Ticks and Tick-borne diseases surveillance with special reference to *Anaplasma* infections in small ruminants on the island of Lesvos, Greece




Research Project Veterinary Medicine

Myrthe de Vries 3833828

May-July 2015

Supervisor Greece: Dr. Smaro Sotiraki

Supervisor Utrecht: Professor F. Jongejan

Contents

1. Abstract

On the island of Lesvos in Greece, 39 sheep farms were visited and ticks (n=1195) were collected between May and July 2015. Furthermore, blood samples were collected from 195 sheep. During the farm visits questionnaires were filled in with questions about farm management, common diseases on the farm and in particular tick-borne diseases. The following ticks were identified: *Hyalomma excavatum* (0,43%), *Hyalomma detritum* (0,17%), *Hyalomma detritum scupense* (0,09%), *Hyalomma marginatum* (1,73%), *Hyalomma rufipes* (0,09%), *Rhipicephalus bursa* (4,92%), *Rhipicephalus sanguineus* (2,67%) and *Rhipicephalus turanicus* (89,9%). *R. turanicus* was the predominant ticks species and is a known vector of a number of tick-borne diseases. As a next step, PCR amplification and Reverse Line Blot Hybridization (RLB) will be performed on the extracted DNA obtained from ticks and blood samples. Once it is determined which tick-borne diseases exist on the island of Lesvos, a suitable strategy for control and prevention can be developed.

2. Introduction

Geography and climate of the island of Lesvos

Geography

Lesvos is an island that belongs to the islands of the north-eastern Aegean and lies near the border of Turkey. With an area of 1630 km^2 and a coastline of 370 km it's the third largest Greek island. Two volcanoes that are nowadays non-active formed the island, but there is still some volcanic activity in the form of hot springs that are found on the island. Because the two volcanoes sunk into the ocean, two bays were formed. The two bays are excellent fishing grounds. The biggest bay is the gulf of Kalloni, which is famous for it's sardines. The smaller bay is the gulf of Ghera in the southeast of Lesvos.[1]

There are three big mountains on Lesvos; mount Olympos (967m), mount Ordymnos (634m) and mount Lepetymnos (908m). In between there are plains stretching around the villages of Kalloni, Ghera and Polichnitos. The wetlands of Kalloni cover an area of 100km^2 and are an important place for migrating birds. There are more than 252 species of birds. Also rare plants and wildlife live in these wetlands. Therefore these wetlands are classified as a CORINE biotope and are included in the NATURA 2000 network. This means the wetland area is protected by the European Union. [2]

Almost the entire western part of Lesvos is treeless, while the rest of the island is rich in vegetation. On the eastern part of the island are forests of pine, plane and chestnut trees. Also olive trees and fruit trees are cultivated. The oil that is produced from the olive trees in Lesvos is one of the finest in Greece.[1]

Figure 1: Treeless western part of Lesvos

Climate

Lesvos has a Mediterranean/dry-summer subtropical climate. This means that the summers are long and dry and the winters are short and have mild temperatures. The seasonality is moderate and the average temperature during the year is 17,8 degrees. In springtime the average temperatures are between 11-19 degrees, in summer between 21-31 degrees, in autumn/fall between15-23 degrees and in wintertime between 8-14 degrees. The total average rainfall is 739 mm per year.[3]

Livestock farms in Lesvos

Before the 20th century production of sheep and goat farms in Lesvos was based on self-sufficiency. After that, the rise of the industrialisation began and self-sufficiency was no longer the only goal. In 1922 there was a major economic crisis in Lesvos. Almost all industrial and trading activities stopped and the population declined. Because of this, the land use changed and almost all of the cultivated areas disappeared except for the olive groves. The cultivated areas changed into grazing land. This is why the number of sheep on the island tripled during this period. [4]

In figure 2 the land use zones of Lesvos are shown. The light coloured areas (Mithymna, Mantamodos and Eresos-Antissa) are grazing land only. The area that lies in the southeast of the island consists mainly of olive trees and pine forests. The darkest areas in the middle of the island are intermediated areas. These areas are used for both olive and pine forests and grazing lands. The grazing lands cover more than 50% of the whole island. [4]

