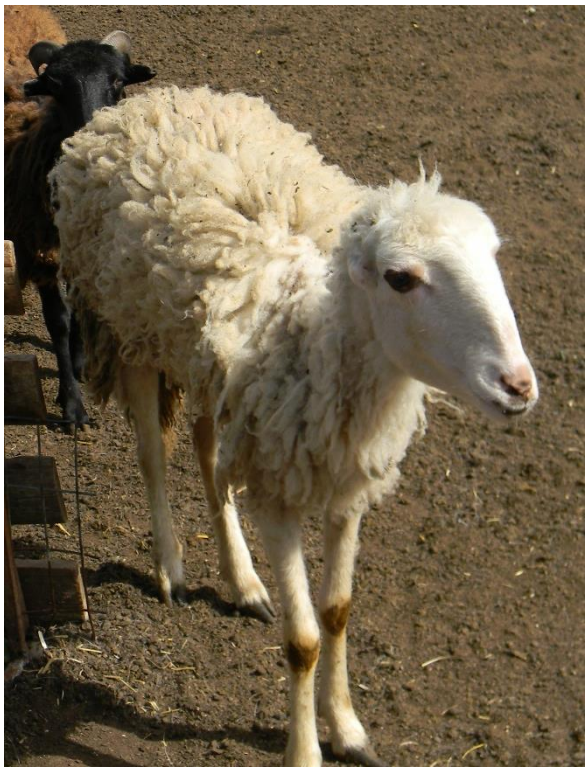
Ticks and Tick-borne diseases surveillance with special reference to *Babesia* infections in small ruminants in northern Greece.



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Contents

1. Abstract

Ticks (n= 530) and blood samples (n= 228) were collected from small ruminants on farms (n= 39) in northern Greece during the period of April-June in 2015. The farms were either sheep farms, goat farms or mixed (shoats). The following prefectures were visited: Epirus, West Macedonia, Central Macedonia, East Macedonia and Thrace. During the visits the farmers were asked questions that were recorded on a questionnaire. There were questions about the management of the farmer, common diseases and tick-borne diseases. The ticks were identified as follows: *Dermacentor marginatus* (0.57%), *Hyalloma marginatum* (0.75%), *Ixodes gibbosus* (1.89%), *Ixodes ricinus* (1.32%), *Rhipicephalus annulatus* (2.08%), *Rhipicephalus bursa* (70.19%), *Rhipicephalus sanguineus* (4.90%), *Rhipicephalus turanicus* (18.30%). *R.* bursa had the highest prevalence and is known to be a vector of *Babesia ovis, Anaplasma ovis* and *Theileria* spp. The blood samples will be used in a subsequent study using PCR reverse line blot (RLB) hybridization and sequencing. Knowledge of the present tick species in Greece gives an opportunity to make a risk analysis about tick-borne diseases and the possible economic impact for the farmers.

2. Introduction

Small ruminant production is very important in Greece as it covers around 27% of the total animal production[1]. Over 90% of the small ruminants in Greece are held for the production of milk and milk products, such as the famous feta cheese or Greek yoghurt[2]. Naturally the farmers want to keep the milk production rates as high as possible. It is important to know that diseases can limit the production. It is likely that ticks and tick-borne diseases can have a negative impact on the production, but no reliable data is available. Tick-borne diseases can however cause morbidity and mortality in small ruminants[3].

There are different tick species that can be found in northern Greece. Table 1 shows tick species found in Greece in previous studies. All tick species have some sort of preferred habitat. This includes the environment and the host. Geographical information of northern Greece will be described below, and also the production systems used in Greece and the most common sheep and goat breeds will be discussed. This information is useful to understand why some tick species are present in northern Greece, while others are not or in lower numbers.

Table 1: Tick species found in Greece

Tick species	**Papazahariadou et al., 2003**	**Chaligiannis et al., 2014**	**V. Pavlidou et al., 2008**	**A. Papa et al., 2011**	**B. Papadopoulos et al., 1996**
Rhipicephalus spp.					
● *turanicus*	X	X	X	X	X
● *sanguineus*	X	X	X	X	X
● *bursa*		X	X	X	X
● *annulatus*		X	X	X	
● *camicasi*		X			
Hyalomma spp.					
● *marginatum*		X	X	X	X
● *anatolicum*		X		X	X
excavatum					X
● *scupense*		X		X	X
● *rufipes*		X			
● *dromedarii*		X			
● *impeltatum*		X			
● *turanicum*					
Ixodes spp.					
● *ricinus*		X	X		
● *gibbosus*		X	X		
Dermacentor spp.					
● *marginatus*		X	X		

2.1. Geographical information

Greece is one of the southern European countries with the Mediterranean Sea as a border and therefore it has a Mediterranean type of climate. The winters are rainy and the summers are dry and warm. The country is divided in several regions shown in figure 1.

