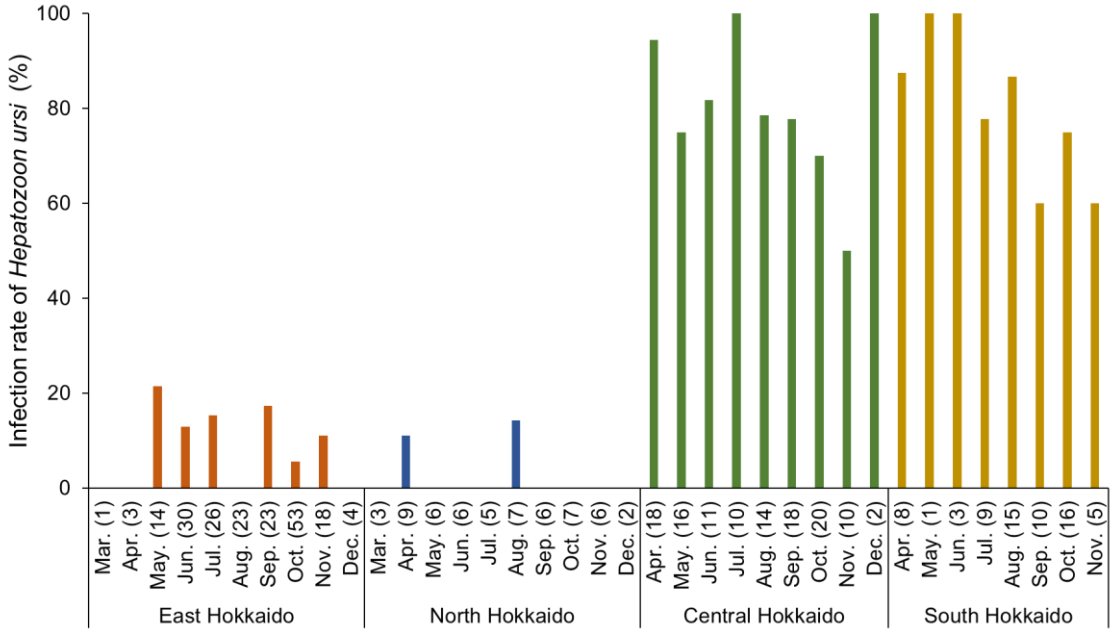

Figure 7. Seasonal changes in the prevalence of *Hepatozoon ursi* in brown bears in Hokkaido. The x-axis indicates the sampling month (number of bears). Different colors represent different regions of Hokkaido (East, North, Central, and South Hokkaido).

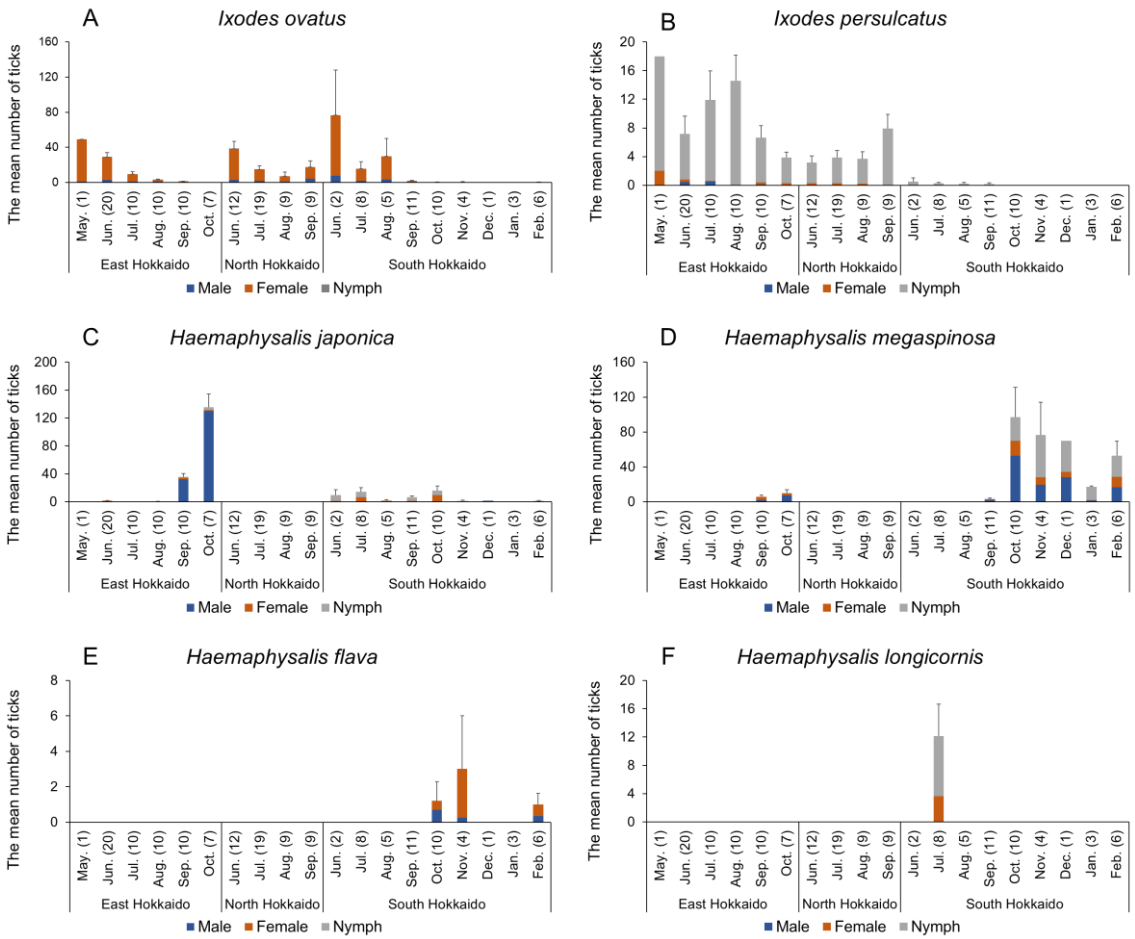

Figure 8. Seasonal dynamics of tick infestation in sika deer in Hokkaido. Error bars represent the standard error of total tick counts, with the x-axis indicating sampling regions and months (number of deer). (A) *Ixodes ovatus*, (B) *Ixodes persulcatus*, (C) *Haemaphysalis japonica*, (D) *Haemaphysalis megaspinosa*, (E) *Haemaphysalis flava*, and (F) *Haemaphysalis longicornis.*