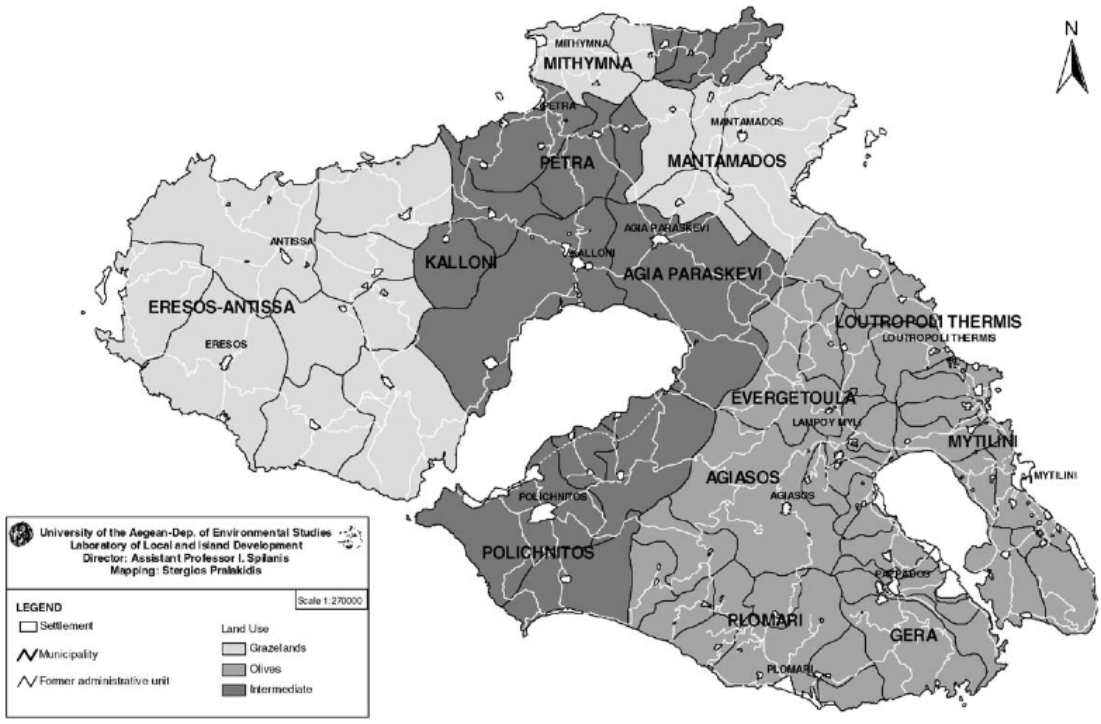

Figure 2: Land use zones of Lesvos [4]

In the northwest of Lesvos almost 80% of the total amount of sheep are kept.[5] There are over 400.000 sheep in Lesvos divided over more than 4000 farms.[6] The flocks have a size of 100-150 sheep per farm, but are sometimes larger. The sheep are kept in simple sheds (‘madria’) at night. Scrap metal, wood and light materials are used to make the sheds, this way the costs of maintaining the shed stay low and the temperature is controlled during the summer.[5]

Figure 3: Typical sheep shed Lesvos

During the day the sheep flocks are grazing on the pastures. In the morning and in the evening the milking process starts. This is done by hand or by milking machines.

Figure 4: Sheep milking system

As mentioned before, mainly sheep are kept for the production of milk and dairy products. 80% of the milk is used to make cheese.[5] Special cheese from Lesvos are Ladotyri, Graviera and Feta. Also meat, leather and wool are important products from sheep.[1]

In order to get good production rates it is important to have a breed that is well adapted to their environment and climate. Therefore breeding selection is done since the last century. Traditional livestock breeds like the Lesvos breed is somewhat smaller in size but better adapted than the modern breeds that are only selected on high productivity. That is why the Lesvos breed is the most common breed in Lesvos.[7] Of course there are also other breeds on the island like Chios, Laucane, Karagouniko and mixed breeds.

Tick-borne diseases in small ruminants

Ticks and tick-borne diseases may have an impact on the production of small ruminants. Several studies have been conducted to determine which tick species are present in Greece on both humans and animals. These studies were conducted on the Greek mainland and therefore very little information is available about ticks and tick-borne diseases on the island of Lesvos.

In Greece there are several tick species identified that have been implicated in the transmission of tick-borne pathogens that can cause diseases in small ruminants. Table 1 shows the tick species that have been reported in Greece on a range of different animals, including cattle, small ruminants, dogs and even humans. The situation on Lesvos may be different from the Greek mainland.

Tick species	B. Papadopoulos et al., 1996[8]	A. Papa et al., 2011[9]	V. Pavlidou et al., 2008[10]	Chaligiannis et al., 2014[11]	Papazahariadou et al., 2003[12]
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	
• *excavatum*	X	X		X	
• *scupense*	X				
• *m. rufipes*	X	X		X	
• *dromedarii*				X	
• *impeltatum*				X	
• *turanicum*				X	
Ixodes spp.					
• *ricinus*			X	X	
• *gibbosus*			X	X	
Dermacentor spp.					
• *marginatus*			X	X	

Table 1: Tick species found in Greece

There are several important tick-borne diseases that could have an impact on small ruminants on the island of Lesbos. *Anaplasmosis* is a tick-borne disease in goats and sheep, caused by *Anaplasma ovis* in many countries in the world and is transmitted by *Rhipicephalus bursa* ticks. According to a recent study that has been performed to detect tick-borne pathogens by PCR in blood samples in Turkey, Sudan and Portugal, there is a high prevalence of *Anaplasma ovis* in small ruminants. It is likely that the situation is similar in Greece.[13] *R. bursa* ticks have been frequently found in several studies conducted in Greece, as shown in table 1.