Figure 1: Districts of Greece

Greece is very mountainous as the mountains have a coverage of more than 80% of the whole surface. The Pindus mountains cover most parts of northern Greece and have some high peaks. Mount Olympos has the highest peak (2.917 m) of Greece.

It is hard to maintain agricultural activities in places that are very mountainous. The mountainous areas are called less favored areas (LFA's) by the European Union, meaning that there has to be some sort of compensation for the farms that are located in these sites. Sheep and goat farming provides employment in the rural areas where other agricultural activities are limited. Sheep and goats can maintain themselves in these less favored areas, because they have a few adaptations for dry and low fertile grounds. Cattle for example cannot live in the mountainous areas because

they drink more water than small ruminants. Sheep and goats can act as a prevention to fire in these dry areas, because they eat the dry woody products [4].

2.2. Production systems

The production systems in the livestock industry are divided according to their feeding management, the infrastructure and the productivity of the animals. The sort of production system that is used on a farm is dependent on the area in which the farm is located. The natural grazing area can be very dry with very little vegetation. This can result in a low intake of nutrients and consequential low productivity. Too many animals held in one area can also affect the quality of the grazing areas. When grazing is not sufficient there needs to be supplementary feeding in order to keep up with the needs of the animals. Unfortunately, supplementing food is highly dependent on the availability and the price instead of the needs of the animals [5].

Picture 1: round milking machine

Picture 2: straight milking machine

Originally there were four types of production systems in Greece: semi-intensive, sedentary extensive, transhumant and small intensive. Since the last ten years the production systems in Greece are mainly semi-intensive or sedentary extensive [4]. The intensive production systems is used for small flocks. Sheep and goats are kept mostly indoors and sometimes graze for a few hours a day in an adjacent pasture. The semi-intensive production system has developed as a replacement for the cotton production as this used to be a large sector in Greece, but is not anymore. The farms are located in plains and give other job opportunities because the flocks need to be guided by shepherds. The milk price is high compared to that of the feed for the animals. These animals have a high production rate. The farms that use the intensive production system invest in a good infrastructure for example by buying a milking machine [4]. There are a lot of different milking machines available in

the market. Some are situated in a circle, which turns around while the sheep or goats are milked. Other are built in straight lines and they can be built for different numbers of animals. Examples of milking machines are shown in the pictures below.

The extensive production system can be divided in transhumance and non-transhumance. Transhumance means that different fields are used for grazing during summer and winter. These farms are dependent on the natural circumstances and are often located in Less Favored Areas. The organic farms originated from this production system. The flocks on these farms consist usually of more than a hundred animals. These are animals of local breeds that have adapted to the circumstances in the areas that are less favored. The production level of these animals is lower than that of the animals from the intensive production systems. Milking is done outside and by hand in most cases. The flocks are kept together by shepherds with their shepherd dogs [1,6]. The sheds for housing the animals during the night, are built with low cost materials. Picture 3 shows how such a shed could look like in a mountainous area. Of course, many variations are possible.

Picture 1: an example of a shed for housing the animals

2.3. Sheep and goat breeds

The breed of the animals and the production system that is used on a farm are two variables that are dependent upon each other. Some breeds are more suitable for the intensive production system, while others are more suitable for the extensive production system. It depends on the productivity of the breed. The breeds that have high production levels can be found on the farms with an intensive production system[4].

There are many local sheep and goat breeds in Greece. Most of the sheep and goat farms in Greece are located in very dry areas where there is not much food for the animals. The local breeds have adapted to the circumstances on the Greek mainland. Sometimes local breeds are less susceptible to diseases then foreign sheep or goat breeds. This can also be the case with tick-borne diseases.

Local sheep breeds that can be found on farms in northern Greece are Katsika (Epirus), Serres (Thrace), Chios (central, eastern and western Macedonia), Tsigai, Zackel. The Chios breed is considered to be having the highest production levels of all Greek sheep breeds. This particular breed can be found on many farms with the intensive production system. On farms that are using the intensive production system, one can come across some local breeds that are cross bred with other breeds to have a higher performance[7].

Less information is available about the local goat breeds of Greece. A lot of farms use cross breeds from local Greek goat breeds and foreign goat breeds. Zaanen and Alpin goats can be found on farms that use an intensive production system. These breeds are typical dairy goats. Skopelos is a Greek goat breed that is used in the semi-intensive production systems. Local breeds, also called Indigenous, are often used for the extensive production systems[8].

There are a few differences as regarding the production of sheep milk or goat milk. Sheep milk is typically used for the production of yoghurt and cheese. The production level of goats can be much higher than that of sheep, respectively up to 500 kg and up to 300 kg. Goats also tend to have a longer lactation period than sheep, respectively 300 days and 250 days[9].