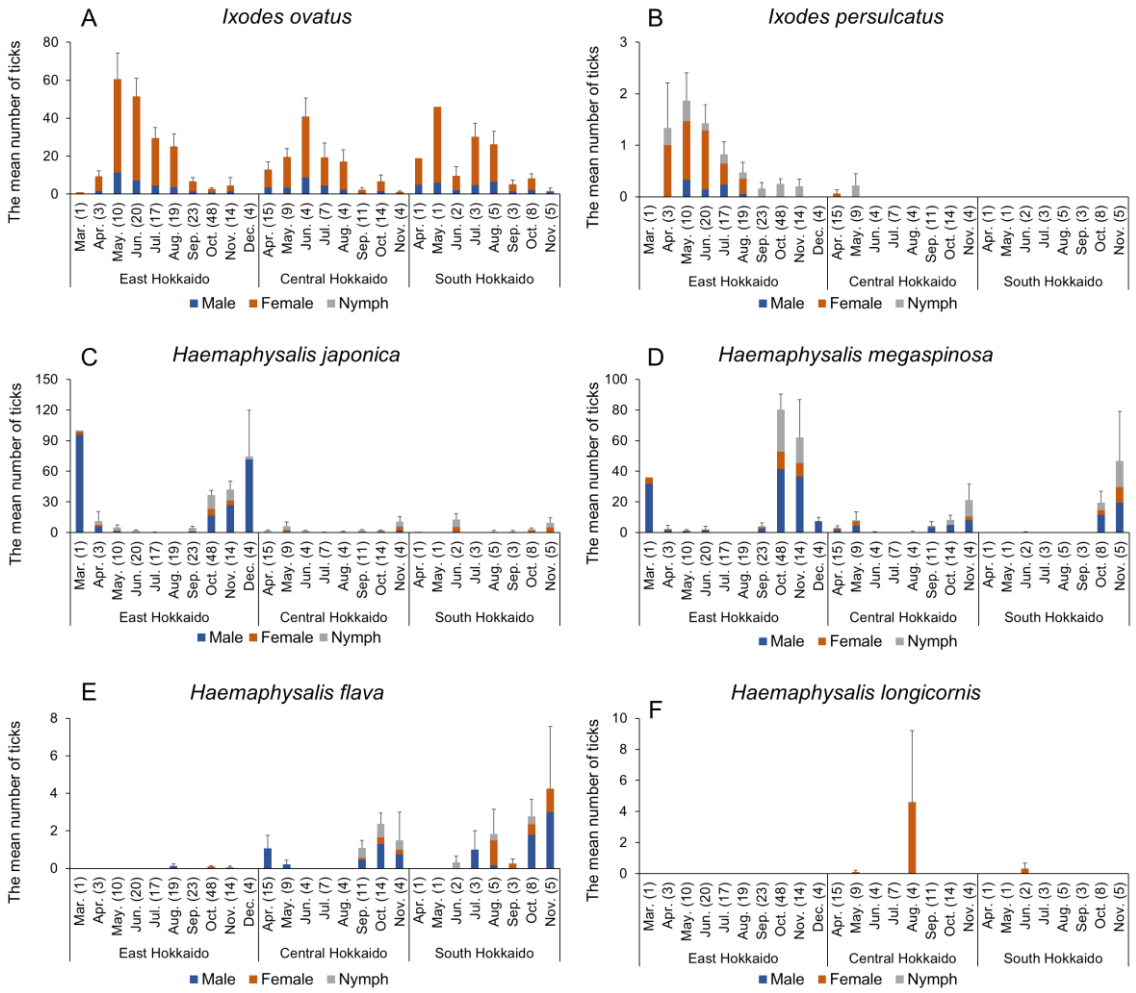

Figure 9. Seasonal dynamics of tick infestation in brown bears in Hokkaido. Error bars represent the standard error of total tick counts, with the x-axis indicating sampling regions and months (number of bears). (A) *Ixodes ovatus*, (B) *Ixodes persulcatus*, (C) *Haemaphysalis japonica*, (D) *Haemaphysalis megaspinosa*, (E) *Haemaphysalis flava*, and (F) *Haemaphysalis longicornis.*

Discussion

This study investigated the ecological dynamics of tick-borne pathogens across Hokkaido and revealed complex interactions between hosts, vectors, and pathogens. The prevalence of AP-sd and *He. ursi* infections were examined, both showing notable regional variations that suggest different transmission mechanisms. AP-sd demonstrated a vector-driven distribution pattern, while *He. ursi* revealed more complex dynamics influenced by both vector distribution and host factors. Six tick species from two genera (*Ixodes* and *Haemaphysalis*) that parasitize sika deer and brown bears were also identified, with each tick species exhibiting distinct seasonal activity patterns and host preferences. These findings highlight that the tick-borne disease ecology in Hokkaido is regulated by a complex interplay of factors beyond simple vector-host relationships.

The findings on tick infestation patterns and pathogen prevalence revealed striking differences in the ecology of AP-sd and *He. ursi* in Hokkaido, particularly regarding the influence of the vector distribution on pathogen transmission. The prevalence of AP-sd showed a clear association with vector distribution (Figures 6 and 8). The high prevalence in South and East Hokkaido corresponds to areas where multiple potential vector species are abundant. This stability in prevalence despite seasonal fluctuations in tick abundance may be attributed to the ability of multiple tick species to transmit the pathogen (Kawahara et al., 2006; Moustafa et al., 2015). The low prevalence in North Hokkaido likely reflects the local tick fauna, where *Ixodes* spp. are present, but *Haemaphysalis* spp. are absent. A previous study in Hokkaido detected AP-sd in questing ticks of both *Ixodes* spp. and *Haemaphysalis* spp., but experimental transmission studies have not been conducted, leaving vector competence unconfirmed (Ybañez et al., 2012). The strong association between AP-sd prevalence and *Haemaphysalis* spp. distribution observed in this study may suggest the importance of *Haemaphysalis* spp. in AP-sd transmission. However, experimental validation is needed to confirm the vector competence of these tick species. The absence of host-related effects on AP-sd prevalence, despite their influence on parasitism patterns of several tick species (Tables 7 and 11), suggests that the occurrence of this infection is more strongly determined by the presence or absence of competent vector species than by the intensity of vector infestation.

Compared to the AP-sd in deer, *He. ursi* demonstrates a more complex relationship with its vectors (Figures 7 and 9). *Hepatozoon ursi* is transmitted by *Haemaphysalis* spp. (Kubo et al., 2008; Tsai et al., 2024), and its low infection rate in

North Hokkaido is likely related to the absence of these vector ticks, as indicated by tick surveys of sika deer. However, it is important to note that tick infestation of brown bears in the North region has not been directly investigated, necessitating caution in interpreting the relationship between vector absence and *He. ursi* infections in this area. Unexpectedly low infection rate in *He. ursi* in East Hokkaido, despite the high abundance of *Haemaphysalis* spp., suggested the presence of additional limiting factors beyond vector availability. This paradoxical situation may be explained by two hypotheses. One possibility is that *He. ursi* was not detected in previous surveys (Moustafa et al., 2020) and may represent a recently introduced pathogen to Hokkaido, and that landscape factors impede pathogen transmission to eastern populations. However, both *Haemaphysalis* spp. (the vectors of *He. ursi*) and their potential hosts appear capable of movement between regions. This is supported by evidence from both major host species: sika deer populations exhibit relatively homogeneous genetic structure across the island (Nabata et al., 2007; Terada et al., 2013). Similarly, while mitochondrial DNA in brown bears shows spatial genetic differentiation reflecting female sedentary behavior (Matsuhashi et al., 1999), spatially homogenized nuclear genes through male dispersal have been reported (Endo et al., 2021; Hirata et al., 2017). These evidence suggest that substantial movement of both host species and their associated vectors occurs between regions, making landscape barriers alone insufficient to explain the observed pathogen distribution patterns. Alternatively, the recent expansion of *Haemaphysalis* spp. into East Hokkaido, independent of the *He. ursi* may have contributed to the observed pattern. This scenario aligns with several previous studies that have predicted tick range expansion due to global warming (Guillot et al., 2022; Nuttall, 2022; Yang et al., 2021). Increased numbers of *Haemaphysalis* spp. in East Hokkaido were observed compared to previous studies (Elbaz et al., 2020; Zamoto-Niikura et al., 2018, 2012), which potentially indicates a recent range expansion. The detection of *He. ursi* in East Hokkaido in this study, where it had not been reported in previous surveys, provides additional support for this hypothesis. However, it should be noted that these previous studies did not specifically focus on tick fauna in this region (Elbaz et al., 2020; Zamoto-Niikura et al., 2018, 2012), limiting our ability to make direct temporal comparisons of population changes. Furthermore, the stable infection rates across seasons, independent of *Haemaphysalis* spp. phenology, support the possibility of chronic *He. ursi* infections as previously suggested (Moustafa et al., 2020). Furthermore, host age class significantly influenced *He. ursi* infection pattern. The analysis revealed that adult bears had lower infection rates for *He. ursi* despite

experiencing higher infestation levels of several *Haemaphysalis* spp. (Tables 9 and 12). This contradictory pattern suggests that acquired immunity for *He. ursi* plays a crucial role in determining infection outcomes for this pathogen. This finding contrasts with that of a study on Japanese black bears on Honshu Island of Japan, where higher infection rates were observed in adults (Moustafa et al., 2020), suggesting potential species-specific differences in immune responses to *He. ursi*.

This study revealed that tick species infesting both animals had distinctive patterns in seasonal activity, host preference, and regional distribution, offering valuable context to understand the pathogen dynamics in study areas. *Ixodes ovatus* was the most dominant tick species throughout the study area in Hokkaido, showing a similar distribution and seasonal patterns in both sika deer and brown bears. Infestation levels decreased from summer to autumn across all regions (Figure 8A and 9A), a trend also observed in raccoons from Central Hokkaido (Ito et al., 2024), suggesting consistent phenology across multiple host species. Most *I. ovatus* collected from sika deer and brown bears were adult ticks, which is consistent with previous studies indicating that adult ticks from two- and three-host species prefer large mammals (McCoy et al., 2003). A survey of rodents in East Hokkaido found only nymphs and larvae (Taylor et al., 2013), further supporting stage-specific host preference. Moreover, medium-sized mammals such as raccoons and raccoon dogs are predominantly parasitized by adult ticks (Ito et al., 2024). These findings indicate that *I. ovatus* in Hokkaido follows a typical host utilization pattern, with larvae and nymphs primarily infesting small mammals and adults targeting medium to large mammals.