Recently, a study was conducted in Italy to determine which tick-borne pathogens were present in sheep. PCR combined with reverse line blot hybridization (RLB) was used to detect these pathogens. *Anaplasma ovis* had the highest prevalence, but there were also sheep that had a co-infection with *Theileria sp. OT3* or *Babesia motasi*. *Anaplasma phagocytophilum* was also highly prevalent, alone or co-infected with *B. motasi* or *Theileria sp. OT3*.[14] *Anaplasma phagocytophilum* is a bacteria that causes granulocytic *anaplasmosis*. In northern Greece this bacteria has been found in *Ixodes ricinus* ticks.[15,16] *A. phagocytophilum* can cause tick-borne fever in ruminants, and occurs also in companion animals and even in humans.

A very recent study conducted in China revealed a novel *Anaplasma* species in humans, named *Anaplasma capra*, which originates in local goats. Blood samples were taken from patients with a history of a tick bite. *A. capra* was found in 6% of the patients according to PCR and sequencing.[16,17] In figure 5 infected cells with *A. capra* are shown.

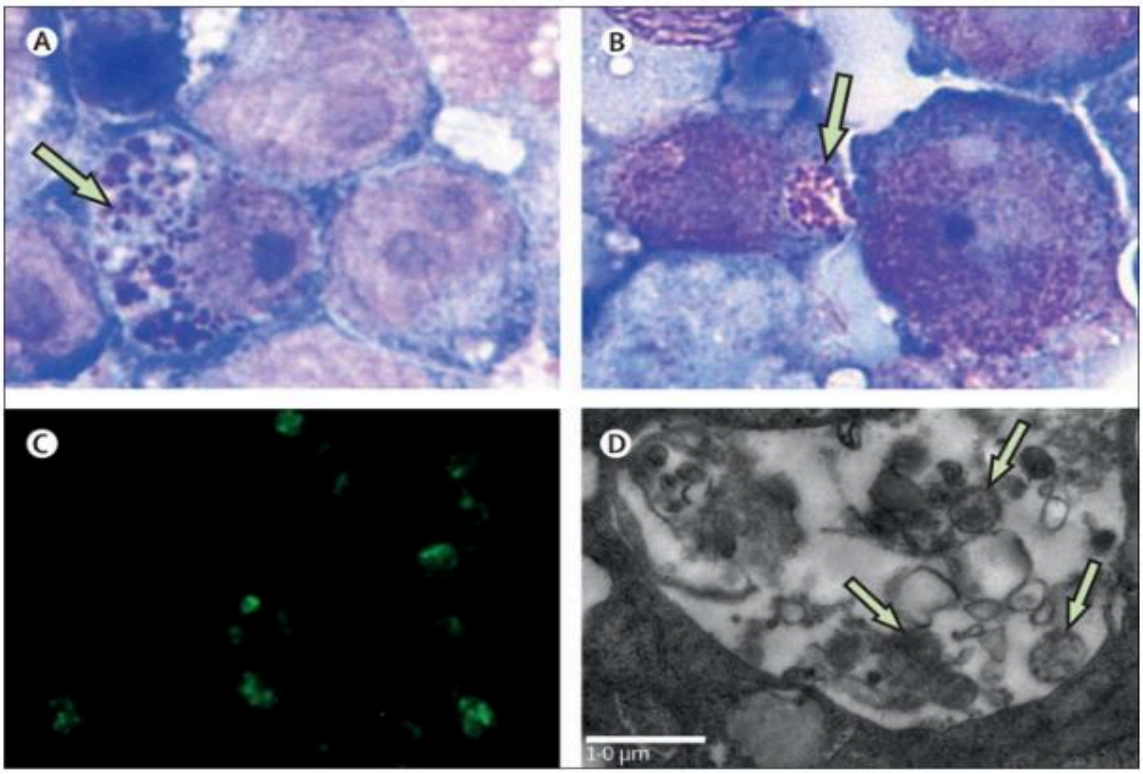

Figure 1.
Photomicrographs of cells infected with "*Anaplasma capra*"
Morulae are indicated by arrows. Panel A shows Wright-Giemsa stained HL-60 cells. Panel B shows Wright-Giemsa stained THP-1 cells. Panel C shows *"A capra"* grown in HL-60 cells and detected on immunofluorescence assay of a serum sample obtained from a convalescent patient. Panel D shows an electron photomicrograph of a THP-1 cell infected with *A capra*.

Figure 5 : Anaplasma capra in human cells[17]

Medication and prevention

Because little is known about ticks and tick-borne diseases in Greece, most of farmers do not know about the possible consequences for their livestock. That is why ticks are underestimated and most farmers do not treat their animals against ticks.