2.4. Ticks and tick-borne diseases

There are two tick families, the Ixodidae and the Argasidea or respectively the hard ticks and the soft ticks. Almost all genera of tick species can be present on sheep and goats worldwide, but the ones with the highest prevalence are: *Rhipicephalus, Haemaphysalis, Hyalomma* and *Amblyomma. Dermacentor* and *Ixodes* are two genera which are also found frequently on sheep and goats[10]. Tick species found in Greece are listed in table 1 and include the genera *Rhipicephalus, Hyalomma, Ixodes* and *Dermacentor. Rhipicephalus* has the highest prevalence in Greece, especially *Rhipicephalus bursa* can be found on small ruminants in Greece. It is known that *Rhipicephalus* and *Hyalomma* tick species are active during spring and summer and can be found all over Greece. *Ixodes* and *Dermacentor* prefer a high altitude and can be found during autumn and winter. The immature stages of *Rhipicephalus bursa* can be found during autumn and winter[11].

Tick-borne diseases can have an economic impact on a morbidity and mortality or due to the costs of the antiparasitic drugs. However, tick-borne diseases may be even more important when it comes to improving the production rates. Local breeds may have adapted to the tick-borne pathogens, but these pathogens can inhibit improvement of the production[12].

The tick species *Rhipicephalus* bursa has the highest prevalence in northern Greece. It is the vector of *Babesia ovis, Theileria ovis* and *Anaplasma ovis*[13]. Ovine babesiosis can be caused by *Babesia ovis, B. motasi* and *B. crassa*. In Greece *B. ovis* is found mostly in sheep[14]. *Rhipicephalus bursa* is considered as main vector of *B. ovis*[15]. One study in the region of Thessaly and Epirus (Greece) was selected to determine the infection rate of *Babesia* in small ruminants. On 43% of the farms in this study there were animals tested positive on *B. ovis.* The prevalence on these farms was 10-61%[16]. Interestingly, another study in a village in northern Greece found a *Babesia lengau*-like organism infection in two sheep flocks. These sheep showed symptoms of haemolytic disease. Until recently *B. lengau* was only detected in a cheetah population in South Africa. It is important to further investigate the presence of *B. lengau*-like organisms in small ruminants in Greece, because there is little information available about this matter[17].

2.5. Aim of the study

The aim of this study was to investigate which tick species are present in the northern region of Greece, with special reference to the Thessaloniki area where approximately 165,000 sheep and 135,000 goats are used for animal production. For this study several regions from northern Greece are visited for sampling, including Epirus, West Macedonia, Central Macedonia, East Macedonia and Thrace.

Knowledge of the present tick species is required in order to make a risk analysis about tick-borne diseases, that may affect the production rates of the small ruminant farming enterprises. When this information is available prevention plans can be made in order to keep the production rates as high as possible.

3. Material and methods

This research study is performed in several different regions of northern Greece; Epirus, West Macedonia, Central Macedonia, East Macedonia and Thrace. 39 farms were visited to collect ticks and blood samples; 20 sheep farms, 14 goat farms and 5 farms that had both sheep and goats.

3.1 Tick and blood collection

Collecting the samples took place in the morning or evening, because the animals were grazing outside during the day. Five to ten animals per farm were used for the sampling. The farmers tried to select the animals with tick infestations.

The farmers helped handling the animals and put them on their back when this was necessary to check for ticks. From each animal that was being examined, ticks were collected using a forceps and a tick vial. Predilection sites were checked for the presence of ticks, including the ears, the udder and the area around the genital organs. Some animals did not have ticks, which can be caused by all different sort of things. The use of ectoparasitica could be the cause of the absence of ticks, but also the climate, the time of the year and the breed of the hosting animal could influence the presence of ticks. From the same animal from which the ticks were collected, blood was taken using EDTA vacutainers. 18G needles were used to take the blood.

Each tick vial was labeled with the farm number and the animal number. Blood vials had the same information written on the label and the date and host species (sheep or goat) was added on these labels. The ticks and the blood were kept in separate bags per farm in a coolbox with icepacks until returning in the lab. After returning in the lab, the ticks were put into Eppendorf tubes containing 80% ethanol and the blood was stored at -20 °C, also in Eppendorf tubes. All samples were labeled with farm and animal number, the date and the host species (sheep or goat).

While the collection took place, the farmers were asked some questions from the questionnaire, which is added as appendix I. It contained questions about the management strategies of the farms, the number of animals and whether or not symptoms of tick-borne diseases were present in the sheep or goat flock. The use of ectoparasitica was also important information, because this can cause the absence of ticks on the animals.

3.2 Tick identification

All the collected ticks (n= 530) were identified using the book *Ticks of domestic animals in the Mediterranean region: a guide to identification of species* of Estrada-Pena et al., 2004. After the identification the ticks were put in separate vials according to their species and the sample number of the animal on which they were found.