Ixodes persulcatus exhibited distinct distributions and host utilization patterns in sika deer and brown bears (Figure 8B and 9B). In sika deer, this species was frequently found in East and North Hokkaido, with stable year-round infestations of nymphs; however, it was rarely collected in South Hokkaido. This distribution pattern of *I. persulcatus* likely reflects its adaptation to cold climates (Uusitalo et al., 2022). In contrast, infestation levels in brown bears were low and primarily restricted to East Hokkaido, with a distinctive stage composition. Adult female ticks were predominant during spring and summer, whereas adult ticks were rarely observed after autumn. These contrasting stage compositions between the two host species (predominantly nymphs in sika deer versus adult female ticks in brown bears) suggest stage-specific host preferences for *I. persulcatus*.

Haemaphysalis spp. demonstrated a more pronounced regional variation than *Ixodes* spp., with a notable absence in North Hokkaido. Among them, *Ha. japonica* exhibited distinct regional and seasonal differences in both host species, particularly in infestation patterns during autumn and winter (Figure 8C and 9C). In East Hokkaido, adult male ticks predominantly increased after autumn and remained prevalent throughout winter, constituting the majority of the total collection. However, in South and Central Hokkaido, nymphal and adult female ticks predominated throughout the observation period, with no autumn–winter increase in the number of adult male ticks. These patterns suggest the existence of various overwintering strategies of *Ha. japonica*. The predominance of adult male ticks in East Hokkaido from autumn onward implies overwintering of host bodies (Doi et al., 2018; Ozawa and Kadosaki, 1996), which is likely linked to the reproductive ecology of *Haemaphysalis* spp. For these tick species, mating occurs exclusively on hosts, and early attachment and prolonged stay of males may enhance reproductive success (Kiszewski et al., 2001; Tsunoda, 2012). Conversely, the low infestation levels during autumn and winter in South and Central Hokkaido suggest tick overwintering in leaf litter and upper soil layers, which is a strategy observed in many tick species (Daniel et al., 1972; Kim et al., 2020; Whitlow et al., 2021). Regional differences in overwintering strategies are likely to reflect variations in winter temperatures and snow cover. The cold and low-snow conditions of East Hokkaido may make soil overwintering unfavorable (Christopher et al., 2008; Okawa, 1992), promoting host-surface overwintering. This aligns with experimental findings showing reduced soil overwintering success in areas where freezing precedes snowfall (Burtis et al., 2016; Diyes et al., 2024; Volk et al., 2022). However, as this study focused only on host-attached ticks, further research incorporating environmental manipulation and cold tolerance experiments is required to validate these hypotheses.

Haemaphysalis megaspinosa exhibited distinct seasonality, with infestations occurring primarily after autumn (Figure 8D and 9D). This pattern has been widely reported in Honshu, Japan (Doi et al., 2020; Inokuma et al., 2002a; Ozawa and Kadosaki, 1996; Sakai and Torii, 2014; Sibata et al., 2020), suggesting that autumn–winter activity is a characteristic of this species. This findings confirm that *Ha. megaspinosa* infestation persisted during the snow-covered period, which is consistent with previous reports (Ozawa and Kadosaki, 1996; Sakai and Torii, 2014; Sibata et al., 2020). However, knowledge of tick ecology in snow-covered environments remains limited. Most studies on questing behavior have focused on snow-free regions, whereas observations under

snow cover have largely been abandoned because of technical constraints (Ozawa and Kadosaki, 1996; Sakai and Torii, 2014; Sibata et al., 2020). Further research on overwintering behavior and activity patterns is required to fully understand wildlife-tick interactions in snowy environments.

Haemaphysalis flava exhibited a geographically limited distribution, being found only in South Hokkaido on sika deer and primarily in South and Central Hokkaido on brown bears (Figure 8E and 9E). Its seasonal pattern, with increasing numbers from autumn to winter, was similar to that reported in Honshu but at noticeably lower levels (Doi et al., 2018; Inokuma et al., 2002a; Sakai and Torii, 2014). These findings suggest variations in host utilization compared with a previous study in Hokkaido (Ito et al., 2024). While adult ticks predominate in sika deer and brown bears, a study on raccoons in Central Hokkaido reported a higher prevalence of nymphs in spring and summer (Ito et al., 2024). These results indicate that *Ha. flava* may shift its host preference depending on the developmental stage.

Across both the host species examined, *Ha. longicornis* showed low infestation levels, occurring only briefly in summer (Figure 8F and 9F). In Hokkaido, this species has been primarily reported in pastures (Maeno et al., 2015; Namba, 1958), with few records from wildlife (Ito et al., 2024; Ozawa and Kadosaki, 1996). These findings suggest that *Ha. longicornis* has a more restricted distribution in Hokkaido than in Honshu and has a limited utilization of wildlife hosts.

The effects of host factors (sex and age class) on tick infestation varied depending on the combination of tick and host species, as revealed by GLMM analysis (Tables 11 and 12). In sika deer, GLMMs revealed higher infestation levels of adult female *I. ovatus* in male sika deer than in female deer, which aligns with previous studies reporting similar male-biased parasitism patterns across various mammalian species (Dhakal et al., 2023; Hillegass et al., 2008; Takatsuki et al., 2024). Male-biased infestation burden is typically attributable to testosterone-induced immunosuppression, larger home ranges, and greater body mass associated with sexual dimorphism (Dhakal et al., 2023; Hillegass et al., 2008; Takatsuki et al., 2024). In contrast, *I. persulcatus* nymphs were notably less abundant in male brown bears. This may be related to tree rubbing, a behavior unique to brown bears. Tree rubbing is particularly common in males during the breeding season and is primarily associated with scent marking and social communication (Sato et al., 2014; Tomiyasu et al., 2017). However, recent studies have suggested that it may also have additional functions, including mechanical removal of ectoparasites and exposure to

tree resins and other plant-derived substances with potential repellent properties (Blaise et al., 2023; Lamb et al., 2017; Revilla et al., 2021). Although the extent to which tree rubbing directly influences tick infestation remains unclear, the consistently low numbers of *I. persulcatus* nymphs in male bears suggest that behavioral factors may contribute to host-parasite interactions. Further research is required to clarify the role of tree rubbing in ectoparasite defense and its potential species-specific effects. Host age also influenced the infestation levels across multiple tick species and stages. Higher tick burdens were observed on adult hosts, with significant age-related differences found specifically in male *Ha. japonica* on sika deer, and female *Ha. japonica* and male *Ha. flava* on brown bears. The higher infestation levels in adult sika deer and brown bears suggest that the increased opportunities for tick acquisition associated with larger body sizes outweighed the protective effects of acquired immunity, particularly in species that exhibited significant age-related growth (Karasuyama et al., 2020; Tabakawa et al., 2018).

Overall, these detailed observations of tick ecology across Hokkaido provide crucial context for understanding the contrasting transmission dynamics of AP-sd and *He. ursi*. The regional distribution patterns of tick vectors largely explain the AP-sd prevalence, whereas *He. ursi* transmission is likely influenced by the complex interplay between vector ecology and host immunity. Furthermore, the tick species infesting both wildlife showed distinct seasonal activity, host preferences, and geographical distributions, shaped by both environmental conditions and host characteristics. This study demonstrated that tick-borne pathogens in the same ecosystem can exhibit fundamentally different transmission dynamics, emphasizing that effective disease management requires pathogen-specific understanding rather than generalized approaches.