Other diseases like mastitis are treated very well and farmers try to prevent diseases, such as Blue tongue, which is one of the concerns of the farmers in Greece. The blue tongue virus is common in Africa, northern Australia, America and southern Asia were the climate is warm or tropical. The blue tongue virus is an *Orbivirus* and

is transmitted by culicoides midges. Because the virus and the adult vectors favour warm temperatures, outbreaks in Europe are seen in summer and autumn. Lesvos lies close to North Africa and Turkey and has therefore a high change of infection. The infected culicoides spread by the wind or are transported during livestock transportation between countries.[18]

To prevent culicoides from biting the sheep, a lot of farmers use ectoparasiticides. ECTOPOR ® is a synthetic pyrethroid used for the control of ectoparasites like lice, scabies, and flies of sheep, goats and cattle. Also ticks are assumed to be sensitive for this pesticide.

Nevertheless, it is important to study ticks and tick-borne diseases in sheep. Once it is determined which ticks and tick-borne diseases exist on Lesvos, a good strategy for control and prevention can be developed.

Figure 6: ECTOPOR ®

3. Materials & Methods

Blood and tick sampling

The sampling of blood and ticks from sheep was mainly done in the morning and in the evening. At this time the sheep were milked and they were inside the sheds. Five animals per farm were selected for sampling of both ticks and blood. Before the sampling started a questionnaire was answered by the farmer with questions about management techniques and information about diseases and their treatments/prevention on the farm. The questionnaires are attached in appendix 1.

After this was completed, the sampling started. Gloves and overshoes were used on the farm at all times to prevent contamination of other farms. To get the ticks of the sheep forceps were used. Predilection sites like ears, the udder and the peri-anal area were checked for ticks. Special tick bottles were made in advance to store the ticks that were collected from the sheep temporarily. On the tick bottles was the farm number and the animal number written. During transportation the ticks were packed in a cool box with ice. After returning to the laboratory, the ticks were immediately put in 80% ethanol in Eppendorf tubes. The Eppendorf tubes were labelled with the farm number and the animal number.

Figure 7: The sampling of ticks and special tick bottles

The ticks were identified under a microscope with external light sources to see more details. The book; *Ticks of domestic animals in the Mediterranean region: a guide to identification of species* of Estrada-Pena et al. (2004) was used during the identification.[19] During the identification the ticks were separated according to their species and their host.

For the blood sampling EDTA-tubes and vacutainers were used. On the tubes the farm number and the animal number was written. The blood was also stored in a cool box with ice during transportation. After returning to the lab in Thessaloniki the blood was split in 2 Eppendorf tubes labelled with the farm number and the animal number. One tube remained for usage in Thessaloniki and the other will be used in Utrecht. The blood in the tubes was stored at -20 °C and the remaining blood in the EDTA-tubes was stored at -80 °C degrees.

DNA-extractions

DNA-extractions were done on all the blood samples (n=194) from Lesvos. The QIAGEN DNeasy blood and tissue kit was used for the DNA extractions. The Quick-Start protocol that had been provided by the company was used. The protocol is attached in appendix 2.

For every DNA-extraction 100 µl of blood was used to gain 200 µl of isolated DNA. After that the isolated DNA was split into tubes, 100 µl of DNA each. One of the tubes stayed in Greece and the other was used for PCR amplification and Reverse Line Blot Hybridization (RLB) in Utrecht.

Data processing

Some collected data from the questionnaires, such as the coordinates of the farm, the altitude and region was entered into a geodata file. This file is attached as appendix 5. The information from the geodata file was used to make a map of all the places that were visited. This was necessary to gain an overview of the coverage of the sampling in Lesvos. The other data from the blood and ticks and DNA-extractions are available in dropbox. The two files give an overview of the most important part of the information that was gathered during the visits to Lesvos.

The SPSS program was used to make a statistical analysis from the information of the tick identification and the geodata.

4. Results

During the sampling in Lesvos, 39 farms were visited in total. 195 sheep were examined. 1159 ticks were collected in total and 194 blood samples were taken. Most of the tick species that were found were *Rhipicephalus turanicus* (89,9%), as seen in table 2 and figure 8. Also *Hyalomma* species were found. An overview of all the tick and blood sampling is available in dropbox.

Tick species	**Lesvos**
Hyalomma	
- *H. excavatum*	5 (0,43%)
- *H. detritum*	2 (0,17%)
- *H. detritum scupense*	1 (0,09%)
- *H. marginatum*	20 (1,73%)
- *H. rufipes*	1 (0,09%)
Rhipicephalus	
- *R. bursa*	57 (4,92%)
- *R. sanguineus*	31 (2,67%)
- *R. turanicus*	1042 (89,9%)