3.3 DNA extraction

The QIAGEN DNeasy blood and tissue kit was used to do the DNA extractions on all the blood samples (n = 228). The protocol of the QIAGEN company was followed to avoid mistakes and is added as appendix II. Before the DNA extraction could begin the tables were cleaned thoroughly with a special cleaning liquid that contained ethanol. The blood was pipetted from the Eppendorf tubes using special filtered tips. For each sample a new tip was used. 100 µL of blood was used per sample and put in a new sterilized Eppendorf tube together with 100 µL PBS and 20 µL Proteinase K. This is according to the steps of the protocol for nonnucleated blood. The protocol is further followed until all steps are completed. The centrifugation after buffer AW2 is added was done for four minutes instead of three to make sure that everything has passed the filter. When all the steps are completed there is 200 µL of DNA eluted from the original sample. This will be used in a following phase of the project for PCR reverse line blot (RLB) hybridization and sequencing to discover pathogens that were present in the blood samples.

4. Results

The following results were collected during the sampling and the tick identification. The total number of farms that were visited was 39. The number of ticks that were collected during this study was 530.

4.1 Questionnaires

The production systems were split in three groups; extensive transhumance, extensive non-transhumance and semi-intensive. The numbers of farms per group are showed in table 2. As can be seen the semi-intensive and extensive non-transhumance have the highest percentage.

Table 2: Production system

Production system	Frequency	Percent
Extensive transhumance	8	20,5
Extensive non-transhumance	15	38,5
Semi-intenstive	16	41,0
Total	39	100,0

4.2 Tick identification

The total number of ticks found on the farms was 530. A list of all the ticks found on the animals is added as appendix III. *Rhipicephalus bursa* (70%) and *Rhipicephalus turanicus* (18%) accounted for most of the identified ticks. Other ticks belonged to the genus of *Hyalomma*, *Dermacentor* and *Ixodes.* The number and the percentage of each tick species that was found during this study are shown in table 3.