Summary

Understanding the complex interactions between hosts, vectors, and pathogens is essential for elucidating tick-borne disease ecology. In this study, environmental (region, season) and host factors (sex, age) affecting pathogen prevalence and tick infestation in sika deer (*C. nippon yesoensis*) and brown bears (*U. arctos yesoensis*) in Hokkaido, Japan were examined. To analyze the host-vector-pathogen relationships, *Anaplasma* and *Hepatozoon* were selected as two prevalent pathogens in sika deer and brown bears, respectively. Between 2021-2024, blood and liver samples from 223 deer and 437 bears were tested for these pathogens and tick infestation patterns were examined in 157 deer and 255 bears across four regions of Hokkaido. The prevalence of *Anaplasma* infection was high in deer from South and East Hokkaido but low in North Hokkaido. *Hepatozoon* infection in bears was higher in South and Central Hokkaido, with lower rates in the East and North. GLMMs indicated that *Anaplasma* prevalence was determined by regional factors, whereas *Hepatozoon* infection presence was influenced by region and host age, with adults showing lower infection rates. Six tick species were identified: *I. ovatus*, *I. persulcatus*, *Ha. japonica*, *Ha. megaspinosa*, *Ha. flava*, and *Ha. longicornis*. *Ixodes ovatus* dominated all regions in both hosts, while *I. persulcatus* was common in deer but rare in bears. *Haemaphysalis japonica* and *Ha. megaspinosa* showed regional differences and increased infestation in autumn. *Haemaphysalis flava* and *Ha. longicornis* had an overall limited distribution. GLMMs revealed male deer had more *I. ovatus* than females. In contrast, male bears had fewer *I. persulcatus* nymphs. Older hosts had higher levels of infestation across multiple tick species. Contrasting patterns between pathogens highlight distinct transmission dynamics; the geographic patterns of *Anaplasma* prevalence corresponded closely with vector distribution, whereas *Hepatozoon* showed complex dynamics influenced by factors beyond vectors. These findings reveal the complex interplay shaping tick-borne disease ecology in wildlife.

Chapter III

Evaluating the vector potential of deer keds *Lipoptena fortisetosa* for selected pathogens in Hokkaido sika deer

Introduction

Chapters I and II provided comprehensive insights into tick-host-pathogen relationships in Hokkaido sika deer and brown bears; however, the broader question of vector-borne disease ecology requires consideration of other potential arthropod vectors within the ecosystem. In addition to hard ticks (Ixodidae), other hematophagous ectoparasites may play distinct roles in pathogen transmission cycles, potentially creating different transmission dynamics and epidemiological patterns. Among such arthropods, deer keds (*Lipoptena* spp.) represent a fundamentally different type of ectoparasite with unique ecological characteristics that warrant investigation as potential disease vectors. In Hokkaido, *L. fortisetosa* is widely distributed and primarily parasitizes Hokkaido sika deer (*C. nippon yesoensis*) (Fukumoto et al., 2000), making it a particularly relevant candidate for vector potential assessment in this ecosystem.

Species of the genus *Lipoptena* have unique lifecycles and have evolved to establish intimate relationships with their hosts (Bezerra-Santos and Otranto, 2020). They have adenotrophic, viviparous reproductive strategies that produce mature larvae that immediately pupate. Adult deer keds emerge from pupae with their wings and initially use their ability to fly to find suitable hosts. This search period is strongly influenced by the local microclimate and insect phenology (Gałęcki et al., 2020). After locating the host, deer keds immediately shed their wings and settle on the host's body and face for the rest of their lives. It has also been documented that adult deer keds can move between hosts via direct physical contact (Samuel and Trainer, 1972). Both sexes are hematophagous and use specialized mouthparts to puncture the host's skin and feed on the blood (Andreani et al., 2019). Although the amount of blood consumed per feed was small, ranging from 0.0002 to 0.0003 g, these flies could feed at a high frequency (up to 20 times daily) (Ivanov, 1974).

Deer keds are highly host specific and typically reproduce only in wild ruminants. However, there are numerous reports of the infestation of nonspecific hosts, including

humans, companion animals, and livestock (Maślanko et al., 2020; Metelitsa, 1989; Sokół and Gałęcki, 2017). Humans bitten by these ectoparasites are at risk of developing dermatitis, allergic rhinoconjunctivitis, or anaphylactic shock (Laukkanen et al., 2005; Madslien et al., 2011; Rantanen et al., 1982). More importantly, deer keds are suspected to act as vectors for several pathogens, as molecular studies have detected pathogens, such as *Coxiella* spp., *Anaplasma* spp., *Theileria* spp., *Trypanosoma* spp., *Borrelia* spp., and *Bartonella* spp. (Buss et al., 2016; Gałęcki et al., 2021a; Hatama et al., 2007; Lee et al., 2016; Sato et al., 2021; J. Werszko et al., 2020; Joanna Werszko et al., 2020).

The genus *Lipoptena* has 32 species, with *L. cervi*, *L. capreoli*, and *L. fortisetosa* being of notable veterinary importance, prevalent in North America, Europe, China, and Siberia, while *L. mazamae and L. depressa* being exclusive to North America (Gałęcki et al., 2020). In Japan, three species, *L. japonica*, *L. fortisetosa*, and *L. sikae,* have been reported in sika deer (*C. nippon*) and Japanese serow (*Capricornis crispus*) in Honshu (Fukumoto et al., 2000). It has been reported that *L. fortisetosa* may be involved in the spread of *Bartonella* and *Trypanosoma* spp. (Hatama et al., 2007; Sato et al., 2021).

As established in previous chapters, Hokkaido sika deer has been reported to be one of the most important reproductive hosts for several tick species, including the genera *Ixodes* and *Haemaphysalis* (Elbaz et al., 2020; Iijima et al., 2022; Tsukada et al., 2014). Several microorganisms such as AP-sd (Moustafa et al., 2015), *Theileria* sp. Thrivae, *Babesia divergens*-like (Elbaz et al., 2017), and *Borrelia lonestari*-like (Lee et al., 2014) have been detected in Hokkaido sika deer. Previous studies on *Ixodes* and *Haemaphysalis* ticks collected from Hokkaido sika deer revealed the presence of these pathogens in the tested ticks (Elbaz et al., 2020; Ybañez et al., 2012), suggesting that ticks play crucial roles in the maintenance and transmission of these pathogens in deer.

In contrast, the vector potential of deer keds parasitizing the same host species has received limited attention. Given the ecological differences between ticks and deer keds outlined above, the question arises whether deer keds might serve as alternative or complementary vectors in pathogen transmission cycles, potentially filling different ecological niches than ticks in wildlife disease ecology in Hokkaido.

Investigating this vector potential is particularly relevant, as *L. fortisetosa*, like other species in the genus *Lipoptena*, is hematophagous and can potentially serve as a vector for specific pathogens (Gałęcki et al., 2021b; Hatama et al., 2007; Lee et al., 2016; Sato et al., 2021; Joanna Werszko et al., 2020). This study aimed to evaluate the potential role of *L. fortisetosa* as a vector for various pathogens in the Hokkaido sika deer.

Specifically, pathogen detection was investigated in deer keds collected from both parasitized sika deer and the environment.

Materials and methods

Study area

This study was conducted in the Rusha area (44°11'N, 145°11'E) of the Shiretoko Peninsula, easter Hokkaido, Japan—identical to the field site described in Chapter I. The central part of the peninsula, including the study area extending to Shiretoko Cape, is designated Shiretoko National Park, a UNESCO World Heritage Site. The area is characterized by a subarctic climate with long snowy winters and short summers. Only Hokkaido sika deer, the reproductive host of *L. fortisetosa*, was observed in this area (Kawamura et al., 2022).

Sampling

This study used a subset of Hokkaido sika deer individuals captured in the Rusha area of the Shiretoko Peninsula between July and October 2022, as described in Chapter I. While Chapter I focused on tick infestation patterns based on pinna samples, this chapter analyzed blood and deer ked specimens that were collected simultaneously from the same individuals. Capture and immobilization procedures followed the same protocol outlined in Chapter I. Approximately 7 mL of blood was collected from the jugular vein using EDTA tubes. Deer blood samples were stored at −80°C until DNA extraction.

Adult deer keds were collected from two sources: the deer mentioned above and the environment during host foraging. The species and sex of the collected deer keds were identified under a stereomicroscope using a classification key (Andreani et al., 2019; Salvetti et al., 2020). For deer-associated samples, 10–22 ectoparasites were randomly collected from each captured deer. Of these, one male and one female were randomly selected for subsequent analysis, resulting in 64 deer keds from deer hosts. A total of 85 deer keds were collected from the environment. These environmental samples were collected by researchers walking slowly through the forest and catching insects that had landed on their clothing. All deer ked sampling, both from the deer and the environment, was conducted within a radius of three kilometers. Collected deer keds were immersed in 70% ethanol and frozen at −30°C upon arrival at the laboratory until morphological identification and DNA extraction.