Table 2: Number of tick species found in Lesvos

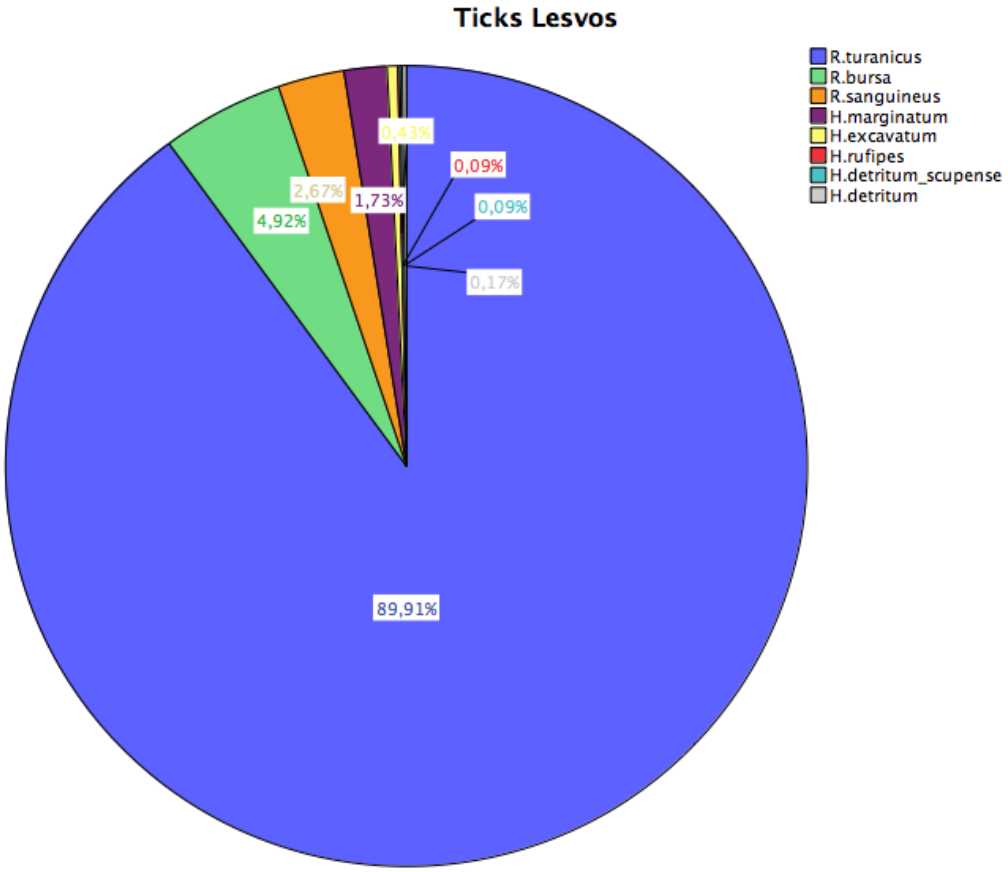

Figure 8: Percentages of ticks found in Lesvos

During the visits to the farms, the coordinates were recorded. Figure 9 shows the exact places of all the farms that were visited on the island.