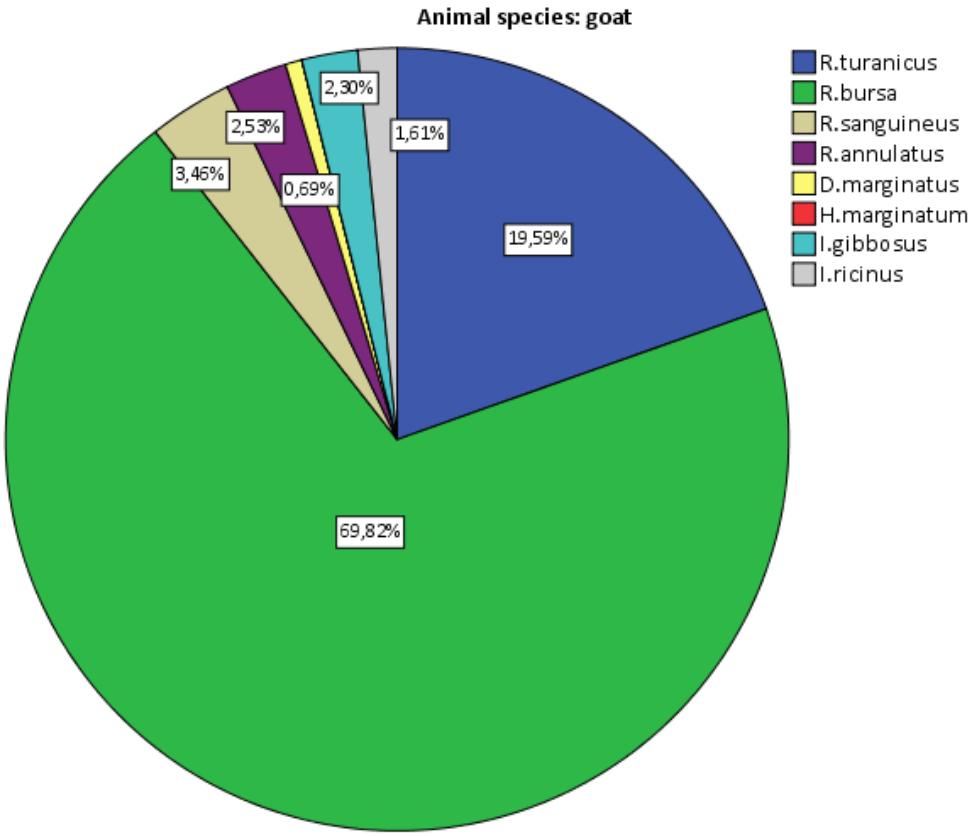
Table 3: Tick species northern Greece

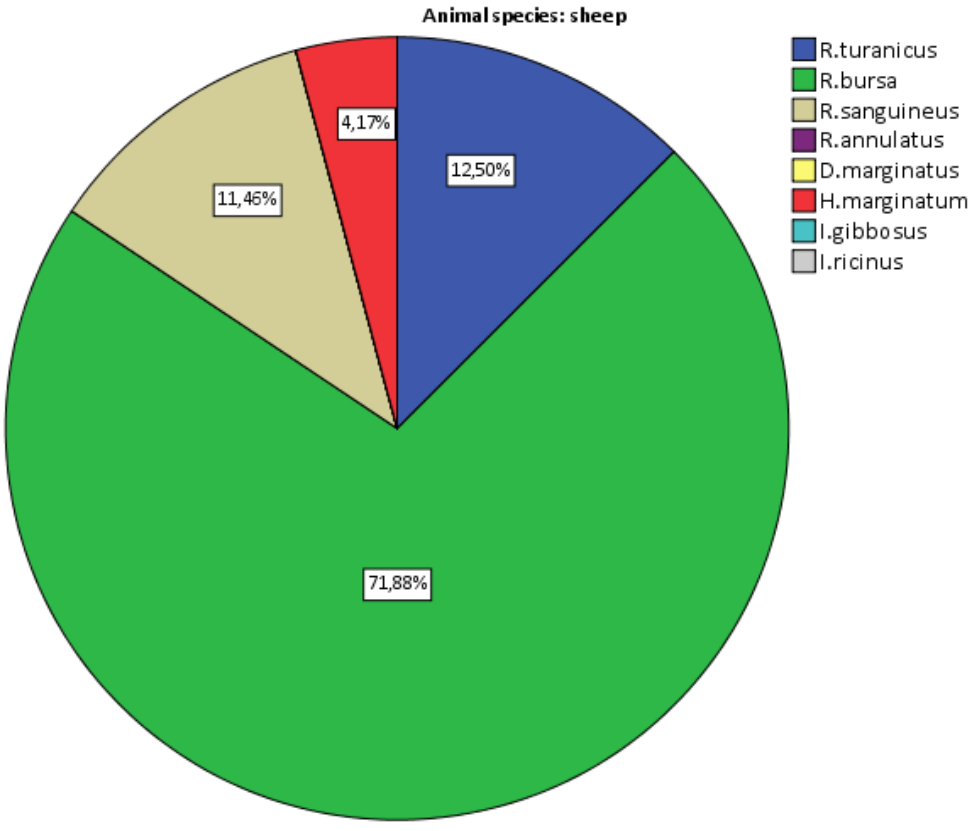
Tick species	Northern Greece
Dermacentor	
- ***D. marginatus***	3 (0,57%)
Hyalomma	
- ***H. marginatum***	4 (0,75%)
Ixodes	
- ***I. gibbosus***	10 (1,89%)
- ***I. ricinus***	7 (1,32%)
Rhipicephalus	
- ***R. annulatus***	11 (2,08%)
- ***R. bursa***	372 (70,19%)
- ***R. sanguineus***	26 (4,90%)
- ***R. turanicus***	97 (18,30%)

The number of ticks per farm is shown in table 4. The minimum and maximum columns are referring to the minimal and maximal number of ticks found on one animal. Sometimes there was a small number of ticks on a farm and sometimes there were no ticks found at all. No ticks were found on 14 farms.

Table 4: Number of ticks per farm

Descriptive Statistics					
Farm no.	No. of animals	Minimum	Maximum	No. of ticks	Mean
1	5	2	7	17	3.40
2	5	10	16	63	12.60
3	6	1	8	22	3.67
4	6	1	14	27	4.50
5	10	2	15	58	5.80
6	5	0	0	0	0.00
7	5	0	0	0	0.00
8	6	0	1	1	0.17
9	5	0	1	1	0.20
10	5	0	2	4	0.80
11	5	0	1	2	0.40
12	5	0	0	0	0.00
13	5	0	0	0	0.00
14	10	0	0	0	0.00
15	5	0	0	0	0.00
16	5	0	0	0	0.00
17	7	0	3	5	0.71
18	5	1	11	26	5.20
19	5	1	31	77	15.40
20	5	0	0	0	0.00
21	5	0	0	0	0.00
22	5	0	0	0	0.00
23	5	0	0	0	0.00
24	5	0	1	1	0.20
25	5	1	3	8	1.60
26	5	0	1	1	0.20
27	10	0	1	5	0.50
28	5	0	0	0	0.00
29	10	0	17	43	4.30
30	5	0	2	2	0.40
31	5	0	1	1	0.20
32	10	0	2	2	0.20
33	5	1	2	7	1.40
34	8	1	12	43	5.38
35	5	1	4	11	2.20
36	5	6	28	90	18.00
37	6	0	0	0	0.00
38	5	0	0	0	0.00
39	5	1	6	13	2.60


Animal species: goat
R.turanicus
R.bursa
R.sanguineus
R.annulatus
D.marginatus
H.marginatum
I.gibbosus
I.ricinus
19,59%
69,82%
3,46%
2,53%
0,69%
2,30%
1,61%


Animal species: sheep
R.turanicus
R.bursa
R.sanguineus
R.annulatus
D.marginatus
H.marginatum
I.gibbosus
I.ricinus
12,50%
71,88%
11,46%
4,17%

5. Discussion

The purpose of this study was to determine whether ticks and tick-borne diseases may play a role on the small ruminant farms in northern Greece. The aim of the study was to investigate which tick species were present on these farms. There is not a lot of information available about this subject, because only a few studies are conducted in this area and the information can be outdated. The presence of ticks and tick-borne diseases can have an impact on the health of the animals and therefore on the production levels.