All procedures involving the capture and sampling of live animals were conducted by the Guidelines for Animal Care and Use, Hokkaido University, and with

the approval of the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Permit Number: 22–0016).

Molecular analysis

DNA was extracted from whole blood and deer keds using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Each deer ked sample was rinsed twice with sterile water after species identification and air-dried prior to DNA extraction. The concentration and quality of the extracted DNA were verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The extracted DNA samples were stored at −30°C until further analysis.

PCR amplification, electrophoresis, purification, and sequencing were performed using the same protocol described in Chapter II. Briefly, a 25-μL reaction mixture including 2× Gflex PCR Buffer, Tks Gflex DNA Polymerase, and 10 μM of each primer was used. Primer sequences specific to this chapter are listed in Table 13. Amplicons were visualized on 1.5% agarose gels, and positive bands were purified and sequenced using standard Sanger methods. Sequence identity was confirmed via BLASTn search against GenBank.

For each pathogen, up to five of the most prominent bands from each sample species were selected for bidirectional sequencing using the same primers used in PCR. The resulting sequences were subjected to phylogenetic analysis using the maximum likelihood method in MEGA 11 (Tamura et al., 2021). Bootstrap confidence values to estimate the reliability of the branches were calculated using 1,000 replicates.

Table 13. List of primers used in this study to detect pathogens in deer blood and deer keds.

Target organism	Target gene	Primer name	Primer sequence (5′−3′)	Expected size (bp)	Reference
Piroplasms	18S rRNA	Piro1-S	CTTGACGGTAGGGTATTGGC	~1,410	(Yang et al., 2014)
		Piro3-AS	CCTTCCTTTAAGTGATAAGGTTCAC		
	18S rRNA	PIRO-A1	CGCAAATTACCCAATCCTGACA	~430	
		PIRO-B	TTAAATACGAATGCCCCCAAC		
Anaplasma sp. AP-sd	16S rRNA	EC9	TACCTTGTTACGACTT	~1,462	(Sashika et al., 2011)
		EC12A	TGATCCTGGCTCAGAACGAACG		
	16S rRNA	AP-f1	CATGCAAGTCGAACGGGTTA	~770	
		AP-r1	CATCAACACGGAGATAAATTATC		
Borrelia spp.	flagellin B gene	Bfla-PAD	GATCARGCWCAAYATAACCAWATGCA	~450	(Sato et al., 1997)
		Bfla-PDU	AGATTCAAGTCTGTTTTGGAAAGC		
	flagellin B gene	Bfla-PBU	GCTGAAGAGCTTGGAATGCAACC	~350	
		Bfla-PCR	TGATCAGTTATCATTCTAATAGCA		

Results

Pathogen DNA detection

PCR analysis and subsequent sequencing revealed the presence of the target pathogens in sika deer and deer keds (Table 14). In sika deer (n = 32), *Theileria* sp. Thrivae was detected in 27 samples (84%), AP-sd in 24 samples (75%), and *Borrelia* sp. (showing high homology with *Borrelia* sp. HM) in one sample (3%). In the deer keds collected from sika deer (n = 64), *Theileria* sp. Thrivae was detected in 54 samples (84%), AP-sd in two samples (3%), and *Borrelia* sp. in one sample (2%). The nine *Theileria* sp. Thrivae-positive deer keds and one AP-sd-positive deer ked were collected from sika deer that tested negative for these pathogens. No pathogens were detected in 85 unfed deer keds collected from the environment.

Phylogenetic analysis

Sequence analysis of *Theileria* sp. Thrivae yielded five sequences each from sika deer and deer keds, representing seven unique sequence types. Among all the sequences, four (LC847943, LC847945, LC847946, and LC847947) were identical. All sequences showed 99.3–100% identity with previously reported *Theileria* sp. Thrivae sequences from sika deer (GenBank accession number MH489010, MH489005, and MH489004). Phylogenetic analysis clustered all *Theileria* sp. Thrivae sequences from the sika deer and deer keds were found together (Figure 10).

In the present study, seven types of sequences of AP-sd (five from sika deer and two from deer keds) were obtained. One sequence each from deer (LC847955) and deer ked (LC847957) showed 100% identity with previously reported AP-sd sequences from sika deer (LC068735). The remaining sequences demonstrated over 99% similarity to the AP-sd sequences reported for sika deer (LC068735 and LC068730). Phylogenetic analysis grouped all AP-sd sequences from this study with previously reported AP-sd sequences (Figure 11).

One sequence of *Borrelia* sp. was obtained from each of the Hokkaido sika deer and deer keds, and the sequences were identical. The obtained sequence showed 100% identity with *Borrelia* sp. HM and has been previously detected in *Ha. megaspinosa* from Honshu, Japan (LC467009). Phylogenetic analysis placed these sequences in the same

cluster as that previously reported for hard tick-borne relapsing fevers (Figure 12).

Table 14. Prevalence of specific pathogens in sika deer and deer keds.

Species	Number of PCR-positive samples		
	Sika deer (No = 32)	Deer keds from deer (No = 64)	Deer keds from the environment (No = 85)
Theileria sp. Thrivae	27 (84%)	54 (62%) (9 were from Negative sika deer)	0 (0%)
Anaplasma sp. AP-sd	24 (75%)	2 (2%) (1 was from Negative sika deer)	0 (0%)
Borrelia sp.	1 (3%)	1 (1%)	0 (0%)

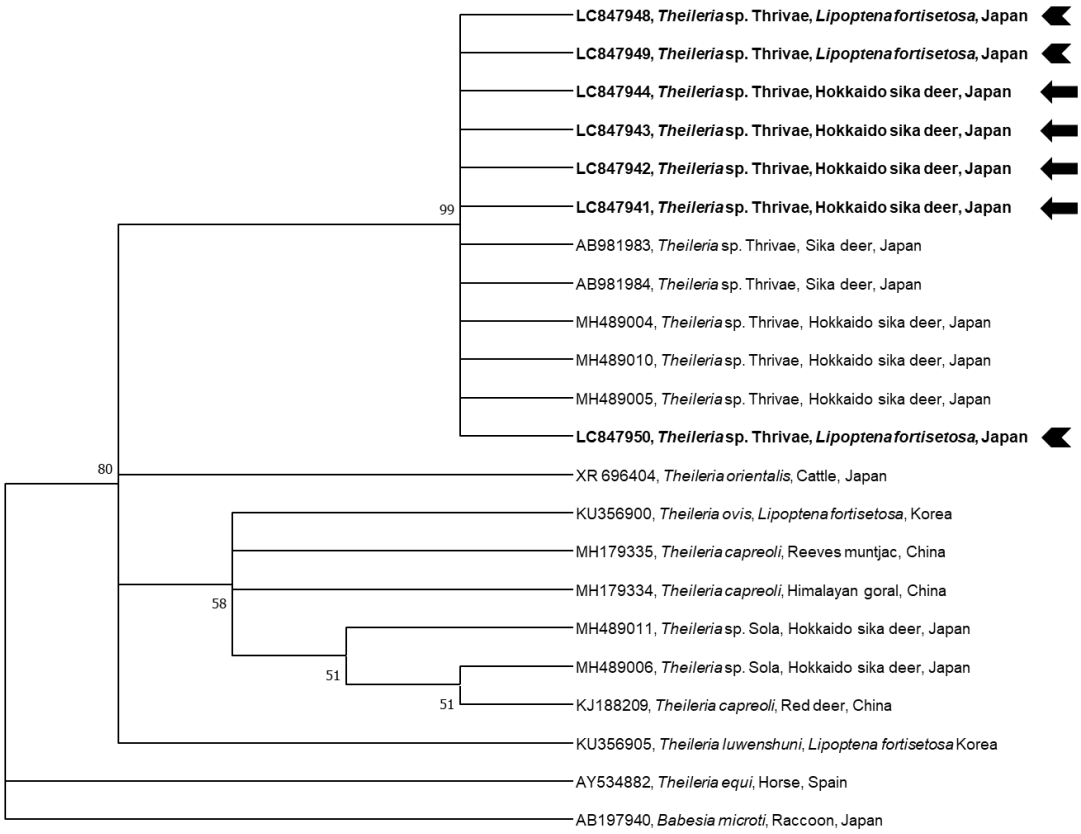

Figure 10. Phylogenetic analysis of 18S rRNA gene of *Theileria* sp. Thrivae in Hokkaido sika deer and *Lipoptena fortisetosa*. The sequences identified in this study are shown in bold. Arrows indicate samples detected from Hokkaido sika deer blood, while arrowheads indicate samples detected from *L. fortisetosa*. A phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates. The scale bar represents the phylogenetic distance between sequences. The species, hosts, isolation regions, and GenBank accession numbers are shown in the figure. LC847943 represents identical sequences: LC847945 (from Hokkaido sika deer, Japan), LC847946 (from *L. fortisetosa*, Japan), and LC847947 (from *L. fortisetosa*, Japan).