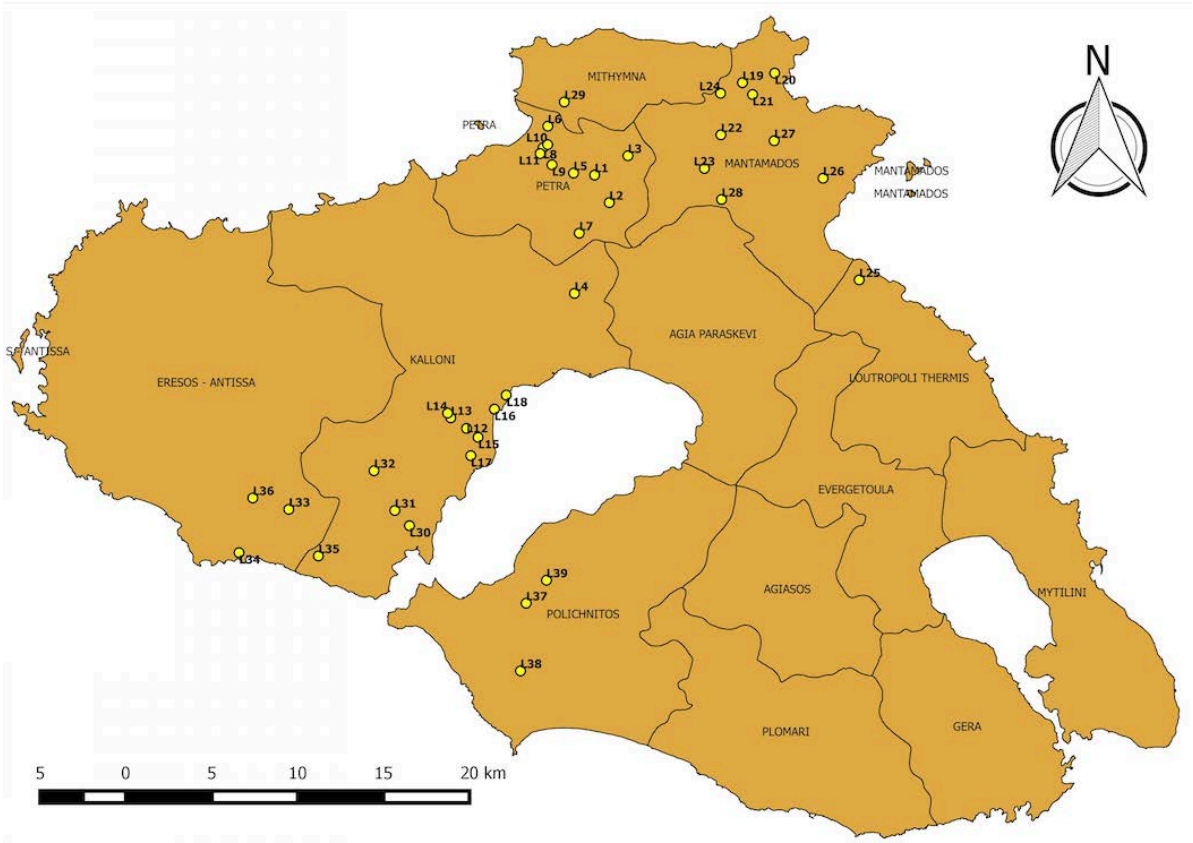

Figure 9: Locations of visited farms in Lesvos

A large part of the information that was gathered from the questionnaires has not yet been translated, so it was not possible to add this to the appendix.

PCR amplification and Reverse Line Blot Hybridization (RLB) on the DNA-extractions of the blood samples, remains to be done.

5. Discussion

During the tick sampling, the farmers assisted to collect the sheep and held them in a steady position so that examining the sheep was more efficient. However, sometimes not the whole flock was infested with ticks. The farmers sometimes picked out the sheep that were infested, so the sampling was not totally random anymore. Also sheep from different ages were examined. It is possible that some species of ticks are only found on lambs or adult sheep. This could have affected the results.

As mentioned before, most of the farmers used ECTOPOR ® against culicoides that is also used against ticks. It is very likely that this has reduced the number of ticks on sheep on this island.

What also could make a difference in finding certain kinds of tick species are the different heights on which the farms are located. Lesvos is mountainous and between farms there is sometimes a big difference in height. Also the vegetation in Lesvos is divers, this could also have an effect on finding ticks and different species of ticks.

6. Conclusion

It is clear that there is a broad range of tick species found on sheep on the island of Lesvos. Not only *Rhipicephalus* species, but also *Hyalomma* species are present. Further research has to be done to determine which diseases these ticks may be able to transmit and whether they cause any problems in sheep or not. Also there should be special reference to *Anaplasma* species that may be infectious for humans as well.

7. Acknowledgements

First of all, I would like to thank professor Jongejan for the wonderful opportunity he gave me to let me do my internship in Greece and for his support and supervision during this experience.

Secondly, I would like to thank Dr. Smaro Sotiraki for all her advice and for giving me the warmest welcome and the best care in Greece. I could not have done this without her.

Furthermore, I would like to thank Tasos Saratsis for being the best guide and friend in Greece. Without his translation skills I would not have been able to communicate with the farmers and veterinarians. I am also grateful for all the help from Tasos and the whole crew in de laboratory in Thessaloniki.

Last but not least I would like to thank Mandy Jelicic for sharing this experience with me and supporting me during the entire internship.

8. References

1. Eleftheriadi M. *Lesvos: The Island of Sappho*.; 2006.

2. Turner RK, Bergh JCJM van den, Brouwer R. *Managing Wetlands, an Ecological Economics Approach*.; 2003.

3. Mytilini, Lesbos Climate Mytilini, Lesbos Temperatures Mytilini, Lesbos Weather Averages. http://www.lesvos.climatemps.com/. Accessed July 28, 2015.

4. Kizos T, Koulouri M. Agricultural landscape dynamics in the Mediterranean: Lesvos (Greece) case study using evidence from the last three centuries. *Environ Sci Policy*. 2006;9(4):330-342. doi:10.1016/j.envsci.2006.02.002.

5. Sossidou E, Ligda C, Mastranestasis I, Tsiokos D. Sheep and Goat Farming in Greece : Implications and Challenges for the Sustainable Development of Less Favoured Areas. 2013;46(2):446-449.

6. Statistics Greece. http://www.statistics.gr/portal/page/portal/ESYE/PAGE-themes?p_param=A1008&r_param=SPK61&y_param=2009_00&mytabs=0.

7. Rare Breeds and Varieties of Greece Atlas 2010. http://www.save-foundation.net/pdf/Breedatlas_Greece_FINAL.pdf. Accessed July 28, 2015.

8. Papadopoulos B, Morel PC, Aeschlimann A. Ticks of domestic animals in the Macedonia region of Greece. *Vet Parasitol*. 1996;63(1-2):25-40. doi:10.1016/0304-4017(95)00877-2.