In order to determine which tick species were present on small ruminant farms in northern Greece, 39 farms were selected and visited to collect ticks. The selection of the farms was not entirely random because there was contact with the veterinarian who supervised the farms to know whether there were ticks on the animals or not. The distribution of the study spreads from the west coast all the way to the east. Visits were always concentrated around a village, because the farms were selected by veterinarians who worked in a certain area which covered all the neighboring farms of that particular village. Veterinarians from all over northern Greece selected farms that were suspected of tick infestations. During the visit on the farms the animals were not selected randomly, but the farmers selected their animals that had tick infestations. In field work situations it is not always possible to select the samples randomly. It can also be difficult to work very precisely when one is working in the field. For example the amount of blood taken from the animal cannot be exactly the same for each animal. Only 100 µl of blood was needed for the DNA extraction, so this was not really a problem as the vials often contained more than 5 ml.

When the ticks were collected and put in vials they were stored in the coolbox or in a freezer at – 20 °C or – 80 °C, until there was time to put them in ethanol. Sometimes this took a little longer than expected and the ticks were in the freezer for a few days. For the identification this was not a problem, but it can be a problem for the further investigation on the presence of pathogens in the ticks.

The tick identification showed that *Rhipicephalus bursa* had the highest prevalence on small ruminants in northern Greece. This was the same result as previous studies conducted by Papadopoulos, et al. and Chaligiannis, et al.

There was not enough time to include the PCR and sequencing in this master thesis. These steps will be conducted by other students who will follow up with this research project. The results of the PCR and the sequencing can be very exciting, because it says a lot about the distribution of tick-borne diseases. The blood samples and the ticks will be screened for the presence of *Babesia* spp, with special reference to *Babesia ovis* infections.

6. Conclusion

There are many different tick species present on small ruminants living in northern Greece. This study gives a good overview of the distribution of several tick species in Northern Greece. *Rhipicephalus bursa* has the highest prevalence of all species. It is still unknown if tick-borne diseases play a role. The follow up study will determine if there are any pathogens present in the collected ticks and in the blood samples that were collected on farms in northern Greece.

7. Acknowledgement

I would like to thank Professor Frans Jongejan for giving me the opportunity to fulfill my research internship abroad. I was one of the first students he sent to Greece and I am very grateful for that. It is nice to be a pioneer and have to discover everything by yourself without other students going there before you. Professor Frans Jongejan was a very good supervisor, who even came to visit us in Greece to keep everyone updated.

Of all the people in Greece who helped during the implementation of this research project, most of the credits go to Dr. Smaro Sotiraki and Tasos Saratsis. I could not have done this without them. Dr. Smaro Sotiraki always gave a pep-talk when I was lost in all the information I had to deal with. Tasos went with me to the farms, and I really needed someone who could speak Greek, because the farmers did not speak English. And of course I want to thank all the other people from the lab in Thessaloniki for helping me out and for the nice atmosphere in the lab. It was really a great experience and I learned a lot from it.

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9. Appendix

Appendix I: farm questionnaire

1. Farm details

Farm: Date:

Name farmer:

Age:

Education level:

Address:

Telephone:

Farm coordinates:

Production system:

A. Extensive (moving)
B. Extensive (non moving)
C. Semi-intensive

Number of workers:

2. Farm characteristics

Sheep

- Breed/breeds:
- Total number of sheep:
- Number of rams:
- Number of ewes milked:
- Number of lambs (replacement stock):
- Mean weight of adults (Kg):

Goats

- Breed/breeds:
- Total number of sheep:
- Number of bucks:
- Number of goats milked:
- Number of kids (replacement stock):
- Mean weight of adults (Kg):

Presence of other animals: YES/NO

If yes, which? dogs/cats/cattle/other:

……..

Animals grazing next/inside wildlife preservation park? YES/NO

3. Farm infrastructure

Stable surface (m^2):

Milking machine: YES/NO

Milking machine capacity:

Warehouse: YES/NO

Electric power: YES/NO

Water facilities: well/water supply network/springs/transfer

4. Grazing/feeding system

Free range grazing?: YES/NO

Pasture per 1000 m^2: A. Private

B. Rented

C. Communal

Rotational grazing applied? YES/NO

Common pasture shared with other flocks? YES/NO

Estimated distance covered from flock per day/radius from stable? (in Km)

- Distance:
- Radius:

Duration of grazing in months/hours (per day)

	Hours: 0-8	**8-16**	**>16**
Month			
January			
February			
March			
April			
May			
June			
July			
August			
September			
October			
November			
December			

5. Most common diseases

	YES	**NO**	**Which diseases are common?**
Infectious			
Parasitic			
Limping			
Abortions			
Low fertility			
Mastitis			

6. Ticks and tick-borne diseases (TBD)

Do you have any knowledge about ticks and tick-borne diseases? YES/NO

Do you know why they can be dangerous? YES/NO

Are your animals infested with ticks? YES/NO

Are your goats and/or sheep infested with ticks? Goats/sheep/both

If yes, which months? ……

On which part of the body are the ticks found?