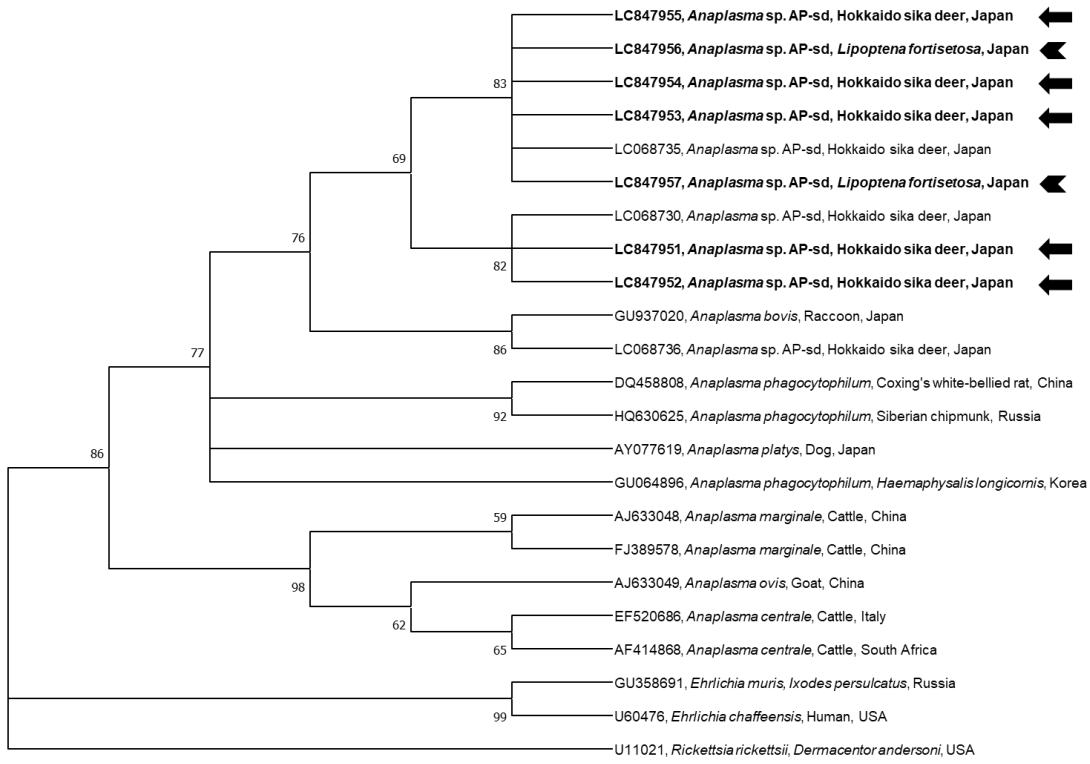

Figure 11. Phylogenetic analysis of 16S rRNA gene of *Anaplasma* sp. AP-sd in Hokkaido sika deer and *Lipoptena fortisetosa*. The sequences identified in this study are shown in bold. Arrows indicate samples detected from Hokkaido sika deer blood, while arrowheads indicate samples detected from *L. fortisetosa*. A phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates. The scale bar represents the phylogenetic distance between sequences. The species, hosts, isolation regions, and GenBank accession numbers are shown in the figure.

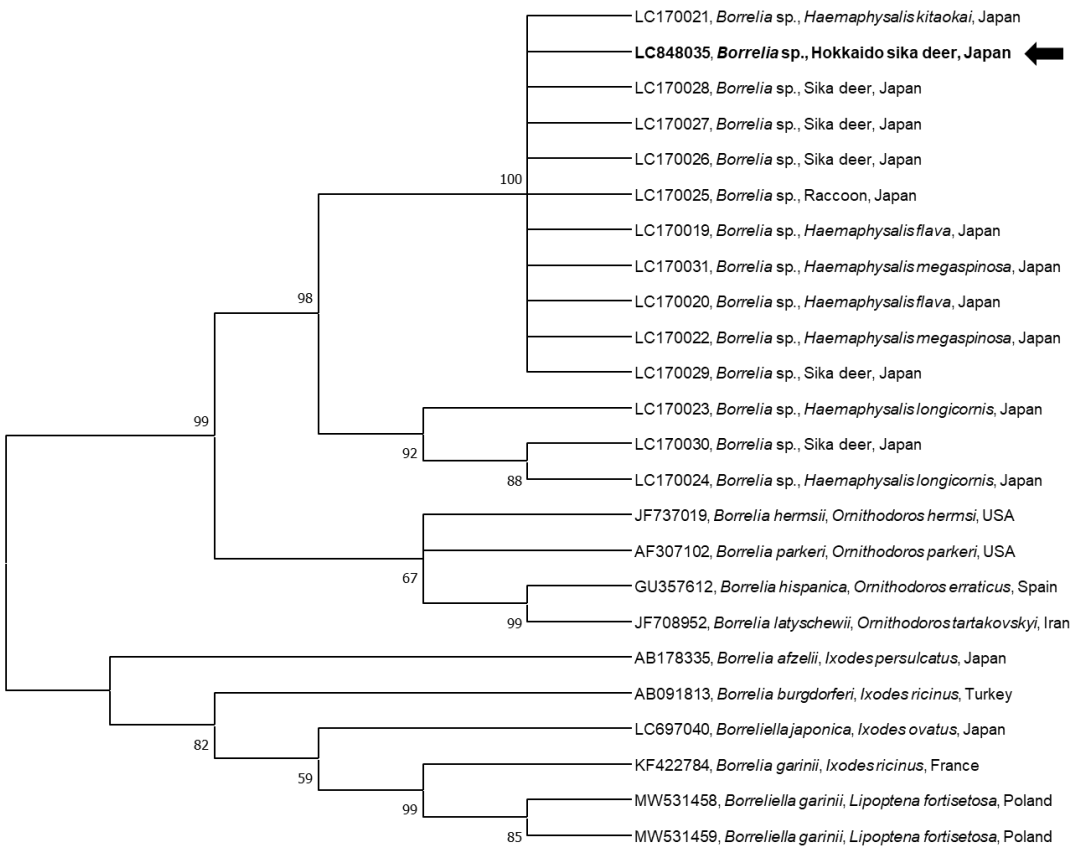

Figure 12. Phylogenetic analysis of the flagellin B gene of *Borrelia* sp. in Hokkaido sika deer and *Lipoptena fortisetosa*. The sequences identified in this study are shown in bold. A phylogenetic tree was constructed using the maximum likelihood method with 1,000 bootstrap replicates. The arrow indicates a sample detected from Hokkaido sika deer blood. The scale bar represents the phylogenetic distance between sequences. The species, hosts, isolation regions, and GenBank accession numbers are shown in the figure. LC848035 represents identical sequences: LC848036 (from *L. fortisetosa*, Japan).

Discussion

In the present study, several pathogens were detected in sika deer and their ectoparasitic deer ked (*L. fortisetosa*). After pupation, deer keds can immediately begin parasitizing hosts and disperse by flying to find new ones, potentially infecting their primary cervid and nonspecific hosts, including humans, companion animals, and livestock (Maślanko et al., 2020; Metelitsa, 1989; Sokół and Gałęcki, 2017). This behavior threatens forest workers and hunters through disease transmission (Härkönen et al., 2009; Maślanko et al., 2020). The pathogens detected in this study have not been confirmed to be pathogenic to humans, livestock, or wildlife, although they may cause clinical symptoms in hosts with weakened immune systems (Furuno et al., 2017; Lee et al., 2019; Yusaku et al., 2016). In this study, *Theileria* sp. Thrivae, AP-sd, and *Borrelia* sp. (showing high homology with *Borrelia* sp. HM) were detected in sika deer and parasitized deer keds. Notably, *Theileria* sp. Thrivae and AP-sd were also found in deer keds collected from sika deer that tested negative for these pathogens. Although the parasitemia or bacteremia levels in the tested deer may have been below the detection limit, these findings suggest that deer keds acquire pathogens from previously infected hosts and potentially function as mechanical vectors for other hosts.