9. Papa A, Chaligiannis I, Xanthopoulou K, Papaioakim M. Ticks Parasitizing Humans in Greece. 2011;11(5):10-13. doi:10.1089/vbz.2010.0036.

10. Pavlidou V, Gerou S, Kahrimanidou M, Papa A. Ticks infesting domestic animals in northern Greece. *Exp Appl Acarol*. 2008;45(3-4):195-198. doi:10.1007/s10493-008-9167-5.

11. Chaligiannis I, Papa a, Sotiraki S. Ticks feeding on ruminants and humans in Greece. *Parasit Vectors*. 2014;7(Suppl 1):O1. doi:10.1186/1756-3305-7-S1-O1.

12. Papazahariadou MG, Saridomichelakis MN, Koutinas a. F, Papadopoulos EG, Leontides L. Tick infestation of dogs in Thessaloniki, northern Greece. *Med Vet Entomol*. 2003;17(1):110-113. doi:10.1046/j.1365-2915.2003.00404.x.

13. Renneker S, Abdo J, Salih DEA, et al. Can Anaplasma ovis in Small Ruminants be Neglected any Longer? *Transbound Emerg Dis*. 2013;60(SUPPL.2):105-112. doi:10.1111/tbed.12149.

14. Giangaspero a., Marangi M, Papini R, Paoletti B, Wijnveld M, Jongejan F. Theileria sp. OT3 and other tick-borne pathogens in sheep and ticks in Italy: Molecular characterization and phylogeny. *Ticks Tick Borne Dis*. 2015;6(1):75-83. doi:10.1016/j.ttbdis.2014.09.007.

15. Kachrimanidou M, Papa A, Chochlakis D, Pavlidou V, Psaroulaki A. Molecular evidence for Anaplasma phagocytophilum in Ixodes ricinus ticks from Greece. *Vector-Borne Zoonotic Dis*. 2011;11(10):1391-1393. doi:10.1089/vbz.2010.0251.

16. Beyer AR, Carlyon JA. Of goats and men: rethinking anaplasmoses as zoonotic infections. *Lancet Infect Dis*. 2015;15(6):619-620. doi:10.1016/S1473-3099(15)70097-6.

17. Li H, Zheng Y-C, Ma L, et al. Human infection with a novel tick-borne Anaplasma species in China: a surveillance study. *Lancet Infect Dis*. 2015;15(6):663-670. doi:10.1016/S1473-3099(15)70051-4.

18. Mellor PS, Carpenter S, Harrup L, Baylis M, Mertens PPC. Bluetongue in Europe and the Mediterranean Basin: History of occurrence prior to 2006. *Prev Vet Med*. 2008;87(1-2):4-20. doi:10.1016/j.prevetmed.2008.06.002.

19. Agustín Estrada-Peña, A. Bouattour, J.-L. Camicas ARW. *Ticks of Domestic Animals in the Mediterranean Region: A Guide to Identification of Species*.; 2004.

8. Appendix

Appendix 1: Questionnaires

Questionnaire form

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:

1

- 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:
(e.g. 8/10/12 animals)

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

Are your animals infested with ticks? YES/NO

Are 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: .....

3

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 2: The QIAGEN DNeasy Protocol

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.

January 2011

QIAGEN®

Sample & Assay Technologies

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.

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 3: Protocol for PCR/RLB

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)
FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

PCR RLB PROCEDURE

Sample description	
Number of samples	

Wear (green) gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

Primers:	***Anaplasma* *Ehrlichia***	***Babesia* *Theileria***	***Borrelia***	***Rickettsia***	**Other:**

Reagent	**1x**	**Number of samples + 10%**	
PCR grade H_2O	15.875µl		
5x Phire reaction buffer	5.0µl		
10mM dNTPs	0.5µl		
Forward primer (20pmol/µl)	0.5µl		
Reverse primer (20pmol/µl)	0.5µl		
2U/µl Phire Hot Start II DNA polymerase	0.125µl		

		Done	
1	Put DNA samples a (few) day(s) before the PCR at 4°C.		
2	Turn on the DNA workstations in the clean room and the dirty room.		
3	Clean workspace in both DNA workstations with sodium hypochloride.		
4	Label the PCR and Eppendorf tubes and put them in the DNA workstation in the clean room		
5	Turn on the UV-light in both DNA workstations for 20 minutes.		
6	**During the UV-light**; thaw the PCR reagents at room temperature, except the polymerase.		
7	Prepare the PCR mix in the Eppendorf tube(s). Multiply the reagent volumes by the number of samples plus 10% of the number of samples: 40 DNA samples + 1 PCR control = 41 + 10% = 45 samples.		