Head/horns/udder/genitals/abdomen/other: ……

Prevention:

Do you use any treatment against ticks? YES/NO

If yes, how often do you use treatment?

A. Once a year
B. Twice a year
C. Three times a year
D. Whenever needed

In which month(s)?

……..

Which drugs do you use?

……

……

How do you calculate the dose?

……

……

What are the costs per year? €…….

Do you apply the same treatment for goats and sheep? YES/NO

If no, explain:

……

Symptoms:

What kind of symptoms do you observe in tick infested animals?

	YES	**NO**
Fever		
Anorexia		
Hematuria		
Swollen lymph nodes		
Breathing problems		
Paralysis		
Death		
Other		

If other symptoms are seen, explain what kind of symptoms:

……

Treatment:

Do you treat the animals showing symptoms? YES/NO

If yes, which drug? ……

Do the animals respond to the treatment? YES/NO

Appendix II: protocol DNA extraction (QIAGEN company)

Quick-StartProtocol

DNeasy® Blood & Tissue Kit

The DNeasy Blood & Tissue Kit (cat. nos. 69504 and 69506) can be stored at room temperature (15–25°C) for up to 1 year.

For more information, please refer to the *DNeasy Blood & Tissue Handbook*, which can be found at www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Redissolve any precipitates in Buffer AL and Buffer ATL.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Equilibrate frozen tissue or cell pellets to room temperature.
- Preheat an incubator to 56°C.
- Refer to the handbook for pretreatment of fixed tissue, insect, bacterial, or other material.

1a. Tissue: Cut tissue (≤10 mg spleen or ≤25 mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, use 1 (rat) or 2 (mouse) 0.4–0.6 cm lengths of tail. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.

1b. Nonnucleated blood: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 50–100 µl anticoagulant-treated blood. Adjust volume to 220 µl with PBS. Proceed to step 2.

1c. Nucleated blood: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 5–10 µl anticoagulant-treated blood. Adjust volume to 220 µl with PBS. Proceed to step 2.

1d. Cultured cells: Centrifuge a maximum of 5 x 10^6 cells for 5 min at 300 x *g* (190 rpm). Resuspend in 200 µl PBS. Add 20 µl proteinase K. Proceed to step 2.

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2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x *g*. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x *g*.
9. Optional: Repeat step 8 for increased DNA yield.

Appendix III: list of all the collected ticks per animal per farm

Animal / Farm	1	2	3	4	5	6	7	8	9	10
1	1 R. bursa ♀, 1 R. turanicus ♀, 1 R. turanicus ♂	1 R. bursa ♀, 1 R.turanicus ♂	1 R. bursa ♀, 5 R. sanguineus ♂, 1 R. bursa ♂	1 R. bursa ♀, 1 R.bursa ♂	2 R. bursa ♀, 1 R.bursa ♂					
2	5 R. bursa ♀, 6 R. bursa ♂	8 R. bursa ♀, 8 R. bursa ♂	2 R. bursa ♀, 1 R. sanguineus ♀, 8 R. bursa ♂	6 R.bursa ♀, 4 R. bursa ♂, 5 R. sanguineus ♂	1 I. gibbosus ♀, 1 I. ricinus ♀, 2 R. bursa ♀, 5 R. bursa ♂, 1 R. sanguineus ♂					
3	4 R. turanicus ♀, 2 R. bursa ♀, 2 R. sanguineus ♂	1 R. bursa ♀	1 R. bursa ♀, 1 R. sanguineus ♀, 1 R. bursa ♂, 1 R. sanguineus ♂	3 R. bursa ♀	5 R. bursa ♀	1 R. bursa ♀				
4	2 I. ricinus ♀, 1 R. bursa ♂	1 R. sanguineus ♂, 1 R. bursa ♂	6 I. gibbosus ♀, 2 I. ricinus ♀, 1 R. bursa ♀, 4 Ixodes ♂, 1 R. turanicus ♂	1 R. bursa ♀, 1 R. bursa ♂	1 R. bursa ♀, 1 R. sanguineus ♀, 1 I. gibbosus ♀, 2 R. bursa ♂	1 R. bursa ♀				
5	2 R. bursa ♀, 4 R. bursa ♂	1 R. bursa ♀, 6 R. bursa ♂	3 R. bursa ♀, 6 R. annulatus ♀, 1 D. marginatus ♀, 4 R.bursa ♂, 1 D. marginatus ♂	5 R. annulatus ♀, 2 R. bursa ♀, 2 R. bursa ♂	6 R. turanicus ♂	2 R. bursa ♂	1 R. bursa ♀, 2 R. bursa ♂	1 R. turanicus ♀, 1 R. turanicus ♂	5 R. bursa ♂	2 R.bursa ♀, 1 R. bursa ♂
6	X	X	X	X	X					
7	X	X	X	X	X					
8	1 R. bursa ♀	X	X	X	X	X				
9	1 R. bursa ♂	X	X	X	X					
10	1 R. bursa ♀	X	1 R. bursa ♀	2 R. bursa ♀	X					
11	1 R. bursa ♀	X	X	X	1 R. bursa ♂					
12	X	X	X	X	X					