Theileria sp. Thrivae was detected at high rates in both sika deer (84%) and deer keds (63%), consistent with previous studies (Elbaz et al., 2017; Lee et al., 2019). This species was considered equivalent to *T. cervi*, which was previously identified in China (Lee et al., 2019; Yusaku et al., 2016). Studies on *Theileria* sp. Thrivae in sika deer and cattle have revealed that the pathogen was present in sika deer and ticks (*I. persulcatus* and *Ha. japonica*) but not in cattle, despite being infected by the same tick species (Shibata et al., 2018).

As discussed in Chapter II, AP-sd is a deer-specific strain of *An. phagocytophilum* commonly found in Hokkaido sika deer. In the present study, AP-sd was detected in 75% of sika deer and 2% of the deer keds, reaffirming the role of deer as a major reservoir of this pathogen.

Borrelia sp., detected in 3% of the sika deer and 1% of the deer ked, had a sequence identical to *Borrelia* sp. HM, a hard tick-borne relapsing fever borreliae. *Borrelia* sp. HM has been detected in wild boars, raccoons, and sika deer in Honshu, with variations in infection rates corresponding to seasonal tick abundance (Lee et al., 2014). Although the sample size employed in this study was insufficient to accurately assess the

prevalence of *Borrelia* spp. in the study area, the prevalence of *Borrelia* spp. may have been markedly lower than that of *Theileria* sp. Thrivae and AP-sd.

To highlight the biological transmission of pathogens via a biological vector, multiple experimental approaches are needed to address several key aspects: (1) pathogen multiplication within the vector body; (2) transstadial and/or transovarial transmission of pathogens in the vector; (3) transmission of pathogens to new hosts via feeding of the vector (Gray et al., 2019; Levin et al., 2017). Although the experimental protocol relying on the PCR detection of pathogen DNA in deer keds is not appropriate for evaluating the aforementioned criteria, the absence of pathogen DNA in unfed specimens suggests that deer keds are unlikely to maintain the pathogens themselves, thus not playing the role of a biological vector of the pathogens. This assumption is supported by previous studies reporting lower detection rates of pathogens in unfed deer keds than in blood-fed keds (De Bruin et al., 2015; Gałęcki et al., 2021a). *Theileria* sp. Thrivae and AP-sd in deer keds parasitizing sika deer tested negative for these pathogens, allowing for several assumptions. First, the deer keds acquired these pathogens from the sika deer that they were parasitizing; however, the levels of parasitemia or bacteremia caused by the pathogens in the deer were below the detection limit. Second, deer keds may have acquired pathogens from previously infected hosts as they can move between hosts through direct contact, especially when deer congregate (Samuel and Trainer, 1972). If this is the case, the detection of pathogens in deer keds of pathogen-negative sika deer may indicate that they act as mechanical vectors for some pathogens.

Considering the mechanical transmission process, the differences in pathogen detection rates between *Theileria* sp. Thrivae and AP-sd in deer keds were noteworthy. The feeding mechanism of deer keds involves a long proboscis that pierces the skin of the host (Andreani et al., 2019; Krenn and Aspöck, 2012), and mechanical transmission can occur via the injection of saliva during feeding or regurgitation of previously ingested blood (Butler et al., 1977). Although digestive fluids and immune mechanisms typically limit the survival of pathogens (Alavi et al., 2003; Cirimotich et al., 2011; Vaughan et al., 1994), some ingested blood may be stored in the esophagus, potentially allowing longer pathogen persistence (Baldacchino et al., 2013; Skvarla et al., 2020). Given that deer keds may not maintain the pathogens themselves, differences in detection rates between pathogens may be attributed to variations in the blood titer levels of the hosts and pathogen persistence in the insect digestive system. These factors affect mechanical transmission efficiency and are influenced by multiple characteristics, including pathogen

structure, physiological properties, and host-pathogen relationships (Baldacchino et al., 2013; Foil and Gorham, 2000). To validate the role of deer ked as vectors, future research should focus on detailed examinations of pathogen expulsion during feeding and broader investigations of host-pathogen-vector dynamics, particularly the mechanisms of pathogen acquisition and transmission.

In the context of vector-borne disease ecology in Hokkaido, these findings on deer keds complement the tick-focused investigations presented in Chapters I and II. While ticks exhibit distinct seasonal dynamics and regional distribution patterns as demonstrated in previous chapters, deer keds represent an additional, yet understudied component in pathogen transmission cycles. The detection patterns observed in this study suggest that while ticks function as biological vectors capable of maintaining pathogens across developmental stages, deer keds likely serve as mechanical vectors with more limited transmission capacity. This multi-vector perspective adds important complexity to our understanding of disease ecology in Hokkaido wildlife.

These findings demonstrate that deer keds (*L. fortisetosa*) can harbor and potentially transmit three distinct pathogens: *Theileria* sp. Thrivae, AP-sd, and *Borrelia* sp. in sika deer populations. The detection of *Theileria* sp. Thrivae and AP-sd in deer keds collected from pathogen-negative sika deer, combined with the absence of pathogens in non-parasitized deer keds, may indicate their possible roles as mechanical vectors. Understanding the role of deer keds in pathogen transmission is crucial for wildlife disease management, given their ability to parasitize multiple host species, including humans and livestock. Future studies should explore the mechanisms of pathogen acquisition and transmission by deer keds to better understand their role in wildlife disease epidemiology.

Summary

Deer keds (*L. fortisetosa*) are hematophagous insects that parasitize various ungulates, including Hokkaido sika deer (*C. nippon yesoensis*). Although deer keds are potential vectors for several pathogens, their role in disease transmission in Japan remains unclear. This study aimed to evaluate the potential of *L. fortisetosa* as a vector for selected pathogens in sika deer. Blood samples were collected from 32 sika deer and 149 deer keds (64 from deer and 85 from the environment) from the Rusha area of the Shiretoko Peninsula, Hokkaido, Japan. Nested PCRs and sequencing were performed to detect the 18S rRNA gene of *Theileria* sp. Thrivae, 16S rRNA gene of AP-sd, and flagellin B gene of *Borrelia* sp. in deer and deer keds. In sika deer, the infection rate was 84% for *Theileria* sp. Thrivae, 75% of AP-sd, and 3% of *Borrelia* sp. The prevalence in deer keds collected from deer was 62% for *Theileria* sp. Thrivae, 2% AP-sd, and 1% *Borrelia* sp. No pathogens were detected in nonparasitic deer keds captured from the environment. *Theileria* sp. Thrivae and AP-sd were detected in deer keds collected from PCR-negative sika deer, suggesting that deer keds acquired pathogens from a previously infested host. The absence of pathogens in non-parasitized deer keds suggests that they do not play as a biological vector for the tested pathogens. This study suggests a potential role for *L. fortisetosa* as a mechanical vector, emphasizing the need for additional experiments, including infection studies.

Conclusion

This study comprehensively investigated host-vector-pathogen dynamics in Hokkaido's key wildlife species, sika deer (*C. nippon yesoensis*) and brown bears (*U. arctos yesoensis*). The investigation revealed complex host-vector-pathogen interactions operating within Hokkaido's unique subarctic ecosystem, providing critical insights for wildlife health management and disease ecology.

In Chapter I, tick attachment site preferences and interspecific interactions among ticks in sika deer from the Rusha area of the Shiretoko Peninsula, Hokkaido were examined. Four tick species were identified (*I. ovatus*, *I. persulcatus*, *Ha. japonica*, and *Ha. megaspinosa*), each displaying characteristic seasonal variation. *Ixodes ovatus* peaked in June and declined in autumn. *Ixodes persulcatus* maintained stable infestation levels throughout the study period, predominantly as the nymph stage. The number of *Haemaphysalis* ticks drastically increased after September, with adult male *Ha. japonica* showing a notable increase. *Haemaphysalis megaspinosa* was observed only in September and October, attaching widely to the head, pinna, and body. Regarding attachment sites, *Ixodes* spp. tended to attach to the pinna, whereas *Ha. japonica* showed seasonal variations, inhabiting the body and legs from June to August and shifting to the pinna after September. Co-occurrence analysis revealed positive co-occurrence patterns within the same species and genera and negative patterns between different genera. The negative co-occurrence pattern between *I. ovatus* and *Ha. japonica* suggests that these species tend to avoid each other, potentially involving interspecific competition or chemical communication interference. These results demonstrate that tick attachment site selection is influenced not only by species-specific attachment site preferences but also potentially by interactions between tick species.