8	Pipet the master mix gently up and down to mix well.		
9	Pipet 22,5µl master mix to each PCR tube and add the leftover mix to an additional tube which will be the negative PCR control.		
10	Close the PCR tubes and remove them from the workstation, clean the workspace with sodium hypochloride and turn on the UV-light for 20 minutes.		
11	Take the closed PCR tubes to the dirty room and place them in the workstation.		
12	Vortex the DNA samples, spin them down briefly at 11,000x g and place them in the workstation.		
13	Add 2.5µl DNA sample to the corresponding PCR tube.		
14	Add 2.5µl of the positive control (, corresponding to the PCR to be performed,) to the positive PCR control tube.		
15	Vortex and spin down briefly.		
16	Clean the workstation with sodium hypochloide and turn on the UV-light for 20 minutes.		
17	Run the corresponding PCR program.		
18	Store the PCR products at 4°C for use within the next few days or store at -20°C for long term preservation.		
19	Turn off both DNA workstations after the UV-light is switched off.		

PCR done:

by _____ on _____ _____

Signature

Comments:

Appendix 4: Tick and blood collection

This Excel file is too large to put into this Word file. However, the file is available in dropbox “Tsiburi project” and click on “Tick & Blood collection Lesvos corrected version”.

Appendix 5: Geodata

Farm ID	Animal	Longitude	Latitude	Altitude (m)	Closest village	Municipality divisions	Date
L1	Sheep	26,223091	39,307378	293,6	Stipsi	Petra	06-05-15
L2	Sheep	26,232939	39,293060	192,2	Stipsi	Petra	06-05-15
L3	Sheep	26,245482	39,317459	432,0	Ipsilometopo	Petra	06-05-15
L4	Sheep	26,209557	39,245842	16,9	Kalloni	Kalloni	06-05-15
L5	Sheep	26,208918	39,308444	338,9	Stipsi	Petra	06-05-15
L6	Sheep	26,191654	39,332816	14,8	Petra	Petra	06-05-15
L7	Sheep	26,212725	39,277158	170,6	Stipsi	Petra	06-05-15
L8	Sheep	26,188504	39,321680	44,5	Petra	Petra	07-05-15
L9	Sheep	26,194430	39,312725	222,8	Petra	Petra	07-05-15
L10	Sheep	26,191539	39,323241	62,9	Petra	Petra	07-05-15
L11	Sheep	26,186468	39,318570	30,4	Petra	Petra	07-05-15
L12	Sheep	26,137028	39,175522	99,6	Parakoila	Kalloni	07-05-15
L13	Sheep	26,126419	39,181030	127,1	Parakoila	Kalloni	08-05-15
L14	Sheep	26,124299	39,183483	162,8	Parakoila	Kalloni	08-05-15
L15	Sheep	26,144789	39,170855	34,0	Parakoila	Kalloni	08-05-15
L16	Sheep	26,155774	39,185549	5,4	Parakoila	Kalloni	08-05-15
L17	Sheep	26,139906	39,161311	42,6	Parakoila	Kalloni	08-05-15
L18	Sheep	26,163725	39,192871	5,5	Parakoila	Kalloni	08-05-15
L19	Sheep	26,322415	39,355418	241,32	Kleio	Mantamados	03-06-15
L20	Sheep	26,344084	39,36039	86,0	Tsonia	Mantamados	03-06-15
L21	Sheep	26,329212	39,349277	146,4	Kleio	Mantamados	03-06-15
L22	Sheep	26,307933	39,328332	335,6	Kapi	Mantamados	03-06-15
L23	Sheep	26,296869	39,310848	235,7	Pelopi	Mantamados	03-06-15
L24	Sheep	26,307712	39,349913	283,0	Kleio-skala Sikami	Mantamados	03-06-15
L25	Sheep	26,400832	39,252861	83,8	Mantamados	Mantamados	04-06-15
L26	Sheep	26,37657	39,30576	70,6	Mantamados	Mantamados	04-06-15
L27	Sheep	26,343739	39,325235	168,0	Mantamados	Mantamados	04-06-15
L28	Sheep	26,308388	39,294713	196,1	Mantamados	Mantamados	04-06-15
L29	Sheep	26,307933	39,328332	111,0	Molivos	Mythimna	04-06-15
L30	Sheep	26,09868	39,124788	138,0	Agra	Kalloni	05-06-15
L31	Sheep	26,088846	39,132735	165,8	Agra	Kalloni	05-06-15
L32	Sheep	26,074847	39,153449	217,8	Agra	Kalloni	05-06-15
L33	Sheep	26,017595	39,133309	204,8	Mesotopos	Eressos	05-06-15
L34	Sheep	25,984147	39,110746	67,5	Mesotopos	Eressos	05-06-15
L35	Sheep	25,999527	39,099075	55,8	Mesotopos	Eressos	05-06-15
L36	Sheep	25,99343	39,139261	203,0	Mesotopos	Eressos	05-06-15
L37	Sheep	26,177086	39,084354	120,4	Polichnitos	Polichinitos	06-06-15
L38	Sheep	26,173281	39,049015	48,7	Polichnitos	Polichinitos	06-06-15
L39	Sheep	26,190744	39,096342	79,3	Polichnitos	Polichinitos	06-06-15