Animal / Farm	1	2	3	4	5	6	7	8	9	10
13	X	X	X	X	X					
14	X	X	X	X	X					
15	X	X	X	X	X					
16	X	X	X	X	X					
17	1 R. turanicus ♂	X	X	X	X	2 R. turanicus ♀, 1 R. turanicus ♂	1 R. sanguineus ♂			
18	3 R. turanicus ♀	4 R. turanicus ♀, 5 R. turanicus ♂, 1 R. bursa ♂	1 R. bursa ♀	5 R. turanicus ♀, 1 R. sanguineus ♀, 5 R. turanicus ♂	1 R. turanicus ♀					
19	1 R. bursa ♀, 3 R. bursa ♂	6 R. bursa ♀, 13 R. bursa ♂	10 R. bursa ♀, 11 R. bursa ♂, 1 R. turanicus ♂	11 R. bursa ♀, 16 R. bursa ♂, 3 R. turanicus ♂	1 R. bursa ♀					
20	X	X	X	X	X					
21	X	X	X	X	X					
22	X	X	X	X	X					
23	X	X	X	X	X					
24	X	X	X	1 R. bursa ♂	X					
25	1 R. bursa ♂, 1 R. bursa ♀	1 R. bursa ♀	1 R. bursa ♂	1 R. bursa ♀	1 R. bursa ♂, 2 R. bursa ♀					
26	X	X	1 D. marginatus ♀	X	X					
27	1 H. marginatum ♂	1 H. marginatum ♂	1 H. marginatum ♂	1 H. marginatum ♂	1 R. bursa ♀	X	X	X	X	X
28	X	X	X	X	X					

Animal / Farm	1	2	3	4	5	6	7	8	9	10
29	1 R. bursa ♂	3 R. bursa ♀, 14 R. bursa ♂	1 R. bursa ♀, 3 R. bursa ♂	1 R. bursa ♀, 3 R. bursa ♂	2 R. bursa ♀, 3 R. bursa ♂	1 R. bursa ♀	1 R. bursa ♀, 1 R. turanicus ♀, 2 R. bursa ♂	1 R. bursa ♀, 3 R. bursa ♂	X	1 R. bursa ♀, 1 R. turanicus ♀, 1 R. bursa ♂
30	X	2 R. turanicus ♀	X	X	X					
31	X	X	X	1 R. turanicus ♀	X					
32	2 R. sanguineus ♀	X	X	X	X	X	X	X	X	X
33	1 R. bursa ♀	1 R. bursa ♀, 1 R. sanguineus ♂	1 R. bursa? ♀	1 R. bursa ♀	1 R. bursa ♀, 1 R. bursa ♂					
34	1 R. turanicus ♂	2 R. turanicus ♂	3 R. turanicus ♀, 3 R. turanicus ♂	3 R. bursa ♀, 1 R. turanicus ♀, 7 R. turanicus♂, 1 R. bursa ♂	2 R. bursa ♀,5 R. bursa ♂	1 R. turanicus ♂	1 R. bursa ♀, 2 R. turanicus ♂	2 R. turanicus ♀, 1 R. sanguineus ♀, 1 R. bursa ♀, 6 R. turanicus ♂, 1 R. bursa ♂		
35	1 R. sanguineus ♀, 2 R. turanicus ♂	3 R. turanicus ♀, 1 R. turanicus ♂	1 R. turanicus ♀, 1 R.turanicus ♂	1 R. turanicus ♂	1 R. sanguineus ♀					
36	6 R. bursa ♀, 22 R. bursa ♂	6 R. bursa ♂	11 R. bursa ♀, 1 R. turanicus ♀ 11 R. bursa ♂	3 R. bursa ♀, 10 R. bursa ♂	3 R. bursa ♀, 17 R. bursa ♂					
37	X	X	X	X	X					
38	X	X	X	X	X					
39	1 R. bursa ♂	1 R. bursa ♀, 5 R. bursa ♂	1 R. bursa ♀	1 R. bursa ♀, 1 R. bursa ♂	1 R. bursa ♀, 2 R. bursa ♂					