In Chapter II, a comprehensive study of tick-borne disease ecology in Hokkaido revealed distinct transmission dynamics between AP-sd in sika deer and *He. ursi* in brown bears across four regional populations. These findings demonstrate that pathogen distribution patterns are shaped by complex interactions between vector ecology, host characteristics, and environmental factors, rather than by simple vector-host relationships. The prevalence of AP-sd showed a clear vector-driven distribution, with high infection rates in South and East Hokkaido, corresponding to areas where multiple tick species (*Ixodes* and *Haemaphysalis* spp.) coexist, while low prevalence in North Hokkaido may reflect the absence of *Haemaphysalis* spp. In contrast, *He. ursi* exhibited more complex

dynamics, with unexpectedly low infection rates in East Hokkaido despite abundant *Haemaphysalis* spp., which serve as vectors. This pattern suggests additional limiting factors, including potential recent vector range expansion, geographical barriers between bear populations, and the effects of host age class. The six tick species (*I. ovatus*, *I. persulcatus*, *Ha. japonica*, *Ha. megaspinosa*, *Ha. flava*, and *Ha. longicornis*) were collected from both host animals and demonstrated distinct seasonal activity patterns, regional distributions, and host preferences. *Haemaphysalis* spp. showed pronounced regional variation with notable absence in North Hokkaido, whereas *Ixodes* spp. were more widely distributed. Host factors, including sex and age, influence tick infestation patterns differently among tick species. In addition, *I. persulcatus* showed different host preferences at each developmental stage, with nymphs predominating on deer and adult stage predominating on brown bears. Collectively, the differences of transmission dynamics AP-sd and *He. ursi* illustrated how environmental conditions, vector distribution, and host immunity interact to create unique epidemiological patterns even within the same ecosystem. These findings emphasize that effective tick-borne disease management requires pathogen-specific understanding rather than generalized approaches.

Chapter III evaluated the pathogen vector potential of deer keds (*L. fortisetosa*), an external parasite that is different from that of ticks. *Theileria* sp. Thrivae, AP-sd, and *Borrelia* sp. were detected in sika deer and deer keds. Importantly, *Theileria* sp. Thrivae and AP-sd were detected in deer keds collected from also pathogen-negative sika deer. In contrast, no pathogens were detected in unfed deer keds collected from the environment. These results suggest that, while deer keds may have limited biological vector capabilities, they potentially function as mechanical vectors for pathogen transmission.

Taken together, these findings demonstrate that wildlife disease transmission operates through complex ecological interactions among hosts, vectors, and pathogens where multiple factors interact to create complex epidemiological patterns. Such complexity cannot be predicted by studying individual components in isolation, and necessitates integrated research frameworks for future disease ecology investigations. While these findings provide important insights, several areas require further investigation to fully understand these complex systems. For instance, Chapter I suggested that similarities in attachment site preferences among different tick species could potentially facilitate co-feeding transmission, whereas Chapter III indicated the possibility of deer keds serving as mechanical vectors. Experimental infection studies are

essential to validate these hypotheses. Additionally, Chapter II emphasizes the importance of research on the vector competence of each tick species and host immune responses.

This study underscores the importance of ecological perspectives in tick-borne disease research, and highlights the need to integrate field surveys with laboratory-based investigations. As the relationship between humans and wildlife changes rapidly and in complex ways, this integrative approach is essential. This study revealed the complex ecological relationships in Hokkaido's unique ecosystem. These findings provide important insights for future directions in tick-borne disease research and a crucial foundation for developing adaptive strategies to protect wildlife and human health.

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Summary in Japanese

マダニ媒介性感染症は、宿主・ベクター・病原体の複雑な相互作用により発生し、野生動物、家畜、人間の健康に深刻な脅威をもたらしている。北海道では、エゾシカやヒグマなどの大型哺乳類がマダニの主要な宿主となっており、多様なマダニ媒介性病原体に感染している。これらの感染症の伝播動態を解明するためには、三者の関係を生態学的観点から統合的に解析することが必要である。しかし、宿主・ベクター・病原体の関係を統合的に検討した生態学的研究は少なく、特に大型哺乳類を対象とした研究は、サンプル採集や長期モニタリングの技術的困難さから、十分に行われていない。本研究では、マダニの重要な宿主であるエゾシカとヒグマを対象に、宿主・ベクター・病原体の関係を解明することを目的とした。この目的達成のため、相互に関連する 3 つの研究項目を設計し、多角的な視点からこの複雑な生態学的相互作用を検討した。

第 1 章では、マダニ種が種特異的な寄生部位嗜好性を有し、そのパターンが種間相互作用によって影響されるという仮説を検証した。具体的には、エゾシカを対象として個体レベルでのマダニ寄生パターンを詳細に調査し、寄生部位の種特異性およびマダニ種間の寄生関係を評価した。その結果、各マダニ種は明瞭な寄生部位嗜好性を示した。ヤマトマダニおよびシュルツェマダニは耳介への寄生を好み、ヤマトチマダニは季節によって寄生部位を変化させる傾向が確認された。オオトゲチマダニは頭部、耳介、体幹部に広く分布していた。また、寄生部位における種間関係の分析により、同種・同属間では同一部位への共寄生という正の関係、異なる属間では同一部位での寄生回避という負の関係が観察された。これらの結果から、マダニの寄生部位選択が種間・種内相互作用の影響を受ける可能性が示唆された。

第 2 章では、病原体の感染率およびマダニの寄生パターンが環境要因と宿主要因によって変動するという仮説を検証した。北海道におけるマダニ媒介性疾患の生態を包括的に解析し、エゾシカにおける AP-sd およびヒグマにおける *He. ursi* の伝播動態を調査した。AP-sd の感染率はベクターの分布と一致し、南部および東部北海道で高い値を示した。これはマダニ属とチマダニ属の複数種が共存する地域に対応していた。一方、北部北海道ではチマダニ属が確認されず、感染率も著しく低かった。これに対し、*He. ursi* の感染率はより複雑な動態を示し、ベクターであるチマダニ属が豊富に存在する東部北海道においても感染率は低かった。この結果は、近年のベクター分布拡大や宿主の発育による獲得免疫など、複数の制限要因が関与していることを示唆している。このように、AP-

sd と *He. ursi* は同一生態系内でも全く異なる感染動態を示し、効果的なマダニ媒介性感染症対策には、病原体ごとに宿主・ベクター・病原体の生態学的相互作用を考慮した個別の管理戦略が必要である。

第 3 章では、マダニ以外の外部寄生虫による病原体伝播への関与可能性を検証した。具体的には、シカシラミバエ（*L. fortisetosa*）の病原体ベクターとしての可能性を評価した。その結果、エゾシカおよびエゾシカから採取されたシカシラミバエから、*Theileria* sp. Thrivae、AP-sd、*Borrelia* sp.が検出された。特に重要な点は、PCR で病原体が検出されなかったエゾシカから採集されたシカシラミバエからも、*Theileria* sp. Thrivae および AP-sd が検出されたことである。これは、シカシラミバエが以前に感染した宿主から病原体を獲得していた可能性を示している。一方、環境中で採集された未吸血のシカシラミバエからは、いずれの病原体も検出されなかった。これらの結果は、シカシラミバエが機械的ベクターとして病原体伝播に関与する可能性を示唆しており、今後は感染実験を含むさらなる研究が必要である。

本研究の各章を統合することで、北海道の代表的な大型哺乳類であるエゾシカとヒグマにおける宿主・ベクター・病原体間の複雑な生態学的相互作用が明らかになった。これらの動物におけるマダニ媒介性感染症の伝播動態は、複数の要因が重層的に関与することで独自の疫学的パターンを生み出しており、個別の構成要素を単独で研究するだけでは予測できない。今後の疾病生態学的研究においては、このような複雑性を踏まえた包括的な研究枠組みの構築が不可欠である。本研究は、マダニ媒介性疾患研究の今後の方向性に関する重要な示唆を提供し、野生動物と人間の健康を増進するための効果的な管理戦略開発に重要な基盤を築いた。